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LINE-1 Methylation Analysis in Mesenchymal Stem Cells Treated with Osteosarcoma-Derived Extracellular Vesicles

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Helsinki, 12 November 2019

Dear Editor,

We hereby submit our revisions to the manuscript titled **LINE-1 methylation analysis in mesenchymal stem cells treated with osteosarcoma-derived extracellular vesicles**, authored by Snehadri Sinha, Bettina Mannerström, Riitta Seppänen-Kaijansinkko and Sippy Kaur.

We thank you and the Reviewers for your feedback. Working on the revisions has proven to be a beneficial learning experience. Thank you for your patience in agreeing to extend the deadlines. We hope the latest draft of our publication meets your approval. We have responded to the peer-review comments in a separate document.

In addition to changes to the manuscript and video, we have made changes to all figures, Table 2 and the Table of Materials. We have also attached a supplementary file that will refer you to the copyright permissions for two publications that we have cited in our paper.

Looking forward to the next stages of the publication process.

On behalf of all the authors,

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TITLE:

LINE-1 Methylation Analysis in Mesenchymal Stem Cells Treated with Osteosarcoma-Derived Extracellular Vesicles

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KEYWORDS:

DNA methylation, LINE-1, epigenetics, extracellular vesicles, EV-depleted fetal bovine serum, osteosarcoma

SUMMARY:

Described here is the use of a methylation-specific probe amplification method to analyze methylation levels of LINE-1 elements in mesenchymal stem cells treated with osteosarcoma-derived extracellular vesicles. Ultracentrifugation, a popular procedure for separating extracellular vesicles from fetal bovine serum, is also demonstrated.

ABSTRACT:

Methylation-specific probe amplification (MSPA) is a simple and robust technique that can be used to detect relative differences in methylation levels of DNA samples. It is resourceful, requires small amounts of DNA, and takes around 4–5 h of hands-on work. In the presented technique, DNA samples are first denatured then hybridized to probes that target DNA at either methylated or reference sites as a control. Hybridized DNA is separated into parallel reactions, one undergoing only ligation and the other undergoing ligation followed by *Hha*I-mediated digestion at unmethylated GCGC sequences. The resultant DNA fragments are amplified by PCR and separated by capillary electrophoresis. Methylated GCGC sites are not digested by *Hha*I and produce peak signals, while unmethylated GCGC sites are digested and no peak signals are generated. Comparing the control-normalized peaks of digested and undigested versions of each sample provides the methylation dosage ratio of a DNA sample. Here, MSPA is used to detect the effects of osteosarcoma-derived extracellular vesicles (EVs) on the methylation status of long interspersed nuclear element-1 (LINE-1) in mesenchymal stem cells. LINE-1s are

repetitive DNA elements that typically undergo hypomethylation in cancer and, in this capacity, may serve as a biomarker. Ultracentrifugation is also used as a cost-effective method to separate extracellular vesicles from biological fluids (i.e., when preparing EV-depleted fetal bovine serum [FBS] and isolating EVs from osteosarcoma conditioned media [differential centrifugation]). For methylation analysis, custom LINE-1 probes are designed to target three methylation sites in the LINE-1 promoter sequence and seven control sites. This protocol demonstrates the use of MSPA for LINE-1 methylation analysis and describes the preparation of EV-depleted FBS by ultracentrifugation.

INTRODUCTION:

DNA methylation is a major epigenetic modification occurring in human cells. DNA methylation refers to the linkage of methyl groups to cytosine residues in CpG dinucleotides. Such dinucleotides are usually found in clusters (CpG islands) at the 5' region of genes[1]. In normal cells, most of these dinucleotides exist in an unmethylated state, which allows DNA transcription. Incidentally, many cancers are associated with hypermethylated CpG islands and transcriptomic silencing[2], especially in tumor suppressor genes, which in turn contribute to various hallmarks of cancer[3].

On the other hand, long interspersed nuclear elements-1 (LINE-1s or L1s) are repetitive, transposable DNA elements that normally have high levels of methylation at CpG islands. Methylation of LINE-1 prevents translocation and helps maintain genome integrity. In several types of cancer, LINE-1 is hypomethylated, resulting in activation and subsequent retrotransposition-mediated chromosomal instability[4]. LINE-1 accounts for nearly 17% of the human genome[5], and its methylation status may serve as an indicator of global genomic methylation levels[6]. Global LINE-1 hypomethylation is considered to precede the transition of cells to a tumor phenotype[7]; therefore, it holds promise as a potential marker for early cancer onset.

Currently, there are several methods for methylation analysis, including pyrosequencing, methylation-specific PCR, microarrays, and chromatin immunoprecipitation[1]. The use of next-generation sequencing has also made it possible to incorporate genome-wide approaches to detection of DNA methylation. Many of these methods rely on bisulfite-treated DNA, in which unmethylated cytosines are converted to uracil and methylated cytosines remain unchanged. However, working with bisulfite-treated DNA has several pitfalls, such as incomplete conversions of unmethylated cytosines to uracil, biased amplification of sequences, and sequencing errors[8].

In methylation-specific probe amplification (MSPA), probes composed of two oligonucleotides target DNA sequences containing a restriction site (GCGC) for the methylation-sensitive restriction enzyme *Hha*I[9]. After the probes hybridize to DNA, each sample is divided into two sets. Probes in the first set undergo ligation, while probes in the second set undergo ligation followed by *Hha*I-mediated digestion at unmethylated CGCG sites. Both sets of samples are then amplified by PCR, and the products are separated by capillary electrophoresis. Probes at

unmethylated sites are digested by *Hha*I and are not amplified during PCR, resulting in no peak signals. By contrast, probes at methylated sites are protected from digestion and are therefore amplified during PCR, subsequently generating peak signals[10].

MSPA has several advantages over alternative methods. First, it requires a low amount of DNA (50–100 ng) and is well-suited for analysis of DNA from formalin-fixed paraffin embedded samples[10]. It does not require bisulfite-treated DNA; in fact, it is unsuitable for DNA that is modified in this way. Many samples can be analyzed at the same time, and MSPA probes can be designed such that they target multiple genes or sequences simultaneously. Additionally, the probes are specific and sensitive for methylated DNA as the *Hha*I restriction site corresponds to a sequence that is typical of CpG islands[10].

This study investigated the effects of osteosarcoma (OS)-derived extracellular vesicles (EVs) on LINE-1 methylation in adipose tissue-derived mesenchymal stem cells (AT-MSCs; **Figure 1)**. EVs are nanoscale, membrane-bound vesicles secreted by most cell types. They carry proteins, lipids, mRNA, microRNA, and additional molecules from parent cells[11,12]. EVs mediate intercellular communication and play important roles in several pathophysiological conditions[13,14]. A recent study showed that cancer-derived EVs may transfer active LINE-1 to recipient cells[15]. It has been reported earlier that EVs from the HOS-143B cell line can alter the methylation status of LINE-1 in MSCs, in addition to other genetic effects[16].

When growing cells for EV isolation, it is important to use EV-depleted FBS in the growth medium, since FBS-derived EVs may interfere with EVs from other sources and hamper the results[17,18]. Ultracentrifugation is one of the most common methods for depleting EVs from FBS. It is a relatively simple and cost-effective procedure compared to alternatives such as ultrafiltration and commercial EV-depleted FBS[19]. Here, the protocol also demonstrates how to prepare EV-depleted FBS by ultracentrifugation.

This article presents a detailed protocol for the aforementioned techniques, from isolation of EVs from an OS cell line to the methylation analysis of LINE-1 in OS-EV treated MSCs (**Figure 1**).

PROTOCOL:

This study was approved by the Ethics Committee of Helsinki and Uusimaa Hospital District (ethical approval D. No. 217/13/03/02/2015).

1. Preparation of EV-depleted FBS by ultracentrifugation

1.1. Take FBS in (ultra)centrifuge tubes and place them in ultracentrifuge buckets. To ensure that the ultracentrifugation runs smoothly and safely, balance the buckets within 10 mg of each other.

1.2. Load the buckets on a swinging rotor (type SW28, k-factor 246). Place the rotor in the ultracentrifuge and run at 100,000 x *g* for 19 h at 4 °C.

1.3. Carefully collect the light-colored upper layer of the supernatant (approximately nine-tenths) and transfer to a 50 mL tube. Do not disturb or pipette the dark brown pellet, as it contains EVs from FBS.

1.4. Pass the supernatant through a 0.22 µm filter into a new 50 mL tube.

1.5. Add the filter-sterilized, EV-depleted FBS to cell culture media when growing cells for EV isolation.

2. Isolation of osteosarcoma-derived EVs

2.1. Plate OS cells (HOS-143B cell line) in a T-175 flask with RPMI 1640 medium, supplemented with 10% normal FBS and 1% antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin). Place the flask in an incubator at 37 °C and 5% CO_2.

2.2. When the flask is 70%–80% confluent, wash the cells with phosphate-buffered saline (PBS) then grow them in media containing 10% EV-depleted FBS (hereafter referred to as EV-depleted media).

NOTE: 1 x 10^6 HOS-143B cells plated in a T175 flask reaches 70% confluency after approximately 60 h.

2.3. During the next 48 h, collect conditioned media from osteosarcoma cells after every 24 h and add fresh EV depleted media.

2.4. Centrifuge the conditioned media at 2500 x *g* for 20 min at 4 °C to remove cells and cell debris. Transfer the supernatant to a new tube, leaving around 2 mL of media at the bottom.

NOTE: If not directly proceeding with EV isolation at this stage, the supernatant can be stored at -80 °C.

2.5. Pour the supernatant into ultracentrifuge tubes and balance them as earlier. Centrifuge the tubes at 100,000 x *g* for 2 h at 4 °C.

2.6. Carefully discard the supernatant, leaving around 1 mL at the bottom. Add around 20 mL of PBS (0.1 µm filtered) to the tube and pipette gently to wash and resuspend the EV pellet.

2.7. Balance the tubes and perform another round of ultracentrifugation with the same settings.

2.8. Carefully remove the supernatant and resuspend the EV pellet in 200 µL of PBS by gentle pipetting. Store the EVs in low binding tubes.

NOTE: EVs can be used straightaway or else stored in -80 °C until they are needed.

3. Characterization of OS-EVs

NOTE: Purified EVs can be characterized by western blotting (WB), nanoparticle tracking analysis (NTA), and transmission electron microscopy (TEM)[16].

3.1. Perform WB as per the standard protocol[16] with EV markers CD63, TSG101, and Hsp70, and with calnexin as a negative control to indicate purity of the EV sample[20].

3.2. For NTA, first dilute the EV sample in 0.1 µm filtered Dulbecco's PBS to obtain (ideally) 30–100 particles per frame.

3.2.1. Take around 500 µL of the sample into a 1 mL syringe and load it into the inlet port of the NTA instrument. Check that there are enough particles per frame, for accurate measurements.

3.2.2. Open the NTA software and record five videos of 60 s duration, using camera level 13 at ambient temperature. During analysis, use detection threshold = 5 and gain = 10.

3.3. Analyze EV samples by TEM as described previously[21].

4. AT-MSC culture

NOTE: Human adipose tissue for mesenchymal stem cell isolation was provided as liposuction aspirate (Department of Plastic Surgery, Laser Tilkka Ltd., Finland). Written informed consent was taken from the lipoaspirate donors, who were undergoing elective liposuction procedures.

4.1. Isolate AT-MSCs from liposuction aspirates using standard mechanical and enzymatic isolation methods[22].

NOTE: MSCs from other sources (including commercial cell lines) may also be used.

4.2. Culture cells in DMEM/F-12 media supplemented with 10% FBS and 1% antibiotics.

5. Treatment of MSCs with OS-EVs

5.1. Plate 15,000 AT-MSCs per well in a 24 well plate.

5.2. After 24 h, remove the old media, wash cells with PBS, and change to EV-depleted media.

5.3. Treat cells with OS-EVs (at a particle concentration of 1×10^6 EVs per cell) on Day 1 (24 h after cell adhesion), Day 3 (48 h after Day 1), and Day 5 (96 h after Day 1).

5.3.1. Stop the OS-EV treatment for timepoint (TP) 0 samples on Day 1, TP 3 samples on Day 3 and TP 7 samples on Day 7 (48 h after Day 5).

NOTE: Other timepoint schedules as per the experimental plans can also be followed.

5.4. Extract DNA from the MSCs using an appropriate method. Include positive and negative control samples for the data analysis.

6. LINE-1 methylation assay

6.1. Design the customized LINE-1 probe primers, as done previously by Pavicic et al.[23]. For methylation probes, select three sequences containing the *Hha*I restriction site within the promoter region of LINE-1. For control probes, select seven sequences lacking the *Hha*I restriction site from the rest of the LINE-1 sequence.

NOTE: The LINE-1 sequence is available at the GenBank database[24] (L1.2, accession no. AH005269.2). Use the MSPA manufacturer's instructions for designing the probes[25].

6.2. Dilute 70 ng of DNA sample in TE buffer to a 5 µL volume.

6.3. Carry out subsequent thermocycling and PCR steps as mentioned in **Table 1**. Heat the samples for 10 min at 98 °C, then cool to 25 °C.

6.4. Add 3 µL of probe hybridization mix to each sample and run the thermocycler to allow the probes to hybridize to the DNA.

6.5. At room temperature (RT), add 13 µL of post-hybridization mix to each sample. Transfer 10 µL to a second tube.

6.6. Place both sets of tubes in the thermocycler and incubate at 48 °C for at least 1 min.

6.6.1. While samples are at 48 °C, add 10 µL of the ligation mix to the first set of tubes (undigested series) and 10 µL of the ligation-digestion mix to the second set of tubes (digested series). Run the next thermocycler program.

6.7. Spin down the tubes and simultaneously set the thermocycler to 72 °C.

6.8. Add 5 µL of polymerase mix to each tube and place the tubes in the thermocycler. Run the PCR program.

6.9. While the PCR program is running, prepare a solution of 1 mL formamide containing 2.5 µL of size standard. Pipette 10 µL of this solution to each well of an optical 96 well plate (with barcode).

6.10. After PCR, dilute the undigested and digested samples to 1:100 and 1:200 respectively in ultrapure water. Add 2 µL of diluted PCR product to the 96 well plate. Centrifuge the plate at 200 x *g* for 15–20 s to remove air bubbles.

6.11. Carry out fragment analysis of samples by capillary electrophoresis.

NOTE: The plate should be stored at 4 °C in the dark until analysis.

7. Fragment data analysis

7.1. Open the capillary electrophoresis results in an electropherogram analysis software.

7.1.1. Under the **Panel** column, for one of the samples, choose **MLPA** from the menu. Click on the **Panel** header and press **Ctrl+D** to apply **MLPA** to all samples.

7.1.2. In the same way, set the **Analysis method** to **Microsatellite default** for all samples.

7.1.3. Select all samples and click on the **Green play** button to analyze the samples as per the chosen settings.

7.1.4. Select all samples and click on the **Graph** button to visualize the probe peaks.

7.1.5. Zoom in on the peak region for higher resolution of the individual probe peaks. Make sure that all 10 peaks corresponding to the probes from the LINE-1 probe-mix are labeled. Discard additional peaks (<95 bp and >160 bp).

7.1.6. In the **Genotypes** tab, export the results in the comma-separated-values (CSV) format.

7.2. Open the CSV file in a data analysis software and sort the data into columns.

7.2.1. Label the three methylation site peaks based on their approximate sizes (L1-1m at 153 bp, L1-2m at 119 bp, L1-3m at 133 bp). The remaining seven peaks correspond to the control probes.

NOTE: Here, values of the L1-2m probe peaks are used, which have a size of 117 bp, since that region has been used in most LINE-1 methylation assays[23].

7.2.2. For each sample (undigested and digested), calculate the sum peak area of all seven control peaks. Divide the peak area of each LINE-1 probe by this sum.

7.2.3. For each DNA sample, divide the value of the digested sample by that of the undigested sample to obtain the methylation dosage ratio (D_M) using the following equation:

$$D_M = \frac{\frac{A_x}{A_{ctrl}}\ digested}{\frac{A_x}{A_{ctrl}}\ undigested}$$

Where: D_M is methylation dosage ratio, A_x is the area under peak *x* (e.g., L1-2m peak), and A_{ctrl} is the sum peak area of all seven control probes.

REPRESENTATIVE RESULTS:

The main goal of this study was to evaluate the epigenetic effects of OS-EVs on MSCs. OS-EVs were isolated from HOS-143B cells using the standard differential centrifugation method. Expression of the typical EV markers CD63, Hsp70, and TSG101 by western blotting confirmed the presence of OS-EVs. (**Figure 2A**). Absence of calnexin signal indicated purity of the OS-EV isolate. Additional indication of purity was observed with TEM, with intact vesicles of various sizes being present (**Figure 2B**). The average OS-EV particle concentration was 7.63×10^{11}/mL (**Figure 2C**). The size distribution of EVs ranged from 50–500 nm, with around 80% of particles falling within 50–200 nm range (**Figure 2D**).

AT-MSCs were treated with OS-EVs and DNA was extracted from the MSCs at different timepoints. LINE-1 methylation was analyzed by MS-MLPA and calculated in terms of methylation dosage ratio. Results from TP 0 were used to determine the baseline methylation levels (dashed line) (**Figure 3**). At TP 3 (green columns), a decrease in average LINE-1 methylation dosage ratio was observed in MSCs when treated with OS-EVs, falling under baseline methylation levels. This hypomethylating phenomenon was more subtle at TP 7 (blue columns), and the average methylation dosage ratio was slightly higher in both untreated and EV-treated MSCs compared to the baseline.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental workflow. EVs were isolated from HOS-143B cells by differential centrifugation and characterized by western blotting, TEM and NTA. AT-MSCs were treated with OS-EVs at different time points (TP 0, 3, an 7). DNA was extracted from MSCs and LINE-1 methylation was analyzed by MSPA.

Figure 2: Characterization of EVs. (A) The presence of EV markers CD63, Hsp70 and TSG101 confirmed the presence of EVs by western blotting, while no bands were observed for calnexin, indicating the purity of the EV isolate. 10 µg of protein was loaded for both HOS-143B protein lysate and OS-EVs. **(B)** TEM confirmed the presence of intact EVs of various sizes in the isolate. **(C)** Particle concentration and **(D)** size distribution of OS-EVs were determined by NTA measurements.

Figure 3: Methylation dosage ratios of LINE-1 from MSCs either untreated or treated with OS-EVs. The dashed grey line represents baseline methylation value, corresponding to TP 0 values.

Green columns represent average methylation dosage ratios without and with OS-EV treatment for TP 3 samples, while blue columns represent the same for TP 7 samples. Both TP 3 and TP 7 samples showed a decrease in methylation levels after treatment with OS-EVs. However, the difference was greater in TP 3 samples, where the methylation level after EV treatment was also lower than the baseline value.

Table 1: MSPA reagent mix recipes and thermocycler/PCR programs. The volumes mentioned represent one DNA sample. It should be noted that the polymerase mix should be prepared for 2x as many samples as previous mixes, since there are two sets of tubes at this stage. Details of the subsequent thermocycling or PCR program are provided to the right.

DISCUSSION:

This study illustrates how MSPA can be used to detect and quantify the methylation status of a specific genetic element. LINE-1 was the focus here, but the probes can be designed to target a range of genes and sequences. Moreover, there is a growing list of probe mixes available for different applications. MSPA is a simple and robust technique for DNA methylation analysis that does not require bisulfite conversion[10]. The complete procedure from sample preparation to data analysis takes around 2 days but only involves 4–5 h of actual hands-on work. It is applicable for small amounts of DNA (as low as 70 ng), as demonstrated here.

The most important part of the methylation analysis protocol is the preparation of custom probe-mixes, such as the LINE-1 probe-mix in this study. Due to their different lengths, LINE-1 probe oligonucleotides were synthesized in the range of 4–40 nM, so the dissolution step and subsequent dilutions had to be performed carefully, as per the manufacturer's instructions[25]. The PCR and several thermocycling programs of the protocol as used here differ from those in the original manufacturer's version.

There are a few precautions relating to the *Hha*I enzyme. First, the volume of enzyme used in the ligation-digestion mix depends on the manufacturer, and versions of the enzyme that are resistant to heat-inactivation are not suitable for MSPA. With some enzymes, there may be instances of incomplete digestion, which would result in the formation of faulty PCR products and aberrant peak signals. Lastly, the extent to which the final PCR products are diluted for capillary electrophoresis depend on the probes. Initial runs are likely to involve optimizations, for which it is recommended to test a range of dilutions.

There are certain limitations of MSPA, such as the selective nature of *Hha*I-mediated DNA digestion. *Hha*I cleaves DNA only at unmethylated GCGC sequences and disregards other instances of CpG dinucleotides that are not enclosed by a G and C, even if they are located within CpG islands. As a result, the methylation status of such dinucleotides (and those that are located outside the target sequence of the probes) cannot be determined. The LINE-1 probe-mix can include more probes containing additional GCGC sequences from the promoter region[24], thereby representing a greater number of methylation sites.

Alternatively, other methylation-specific restriction enzymes, such as *Sac*II and *Mlu*I, that have a different restriction site than that of *Hha*I can be additionally used in MSPA, which may provide a broader representation of global methylation levels[26]. Secondly, as observed with TP 7 samples, the differences in methylation dosage ratio can be quite subtle. This may be due to the high copy number of LINE-1s in the genome[5], which have high levels of global methylation under normal conditions and will largely be unaffected by transient hypomethylation events in only a few CpG sites. Moreover, the MSCs are heterogenous in terms of the stage of differentiation and of the cell cycle, which can affect the baseline methylation value. Lastly, MSPA can only detect relative differences in DNA methylation and requires reference samples for this reason. In such instances, methods that directly detect local methylation may be more appropriate, though they may require the bisulfite conversion step[27].

Since this study involved the use of extracellular vesicles, we also demonstrated the preparation of EV-depleted FBS, which is an essential component of cell culture media for EV isolation. Ultracentrifugation is an inexpensive process that can effectively separate EVs from the rest of the FBS. However, the 19 h of centrifugation makes it a more time-consuming method than alternatives, such as ultrafiltration and commercial versions[19]. Ultracentrifugation also requires careful handling of the sample, such as when balancing the tubes before centrifugation and collecting the supernatant afterwards. Despite these challenges, it is a popular method for separating EVs from biological fluids.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

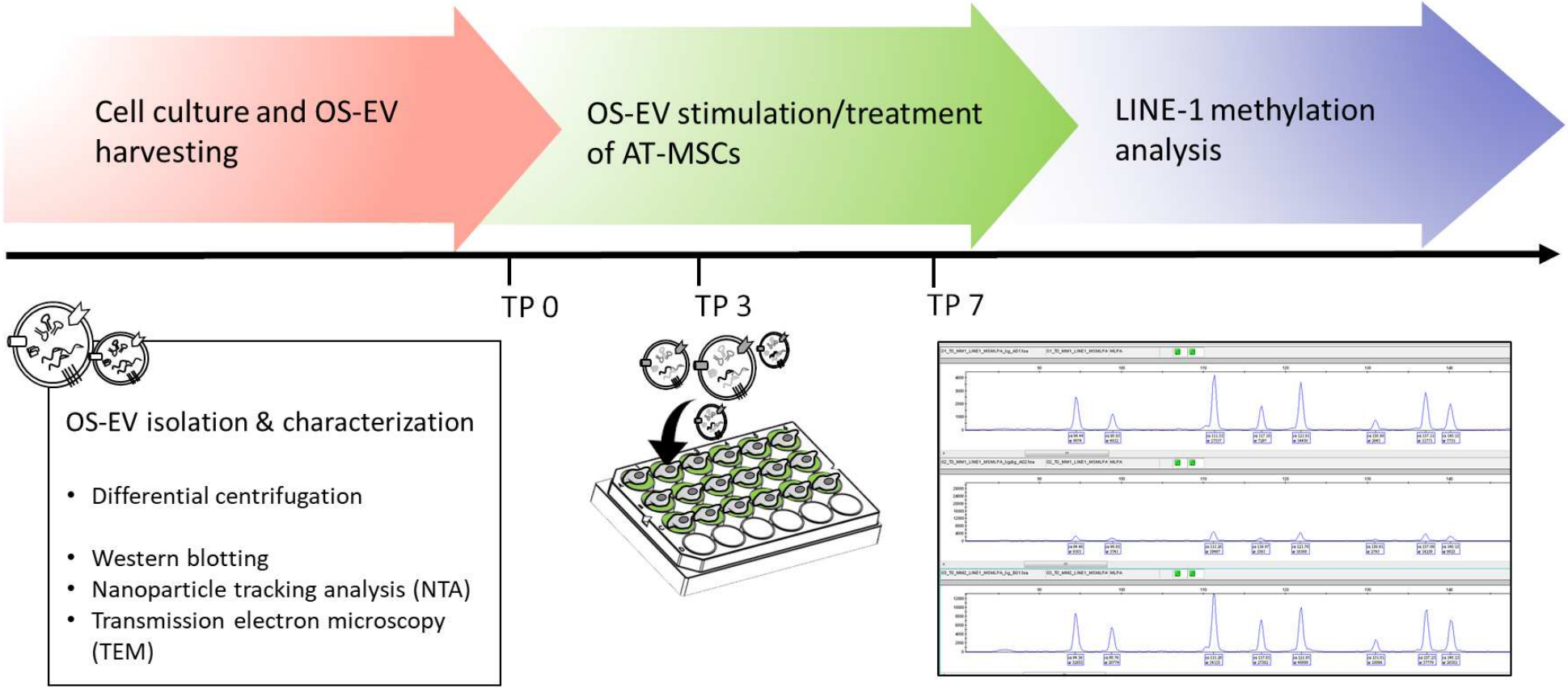


Figure 2

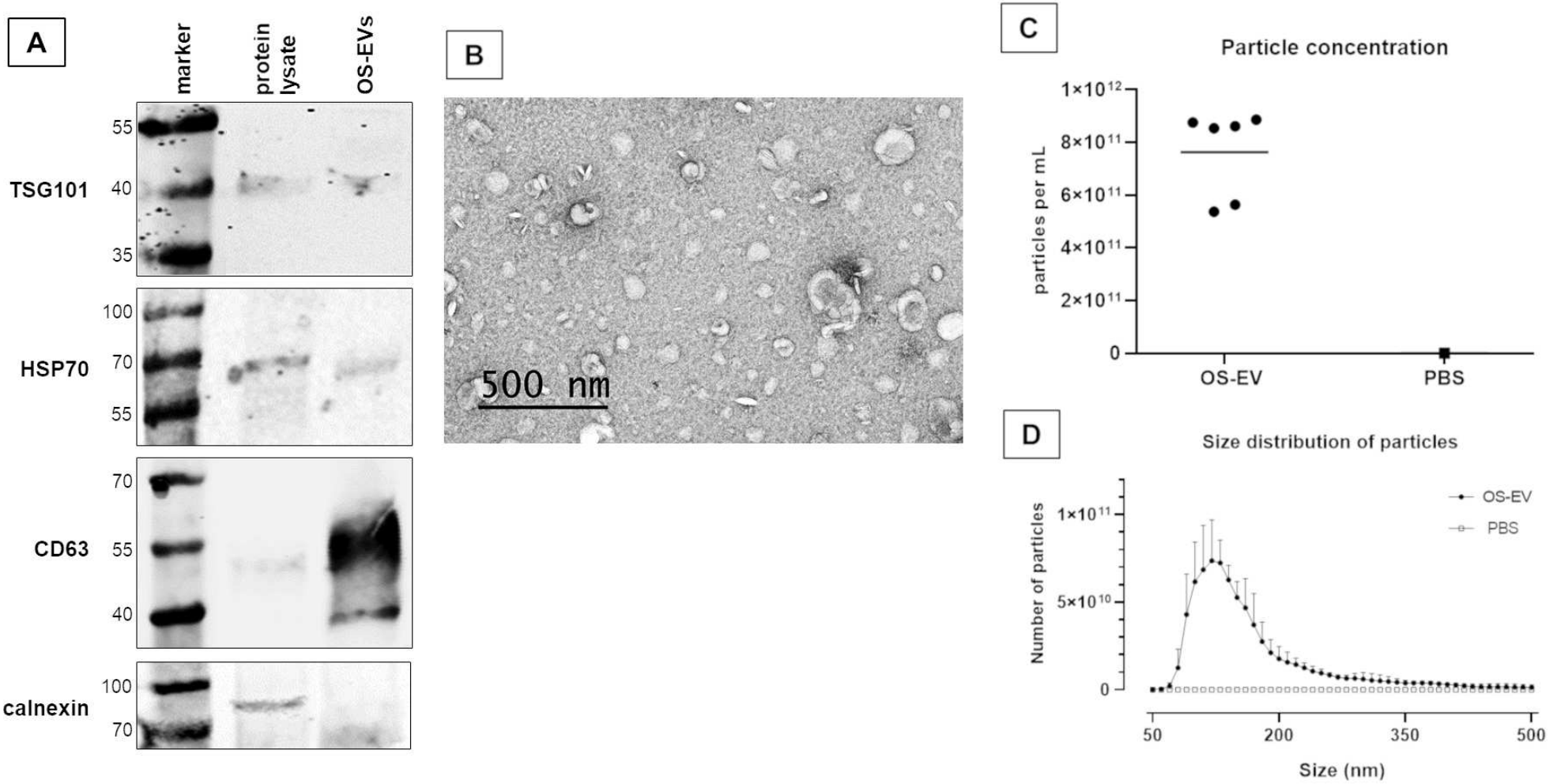


Figure 3

Click here to access/download;Figure;Figure 3.eps

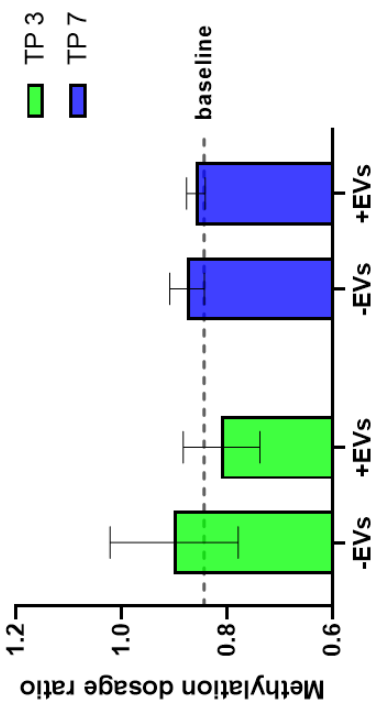


Table 1

MSPA reagent mix recipes				Thermocycler program	
No.	**Mix**	**Reagent**	**Volume (1x, µl)**	**Temperature (°C)**	**Time**
1	Probe hybridization mix	Reference probe-mix (black cap)	1.0	95	60 s
		Custom LINE-1 probe-mix	0.5	60	18 h
		Hybridization buffer (yellow cap)	1.5	8	hold
		Total	**3.0**		
2	Post-hybridization mix	Ligase buffer A (black-ringed cap)	3.0	48	hold
		H_2O	10.0		
		Total	**13.0**		
3	Ligation mix	Ligase buffer B (white cap)	1.50		
		H_2O	8.25		
		Ligase enzyme (green cap)	0.25	48	50 min
		Total	**10.00**	98	5 min
4	Ligation-Digestion mix	Ligase buffer B (white cap)	1.50	20	hold
		H_2O	7.75		
		Ligase enzyme (green cap)	0.25		
		Hha I enzyme	0.50		
		Total	**10.00**		
5	Polymerase mix	PCR primer mix (brown cap)	1.00	72	2 min
		H_2O	3.75	95	30 s ◄ 24 cycles
		Polymerase enzyme (orange cap)	0.25	60	30 s
		Total	**5.00**	72	60 s
				72	20 min
				8	hold

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1 mL syringe	Terumo	SS+01T1	for NTA
24-well plate	Corning	3524	MSC cell culture
3730xl DNA Analyzer	Applied Biosystems, ThermoFisher Scientific	3730XL	
50 mL centrifuge tube	Corning	430829	
Beckman Optima LE-80K Ultracentrifuge	Beckman		
BlueStar Prestained Protein Marker	Nippon Genetics	MWP03	WB: protein marker
Calnexin (clone C5C9)	Cell Signaling Technology	2679	WB, dilution 1:800
CD63 (clone H5C6)	BD Biosciences	556019	WB, dilution 1:1000
Centrifuge 5702 R	Eppendorf	5703000010	For conditioned media and cells
Centrifuge 5810	Eppendorf	5810000010	For spinning down 96-well plate
Centrifuge tube (polyallomer, 14x95 mm)	Beckman	331374	Ultracentrifugation
DMEM/F-12 + GlutaMAX medium	Gibco, Life Technologies	31331-028	For AT-MSC culture
Fetal bovine serum	Gibco, Life Technologies	10270-106	
GeneScan 500 LIZ size standard	Applied Biosystems, Life Technologies	4322682	for capillary electrophoresis
GenomePlex Complete Whole Genome Amplification (WGA) Kit	Sigma	WGA2-10RXN	for MSPA negative control
Hi-Di formamide	Applied Biosystems, Life Technologies	4311320	for capillary electrophoresis
HOS-143B cell line	ATCC	CRL-8303	
Hsp70 (clone 5G10)	BD Biosciences	554243	WB, dilution 1:1000
IRDye 800CW Goat anti-mouse	Li-Cor	926-32210	WB: secondary
IRDye 800CW Goat anti-rabbit	Li-Cor	926-32211	WB: secondary
LINE-1 probe-mix primers	IDT		Sequences in Table 1
MicroAmp Optical 96-well reaction plate with barcode	Applied Biosystems, Life Technologies	4306737	also requires sealing film
Micro BCA Protein Assay kit	ThermoFisher Scientific	23235	measure protein concentration

MiniProtean TGX 10% gels	Bio-Rad	456-1034	WB: gel electrophoresis
NanoSight LM14C	Malvern Instruments		for NTA
Nitrocellulose membrane 0.2 µm	Bio-Rad	1620112	WB: protein transfer
NucleoSpin Tissue XS	Macherey-Nagel	740901.50	for DNA extraction
Odyssey Blocking Buffer	Li-Cor	927-40000	WB: blocking, antibodies
PBS, 1X	Corning	21-040-CVR	
Penicillin-streptomycin	Gibco, Life Technologies	DE17-602E	Antibiotics for culture media
Protein LoBind tube, 0.5 mL	Eppendorf	22431064	For storing Evs
REVERT Total Protein Stain and Wash Solution Kit	Li-Cor	926-11015	WB: total protein staining
RKO cell line	ATCC	CRL-2577	for MSPA positive control
RPMI medium 1640 + GlutaMAX	Gibco, Life Technologies	61870-010	For HOS-143B cell culture
SALSA MLPA HhaI enzyme	MRC-Holland	SMR50	
SALSA MLPA reagent kit	MRC-Holland	EK1-FAM	
SALSA MLPA P300 probe-mix	MRC-Holland	P300-100R	
Swinging rotor SW-28	Beckman Coulter	342207	Ultracentrifugation
Syringe filter, 0.22 µm	Jet Biofil	FPE-204-030	sterile filtering FBS
Tecnai 12	FEI Company		equipped with Gatan Orius SC 1000B CCD-camera (Gatan Inc., USA); for TEM
TBS, 1X tablets	Medicago	09-7500-100	WB: buffer
Trans-Blot Turbo	Bio-Rad		WB: transfer
Thermal cycler	ThermoFisher Scientific	TCA0096	
TrypLE Express	Gibco	12604-021	for trypsinization of cells
TSG101 (clone 4A10)	Sigma	SAB2702167	WB, dilution 1:500

JoVE 60705R1 : Editor and peer review comments – responses from the authors

Dear Editor and Reviewers,

Thank you for your insightful comments and suggestions. There were some oversights on our behalf in the earlier draft of our manuscript, especially with regard to commercial terms. With your feedback we have now improved the quality of our revised draft. Please find our replies in bold to each of your comments below. I hope the revision addresses your concerns adequately.

Sincerely,

The Authors

Editorial and production comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

   We have taken care to revise and proofread our manuscript thoroughly. We vouch for its linguistic accuracy to the best of our abilities.

2. Please expand all abbreviation during the first-time use.

   All abbreviations have now been expanded at the first instance of their use.

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, please remove MS-MLPA (MLPA is copyrighted term), MRC Holland throughout the manuscript. Also reword the line 81-82 to reflect the science instead.

   We had overlooked some of the commercial terms earlier. References to MLPA, MS-MLPA and MRC Holland have now been removed from the manuscript. Lines 81-82 have been modified and the method is being referred to as MSPA in the manuscript.

4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: MLPA, MRC Holland, GlutaMAX, Nanosight LM14C, FEI Tecnai 12, TrypLE Express, NucleoSpin tissue XS kit 221 from Macherey-Nagel, GeneScan LIZ-500, Hi-Di formamide, ABI 260 MicroAmp Optical, Milli-Q water, Centrifuge 5810, Eppendorf, Protein LoBind Tube, ABI well plate, etc ABI 3730xl DNA analyzer, G5 dye set, Gene Mapper, MLPA, etc.

   All instances of commercial language have now been removed from the manuscript and the video.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

The text in the protocol section has been corrected to be in the imperative tense throughout. Notes have been mentioned only where necessary.

6. The Protocol should contain only action items that direct the reader to do something.

   The text in the Protocol section has been modified accordingly to contain only actionable items.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

   The Protocol text has been edited to contain a maximum of three actions per step.

8. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

   The ethics statement was provided earlier as well but now it has been titled so that it is easier to recognise.

9. Please ensure you answer the "how" question, i.e., how is the step performed?

   We have made sure to describe how the steps are being performed. In other cases we have provided references to previous work.

10. 1.5: This is not an action step and can be converted to a note instead.

    The step has been converted to an action step.

11. 3.1: Please include citations for EV markers studied.

    Citations for the EV markers have now been added.

12. 3.2, 3.3: Please include how is this done. Please include the actions associated with it.

    The actions for NTA have been added to a more detailed extent. For TEM we have provided a reference article.

13. 4.4: Please include citations for the markers used.

    The MSC characterization step has been removed after considering Reviewer #1's comments. Hence the markers are not mentioned.

14. 6.5.1: Please include the thermocycler program used for the assay.

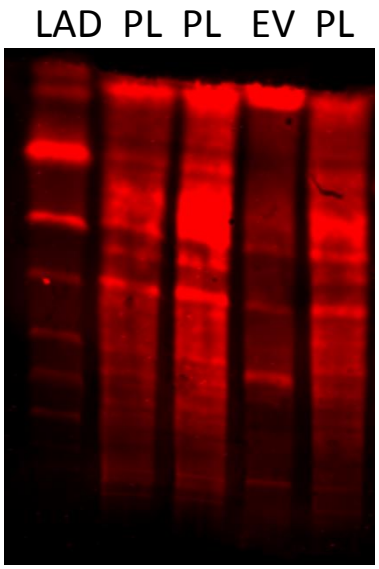
    The entire thermocycler program has been detailed in Table 2 now.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

    Explicit copyright permission was obtained from corresponding with copyright department of the journals. A copy of the correspondence has been attached to this document.

16. Figure 2: Please include a protein loading control for panel A.

The Western blot in Fig. 2A has been repeated with equal amounts of protein (10 µg) loaded for HOS-143B protein lysate and OS-EVs. Protein loading controls have been tested in Western blotting. Please see one of results are below:



Legend
LAD = protein marker
PL = HOS-143B protein lysate (10 µg)
EV = OS-EV (10 µg)

Protein concentrations were measured with Pierce microBCA assay. After protein transfer, membrane was stained with Li-Cor's REVERT Total protein stain, washed, and fluorescence measured at 700 nm. Despite loading equal amounts of protein in all lanes, protein lysates had higher protein content than the EV sample. This may be due to the Micro BCA assay not accurately reporting the protein concentrations. Despite having less total protein, EVs had a stronger CD63 signal than PLs.

17. Table 1 and 2: Please ensure that the tables are not reprinted from MRC-Holland assay. If yes, then please obtain a reprint permission. Please also remove all commercial terms from Table 2. E.g., SALSA, etc.

    The tables are not direct re-prints of the MRC-Holland assay. Table 1 is adapted from Pavicic et al., 2012, while Table 2 is based on personal correspondence with Walter Pavicic, who modified the MRC-Holland assay. Commercial terms have been removed from Table 2. Copyright permission details have been attached to this submission.

Video:

1. Please ensure that protocol section titles are same in the video and in the text.

   The protocol section titles are now matching in the video and in the text.

2. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text.

   The narration has been modified to be more similar to the written protocol text.

3. Please include an ethics statement before AT-MSC culture section.

   The ethics statement has been provided at the beginning of the protocol section, to match how it has been done in other videos published in JoVE. The AT-MSC culture section in the video does not include information on how the MSCs are isolated from the lipoaspirates.

4. Please remove all the commercial language from the narration in the video as well. Please see the list above. MLPA is a copyrighted term as well.

All commercial language has now been removed from the narration and video components.

5. Please include all the results figures together in the representative result section of the video. Please ensure that all the figures from the text are present in the video as well.

   All results are now shown in the Representative Results section.

Production QC notes 9/27/19:

- 4:09, 6:58 - Jump cuts (https://en.wikipedia.org/wiki/Jump_cut), like the one here, where the angle is not changed between shots, should be avoided. Jump cuts tend to have a jarring effect on the audience, and don't fit with our visual style. Please use cross dissolves (aka fades) to cover those edits.

  The instances of jump cuts have been fixed. Cross dissolves have been used to cover some edits in the Conclusion section, hopefully it is up to the standards.

- 6:11, 6:14, 7:57, 7:59, 8:01 - These images should be scaled up to fill the frame.

  These images have either been removed or scaled up to fill the screen.

- 7:13-7:24 - HiDi Formamide, GeneScan, Liz 500 are brand names. These are mentioned in the narration and seen on screen here. Based on the visual, these are brand names. The narration should be changed to more generic terms. The whole sentence should be rerecorded.

  References to commercial names have been removed and replaced with generic terms in the narration.

- 7:30-7:33 - ABI MicroAmp optical 96-well plate is mentioned here. This should probably be replaced with a generic term.

  The product name has been changed to a generic term ("optical 96-well plate").

Reviewers' comments

Reviewer #1

Manuscript Summary:

Authors described clearly and in detailed manner the proposed protocol.

Major Concerns:

none

Minor Concerns:

1) As far as EV isolation technique is concerned, authors applied only two centrifugation steps (2500 g for 20 min and 100000 g for 2h). However, the majority of papers related to EV isolation from cells supernatants reported more centrifugation steps:

* 300-400 × g for 10 min to sediment a main portion of the cells
* 2000 × g to remove cell debris
* 10,000 × g to remove apoptotic bodies, and the other structures with the buoyant density higher than that of EVs.

Please, discussed or explained the choice.

While it is true that many papers on EV studies report multiple centrifugation steps in order to remove not only cells and cell debris but also apoptotic bodies, we did not want to discount large oncosomes from our EV samples. With similar size as apoptotic bodies, these large oncosomes are functionally active vesicles and are likely to have an impact on target cells.

2) Authors explain clearly the amount of EVs used to treat MSC, but they did not explain if EVs have been sterilized and how.

EVs have not been sterilized in this study. may be filter-sterilized but that may incur loss of EVs if they stick to the membrane. We are yet to test the possibility of using specialized membranes, e.g. cellulose acetate (CA) membranes, as low attachment membranes to minimise EV loss during filter-sterilization.

3) The characterization of MSC phenotype could be deleted as it is not explained in details and it is not crucial for the protocol described in the paper.

We agreed that the MSC characterization aspect is not essential for this paper and have removed it.

4) Results: Fig 2A It could be more appropriated to use HOS lysate and EV lysate, instead of MSC lysate and OS-derived EVs lysate. To assess the purity of EVs, EVs and they cells of origin need to be compared.

This was an oversight. We have now included new Western blot results for expression of same markers in HOS-143B protein lysate and HOS-EVs. Protein loading controls were also used and results of total protein staining can be found towards the end of this document.

Reviewer #2

Manuscript Summary:

MS-MLPA is a useful tool for reporting the CpG methylation status of a given locus that utilizes custom probes specific to that locus. The probes containing the CpG to be tested are first hybridized to the sample DNA to be tested. After the hybridization of these probes, the sample is divided into two reactions- one that is treated with a methylation-sensitive restriction enzyme HhaI followed by ligation and the other that is not digested but only ligated. After ligation, the probe:DNA hybrid is used as a template in PCR that follows. The HhaI restriction enzyme that cleaves the unmethylated CpGs in the probe-DNA hybrid at GCGC restriction sites (present in the probe) eliminates the hypomethylated population. By comparing the PCR products from digested and undigested samples using capillary electrophoresis, the authors infer the methylation changes. The authors report these methylation changes as "methylation-dosage ratio" which is the ratio of normalized peak-area obtained after capillary electrophoresis of HhaI digested PCR products to that of its undigested counterpart. In an ideal condition, if the locus assayed is completely methylated this ratio should be 1 and if the locus is hypomethylated the methylation dosage ratio should be less than 1. The authors utilize this method to assay the methylation status of CpGs in LINE-1 retrotransposon promoters which is highly methylated in normal cells. Additionally, the authors also demonstrate the preparation of extracellular vesicles depleted media and isolation of extracellular vesicles from osteosarcoma cells and its

characterization. They have presented all the key steps of these methods in a clear and easy to follow video and text protocol. This can be helpful for the audience planning to do similar experiments in their laboratory.

Major Concerns:

1) As the authors have highlighted this is a rather simple method in comparison to bisulfite conversion that is essential for most of the methods used to study DNA methylation. However, this method in general, and when used to study the DNA methylation status of LINE-1 promoters, has some pitfalls that the authors have pointed out. It would be helpful for the readers if the authors discuss possible solutions that could help the audience and hence the future adopters of this method to design their experiment.

a) First is a technical limitation of assaying CpGs that are in an HhaI restriction site. One possible solution can be to select other methylation-specific restriction enzymes that involve CpG but have a different restriction site and digest the probe:DNA hybrid with it. This might be helpful if a potential user wants to investigate other CpG in the promoter that is not necessarily in an HhaI restriction site.

This is a good suggestion, thank you. We have mentioned some alternative restriction enzymes that could be used.

b) Second is inherent to LINE-1's abundance in the human genome; there are ~6k identical LINE-1 promoters just considering younger retrotransposition competent LINE-1s (Deininger et al., https://www.ncbi.nlm.nih.gov/pubmed/27899577). As Phokaew et al. (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2553567/) point out there can be locus-specific differences in the level of LINE-1 promoter methylation. Perhaps this is the reason why the authors observe a modest LINE-1 hypomethylation effect of OS EVs on MSCs at T7 time-point. Although this method might be appropriate to study global hypomethylation observed in cancers, it might not be sensitive enough to detect these local LINE-1 methylation changes. In that case, instead, assays quantifying unmethylated CpGs on LINE-1 directly as done by Pisanic et al. (https://www.ncbi.nlm.nih.gov/pubmed/30553834) can be a better approach.

Thank you for bringing up these points and for the suggestion. We agree on their validity, and have addressed them in our discussion.

2) Different dilutions for the PCR product of undigested and digested samples (to 1:100 and 1:200 respectively) in step 6.8 is confusing given that same probes and PCR reagents were applied to both of them.

This is a fair question. Dilutions of undigested and digested samples usually require optimization in order to generate well-resolved peaks for analysis in the software. The same dilutions should work for other users but there may yet be a need for optimization due to some inter-laboratory differences.

Minor Concerns:

1) Although the MS-MLPA method is nicely described in the fourth paragraph of the introduction, readers might benefit from a figure illustrating the method.

Thank you for the suggestion. We think that adequate illustrations of the method can already be found in publications (e.g. reference no. 10 in our article).

2) Is "Microsatellite default" a setting that is present on Gene Mapper (v.5.0)? If yes what could be a general analysis method if another software is to be used to analyze the results obtained?

"Microsatellite default" is indeed present on GeneMapper® (v.5.0). In other similar software, such as Geneious® and GeneMarker®, microsatellite analysis settings are pre-installed or can be installed as a plugin.

Reviewer #3

Manuscript Summary:

This manuscript and the accompanying video provide an in depth description of a technique that allows to study the epigenetic effects of osteosarcoma-derived extracellular vesicles on Mesenchymal Stem cells. In particular, authors provided a detailed description of the whole process to obtain EV-depleted FBS and to collect EVs from conditioned media, as well as the procedure of MS-MLPA technique. As mentioned by the authors, both methods are not novel in itself, as they are commonly used. Yet, a video-supported description of the protocols is not present in literature.

Major Concerns:

- Line 147: authors declare that once reached the 70-80% of confluence, they change medium and let the cells grown in the EV-depleted media for 72 hours. Given the very high rate of 143B cell proliferation and their detachment at over-confluence, I have some concern about the viability of these cells following further 72 hours of culturing. Authors should clarify the confluence status of cells when they collect EVs, by providing the amount of plated cells at the beginning of the procedure and the time needed to reach the desired confluence, or they should provide some pictures of the cells at the time-point 0 (the declared 70-80% of confluence) and at the end of the 72h treatment.

We should have worded the protocol more clearly. The EV-depleted media is replaced once every 24 hours to provide adequate nourishment to the rapidly growing cell population. Upon reflection, we have reduced the cell harvesting time to 48 hours as we realized that the cells are actually already 100% confluent by that time. Nevertheless, we have also provided a note in the protocol that plating 1 million HOS-143B cells gives rise to a 70% confluent flask in around 60 h.

-In the introduction section, authors should better described EV isolation methods other than ultracentrifuge, as too quickly mentioned in the discussion.

We have mentioned two other sources of EV-depleted FBS in the introduction. We are not focusing on EV isolation in this article, except for a part in the protocol. Hence, we thought that describing additional EV isolation methods is not so necessary in this publication.

Minor Concerns:

-In their experiments, authors used HOS-143B cells (as declared at line 143), a very aggressive and metastatic osteosarcoma cell line. In the whole manuscript they call the cells just "HOS". This is a great mistake, since HOS are the parental cells and they are very different from HOS-143B cells. I strongly suggest to call them HOS-143B or just 143B (commonly used).

We acknowledge our error in misrepresenting the cell line. We have corrected this and now the proper name (HOS-143B) is mentioned throughout the manuscript, while it is to be understood that OS-EVs are derived from HOS-143B cells and not HOS cells.

-Line 136/Video at 01:51: although the EV-depleted FBS is eventually passed through a 0.22um filter, the whole procedure should be done in a laminar hood, including the steps 1.1 and 1.3.

The balancing step is challenging to perform inside a hood, since the balance requires a sturdy, anti-vibration surface (e.g. marble tables) for milligram-level measurements and this is not available inside a laminar hood. Therefore, this step has to be carried out outside. We accept that the other steps should have been carried out in the laminar hood, and we have removed the specific mention of laminar hood in point 1.4 so that the reader does not think the actions should be carried out outside the hood up until that point.

-Line 130/Video at 03.06: the preparation and the EV collection from the conditioned media should be done in a laminar hood.

You are right, but please see our answer to your previous comment. The balancing step is necessary here too.

-Video at 03.38: authors should use tweezers to take the ultracentrifuge tube from the rotor buckets.

Tweezers are surely more appropriate for handling the tubes. Since the tubes are not filled to the brim but at a comfortable height away from the top, we felt that clean gloved fingers could be used in a careful way. Moreover, some people may feel more secure in holding the tube with their fingers than with tweezers, especially if the tubes are to be handled carefully with minimal disturbance to their contents.

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Author(s): Snehadri Sinha, Bettina Mannerström, Riitta Seppänen-Kaijansinkko, Sippy Kaur

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