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Bovine mRNAs in small extracellular vesicles from cow's milk are not bioavailable in mice and translation products are not detectable in reticulocyte lysates and human U937 cells

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Abstract: Aim: Small extracellular vesicles from bovine milk (BEVs) have garnered attention as vehicles for delivering therapeutics to pathological tissues. Theoretically, mRNAs in BEVs might be translated into proteins, thereby eliciting immune responses in patients. The objectives of this study were to provide a comprehensive analysis of mRNAs in BEVs, assess mRNA bioavailability, and determine whether mRNAs are translated into proteins. **Methods:** BEVs were purified from raw cow's milk (RM) by ultracentrifugation and treated with RNase to remove mRNA adsorbed to BEVs. BEVs were also isolated from store-bought milk (SBM) and analyzed with (SBM+) and without (SBM–) RNase treatment. mRNAs were analyzed using the Illumina HiSeq2500 platform. Bioavailability was assessed by administering BEVs loaded with IRDye-labeled bovine Casein Kappa (CSN3) mRNA by oral gavage in Balb/c mice. Translation was assessed using a rabbit reticulocyte lysate system (using RNA purified from BEVs) and human U937 monocytes (cultured with BEVs). **Results:** We identified transcripts of 4,858, 2,680, and 4,554 genes in BEVs from RM, SBM+, and SBM–, respectively. IRDye-labeled CSN3 mRNA, encapsulated in BEVs and delivered by oral gavage, was not detectable in murine tissues. Upon incubation with RNA and BEVs, bovine proteins were not detected in rabbit reticulocyte lysates and U937 monocytes, respectively. **Conclusion:** We conclude that bovine CSN3 mRNA, encapsulated in BEVs and delivered by oral gavage, is not bioavailable in mice, and translation products of RNA are not detectable in reticulocyte lysates and human U937 cells.

Keywords: drug delivery; extracellular vesicle; milk; mRNA; translation

1. Introduction

Small extracellular vesicles (sEVs) facilitate cell-to-cell communication by transferring lipids, proteins and various biotypes of RNA from donor to adjacent or distant recipient cells and by binding to receptors on the outer surface of recipient cells [1–4]. sEVs are being explored as vehicles for delivering therapeutics to pathological tissues because they can cross tissue barriers and are internalized by cells through endocytosis [5–7]. There is precedent for the great potential of delivering therapeutics by sEVs. For example, self-derived dendritic cells were genetically engineered to express a brain-targeting peptide derived from rabies virus glycoprotein in sEVs [8]. When the sEVs were transfected with GAPDH small interfering RNA (siRNA) and administered to mice by intravenous injection, the siRNA accumulated primarily in neurons, microglia, oligodendrocytes, and their precursors and knocked down GAPDH expression. Independent laboratories successfully applied the technology to knock down a variety of targets including allele-specific knockdown [9–11]. When sEVs were loaded with $KRAS^{G12D}$ siRNA, tumor growth decreased by more than 60% compared to controls receiving scrambled RNA and survival doubled from 250 days to 500 days after tumor induction in mouse models of pancreatic cancer [11].

More recently, researchers began to recognize the potential merit of using bovine milk sEVs (BEVs) for delivering therapeutics to pathological tissues. BEVs offer the following advantages compared to cell culture-derived sEVs in the delivery of therapeutics to pathological tissues. First, the production of BEVs is a scalable and cost-efficient process. One milliliter of milk contains 1.4×10^{14} BEVs and a cow produces approximately 10,800 kg of milk per season for an annual yield of 10^{21} BEVs [12,13]. Second, encapsulation of RNAs in BEVs confers resistance to degradation by low pH and RNase, i.e., conditions encountered in the gastrointestinal tract [14]. Third, BEVs and murine milk sEVs and their microRNA cargo are bioavailable following oral administration and cross barriers such as intestinal mucosa, vascular endothelia, placenta and blood-brain barrier [15–20]. The oral bioavailability of BEVs is approximately 50% in mice [21]. Fourth, BEVs and other sEVs have been used successfully to deliver paclitaxel to human lung tumor xenografts in nude mice as well as deliver $KRAS^{G12S}$ siRNA to human lung cancer A549 cells [22,23]. Fifth, BEV administration did not impair liver and kidney function and erythropoiesis; physical activity, and food and water intake in rats and mice [24,25].

The immunotoxic potential of drugs and biologics needs to be assessed before a drug can be considered for use in humans [26]. Previous studies suggest that BEVs contain mRNAs [12,14]. Informed by these studies we conducted a comprehensive analysis of the mRNA content in BEVs, and tested the hypothesis that the mRNA cargo in BEVs is bioavailable in mice and translated into proteins in rabbit reticulocyte lysates in vitro and human U937 monocyte cultures upon differentiation into macrophages. The translation of mRNA in sEVs into proteins is controversial [2,27]. Theoretically, the translation of bovine mRNAs in non-bovine species might elicit an immune response in the host and raise safety concerns regarding the use of BEVs (and sEVs) in patients [28].

2. Materials and methods

2.1. Animals

Male and female Balb/c mice (Jackson Laboratory, stock number 000651) age 8 weeks were used in this study. All procedures involving animals were conducted in accordance with the University of Nebraska-Lincoln's Institutional Animal Care and Use Committee and approved by the Institutional Animal Care Program (September 28, 2021, protocol 2152).

2.2. BEV isolation

BEVs were isolated from raw milk (RM, *Bos taurus*) and from pasteurized (71.6 ℃ for 15 seconds) fat-free milk from a local grocery store (store-bought milk, SBM). The RM was obtained from the dairy herd in the Department of Animal Science, University of Nebraska-Lincoln. We minimized bacterial contamination and growth in RM by adding gentamicin (0.25 μg/mL final concentration) and ceftazidime (3 μg/mL) during milking [29]. RM was collected and transported on ice and BEV isolation was initiated within less than 30 minutes of milking. BEVs were isolated by differential ultracentrifugation as previously described using a F37L-8x100 rotor with minor modifications [16]. The final BEV pellet was suspended in phosphate-buffered saline and diluted as appropriate in downstream analyses. Experimental details have been deposited in the EV-Track database and can be accessed through ID EV210119.

2.3. RNase treatment of BEVs

RNA on the BEV surface was removed by sequential treatment with proteinase and RNase. BEVs (10 mg protein) were treated with 100 μg proteinase K in a volume of 1 mL at 37 ℃ for 30 minutes when proteinase K was heat-inactivated (90 ℃ for 5 minutes). Samples were incubated with 10 units of RNase ONE (Promega) in RNase buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, and 1 mM EDTA) at 37 ℃ for 10 min. Prior to isolating RNA from BEVs, RNase was inactivated by treating samples with dithiothreitol [30].

2.4. RNA sequencing analysis (RNAseq)

RNA was extracted from RM treated with RNase, and from SBM with (SBM+) and without (SBM-) RNase treatment by using the miRNeasy plasma/serum kit (Qiagen Inc., Germantown, MD, USA) following the manufacturer's recommendations. Three independent biological replicates were analyzed. cDNA libraries were prepared by using Truseq Stranded mRNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) and sequenced at the University of Minnesota Genomics Center by using a 50 base-pair paired-end protocol. RNAseq data quality was assessed by using FastQC 0.11 [31]. Adaptor sequences and reads with quality score of <25 were removed by using TrimGalore 0.4 [32]. The remaining high-quality reads were aligned to bovine reference genome ARS-UCD1.2 using STAR 2.7, and expression was quantified by RSEM 1.3 [33–35]. Mapped reads were extracted by using

SAMtools 1.3 [36]. Reads containing a Kozak sequence (5'-GCCGCCACCATGG-3' or 5'-GCCGCCGCCATGG-3') were identified by grep function in Linux and interpreted by BLAST. The raw RNA-seq data were deposited in the BioProject database (accession numbers ID PRJNA715225 and PRJNA715226).

2.5. mRNA bioavailability and distribution

CSN3 mRNA was among the three most abundant mRNAs in all samples and used to assess bioavailability and tissue distribution of bovine mRNA in mice. We loaded BEVs from SBM- with a synthetic IRDye-labeled fragment of CSN3 mRNA (5'IRDye® 800 CW-AUU UAU GGC CAU UCC ACC AA-3'; IDTDNA, Inc., Coralville, IA) using calcium chloride and ethanol as previously described [18]. CSN3-loaded BEVs were administered to mice by oral gavage (1×10^{12} BEVs per g body weight) and tissues were harvested 12 hours after administration; controls received unloaded BEVs. Doses and time of sample collection were informed by previous studies [21]. While the dose exceeds the amount of BEVs ingested by an adult consuming 0.25 liter milk, it is well below the 176 trillion human milk sEVs ingested per day by an infant consuming 800 mL milk [37,38]. Stability of IRDye-labeled RNA was demonstrated in previous studies [18]. Tissue fluorescence was measured using the 800-nm channel of an Odyssey Clx system (LI-COR Biosciences Inc. Lincoln, NE, USA) and quantified using Image J.

2.6. Translation of mRNA

Translation of BEV mRNA (10 μg) was assessed using a reticulocyte lysate protocol following the manufacturer's recommendations (FluoroTect™ GreenLys; Promega Corp.), and in human U937 monocyte cultures. We used mRNA encoding luciferase as a control in the reticulocyte protocol. U937 monocytes were differentiated into macrophages by incubation with 10 nM phorbol 12-myristate 13-acetate for 48 hours [39]. Macrophages were used as cell model because of their high capacity for internalizing BEVs [40]. Briefly, human U937 monocytes (American Type Culture collection CRL-1593.2) were cultured in RPMI-1640 containing 100 international units penicillin and 100 mg streptomycin per liter; media were supplemented with 10% (by volume) sEV-depleted (120,000 g, 4 ℃, overnight) human serum [41]. BEVs in phosphate-buffered saline were added to culture media (2,000 μg BEV protein per 7×10^6 cells) at time of seeding the cells or timed intervals after seeding, Controls were treated with vehicle. Cells were cultured for 12 hours and harvested by centrifugation at 12,000 g for 8 minutes. Protein was extracted from cells by using radioimmunoprecipitation buffer containing a protease inhibitor cocktail (cat. no. P8340-1ML; Sigma-Aldrich). Total protein was quantified by using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) and stored in –20 ℃ freezer until analysis.

Proteins from reticulocyte lysate assays and U937 cells were precipitated using four volumes of acetone at –20 ℃ for 60 minutes and collected by centrifugation at 12,000 g (4 ℃, 10 minutes) for analysis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [42]. In the first dimension, samples were run in 7 cm strips of premade gel containing immobilized

ampholytes with a pH range from 3 to 10. The second dimension of the electrophoresis was conducted using 15% (w/v) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Gels were fixed in 10% acetic acid and 40% ethanol (v/v) at room temperature for 60 minutes. BODIPY fluorescence is reticulocyte assays was measured using a Typhoon FLA 7000 scanner and Alexa Fluor™ 488 filter. Proteins in U937 cell extracts were visualized by using Coomassie Blue in a Gel Doc™ EZ Gel Documentation System (Bio-Rad Inc., Hercules, CA, USA). Progenesis SameSpots 4.5 (La Jolla, CA, USA) was used to identify spots unique to samples treated with BEVs compared to BME-free samples. Cut-out pieces of apparently unique spots were analyzed using tandem liquid chromatography mass spectrometry analysis as previously described [12].

2.7. Statistical analysis

Homogeneity of variances was assessed by Levene's test. The Shapiro–Wilk test was used to assess normality of distribution. IRDye fluorescence intensity data was analyzed by Student's t-test when comparing treatment group to control group. All statistical analyses were conducted using R version 4.2 (The R Project for Statistical Computing). Data are presented as mean ± SD. Differences were considered significant if $P < 0.05$.

3. Results

3.1. mRNA expression in BEVs

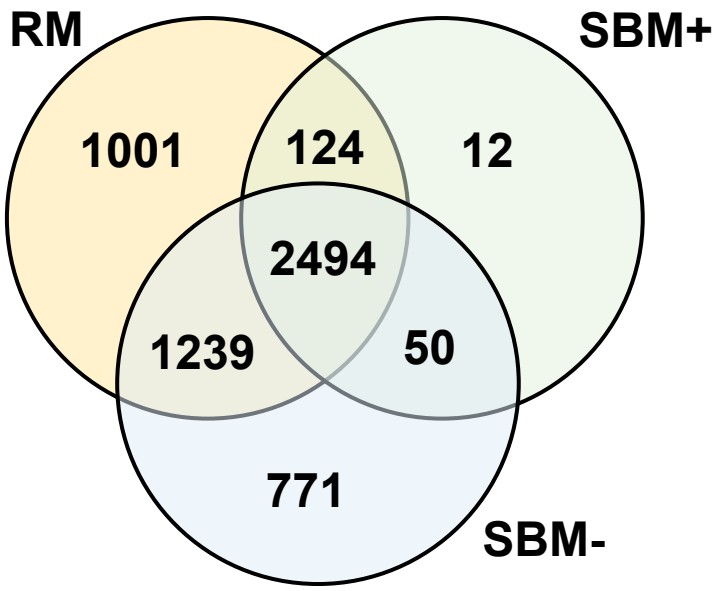

Figure 1. Venn diagram of mRNAs in BEVs from RM, SBM+, and SBM– (n = 3).

We identified mRNAs from 4858, 2680, and 4554 genes in BEVs from RM, SBM+, and SBM–, respectively (Supplementary Table S1). Trace amounts of ribosomal RNA were detected in all samples, and their abundance was slightly higher in SBM– (0.01% of total reads) compared to RM (0.0002%) and SBM+ (0.0005%). Two thousand four hundred ninety-four

mRNAs were detected in all biological replicates in all three sources of BEVs (Figure 1). The 10 most abundant mRNAs accounted for 63 ±6.2%, 44 ±12%, and 55 ±8.0% of all transcripts in RM, SBM+, and SBM– BEVs, respectively (Figure 2), and were largely identical in the three input materials (Supplementary Table S1). Twenty, 13, and 6 mRNAs contained a Kozak sequence in BEVs from RM, SBM+, and SBM–, and therefore are prime candidates for translation (Supplementary Table S2). That said, most mRNAs that contained a Kozak sequence were rare and ranked in the bottom 2000 of transcripts detected. The only exception was the *GNAS* complex locus which ranked among the top 100 most abundant transcripts [43].

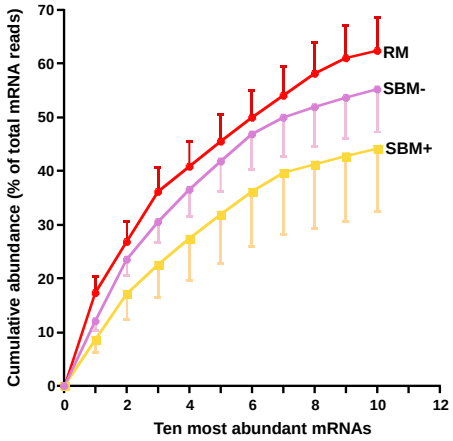

Figure 2. Cumulative abundance of transcripts from the ten most abundant mRNAs in BEVs from RM, SBM+, and SBM– relative to the total reads in input materials. Values are means ±SD, *n* = 3.

3.2. mRNA bioavailability and distribution

IRDye-labeled CSN3 mRNA, encapsulated in BEVs and delivered by oral gavage, did not increase tissue fluorescence compared to controls receiving unloaded BEVs (Figure 3, Table 1).

Table 1. Densitometry analysis of tissue fluorescence in mice receiving BEVs loaded with IRDye-labeled CSN3 mRNA and controls receiving unloaded BEVs.

Tissue BEVs	Loaded BEVs	Unloaded
Liver	84 ±107	98 ±9.6*
Heart	17 ±9.4	18 ±2.7
Spleen	14 ±5.8	14 ±1.2
Kidneys	25 ±2.33	23 ±10
Lungs	41 ±55	42 ±17
Gut	7.0 ±12.9	7.1 ±3.3

* $P < 0.05$ compared to Loaded BEVs, $n = 3$.

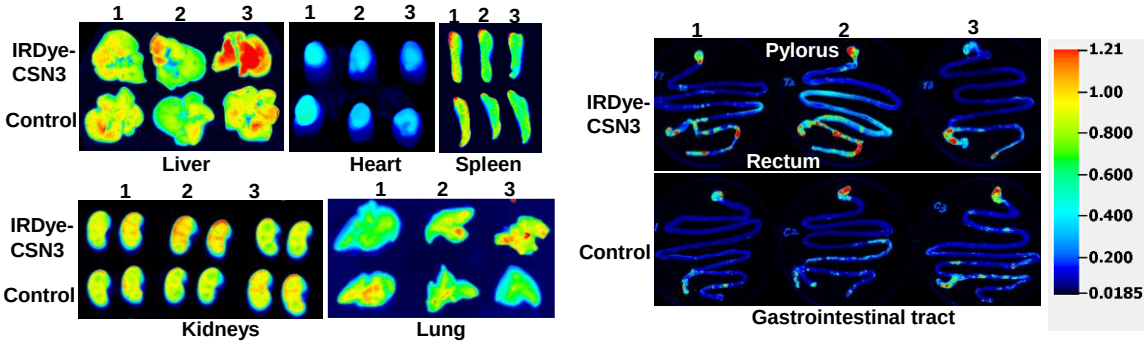

Figure 3. Tissue distribution of IRDye-labeled Casein Kappa (CSN3) mRNA, encapsulated in bovine milk small extracellular vesicles and delivered by oral gavage. Numerals denote independent replicates (mice, $n = 3$).

3.3. Translation of BME mRNA cargo

Translation of mRNA in bovine milk sEVs was below the limit of detection using a rabbit reticulocyte lysate protocol (Figure 4), including the analysis by tandem liquid chromatography mass spectrometry. The functionality of the system was demonstrated by using luciferase control RNA as substrate (Supplementary Figure S1). Likewise, no unique proteins were detected in BEV-supplemented cultures of U937 cells compared to BEV-free control cultures by using 2-D gel electrophoresis (Figure 5). For example, we quantified the data from two of the same spots (marked in Figure 5) using densitometry analysis. Values (arbitrary units) were 16.4 (2 hours), 15.5 (5 hours), 15.7 (12 hours), and 16.8 (vehicle) for spot 1, and 13.2 (2 hours), 13.7 (5 hours), 15.7 (12 hours), and 14.5 (vehicle) for spot 2.

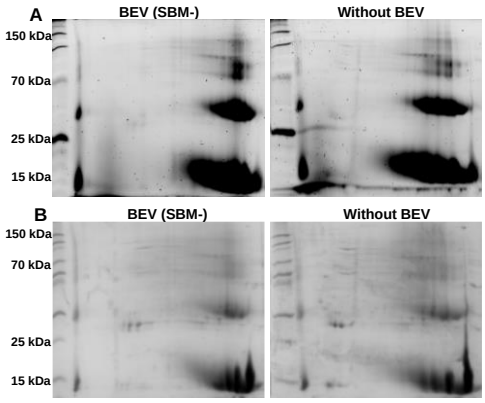

Figure 4. Translation of mRNAs in BEVs in a rabbit reticulocyte lysate system. (**A**) BODIPY fluorescence on a 2-D gel. (**B**) Coomassie blue stain on a 2-D gel.

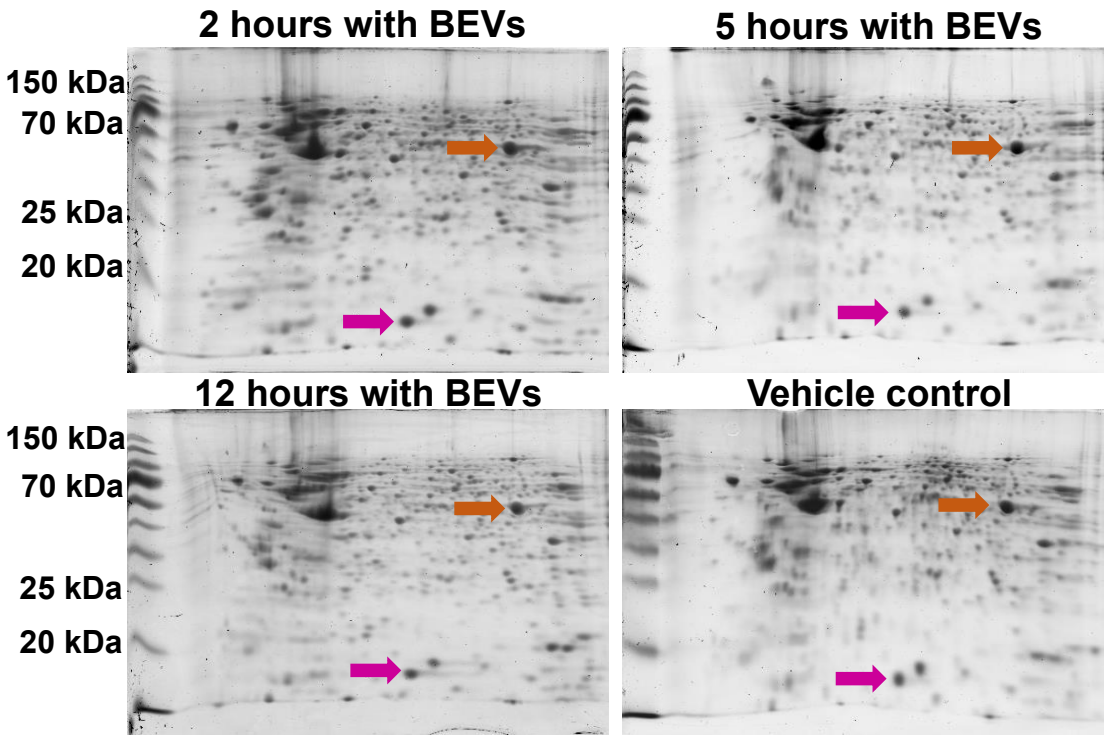

Figure 5. Coomassie blue stained 2-D gels of protein extracts from U937 cells harvested 2, 5 and 12 hours after addition of BEVs to culture media. Arrows indicate spots used in densitometry analysis (brown, spot 1; purple, spot 2).

4. Discussion

This is the first comprehensive analysis of mRNA cargo in sEVs from raw and store-bought bovine milk with and without removal of surface-bound transcripts by RNase, as well as bioavailability and translation of mRNA cargo. Transcripts of close to 5000 genes were identified in RNase-treated RM, and a comparison of SBM+ to SBM– suggests that a considerable amount of mRNAs may be attached to the BEV surface. The apparent bioavailability of bovine mRNA was modest compared to BEVs for which a 50% bioavailability has been reported [21]. Low bioavailability and absence of detectable translation are important observations because of the immunogenic potential of mRNAs, as demonstrated by the successful use of mRNA vaccines in the COVID-19 pandemic [44]. While an immune response is the desired outcome in vaccination settings, the expression of endogenous mRNA cargo in BEVs may elicit adverse reactions in patients treated with therapeutics loaded BEVs, and therefore is not desirable.

At first sight, the absence of translation might come as a surprise when considering that mRNA COVID-19 vaccines elicit an immune response. The total amount of mRNA per dose of COVID-19 vaccines range from 30 μg (Pfizer-BioNTech Bivalent and Monovalent) to 50 μg (Moderna Bivalent) to 100 μg (Moderna Monovalent). The input of BEV total RNA in our *in vitro* translation experiment was 10 μg representing more than 4500 different RNA molecules, which translates into exceedingly low levels of distinct mRNA sequences. We cannot formally exclude the possibility that a larger amount of input mRNA might lead to

detectable quantities of bovine proteins. Increasing the amount of input material seems physiologically irrelevant because it required 13.4 L milk to purify 10 μg RNA from BEVs in this study. In addition, it has been reported that a large fraction or mRNAs in sEVs is present in truncated form which likely triggers nonsense mediated RNA decay [45,46]. Consistent with these considerations no "cytokine storm" was observed when human peripheral blood mononuclear cells were incubated with BEVs *ex vivo* [47]. We acknowledge a report suggesting that mRNA in mouse mast cell sEVs are translated in human mast cells and the limitation that protease K inactivation at 90 ℃ might have cause a loss of BEVs in this study [2]. We consider this a minor concern because RNA preparations were obtained from the same number of BEVs in the three types of milk (RM, SBM+, SBM–). We speculate that mRNA translation may be more efficient if transcripts are transferred within the same lineage of cells, as opposed to transfer between different cell lineages. It has been proposed that BEVs originate in the mammary gland, although the evidence is circumstantial [48].

5. Conclusion

While this study constitutes an important contribution to the assessment of biological safety of BEVs in the delivery of therapeutics, it may not be construed as BEVs not having the potential to elicit adverse reactions in patients. Future studies need to assess the shelf-life of BEVs and their therapeutic cargo, the pharmacokinetics of BEVs and therapeutic cargo, and the biological safety of the therapeutic cargo.

Acknowledgments

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Supplementary data

The authors confirm that the supplementary data are available within this article. Figure S1. Translation of luciferase mRNA in a rabbit reticulocyte lysate system; Table S1. mRNAs detected in BEVs isolated from RM, SBM+, and SBM- (n = 3 biological repeats per inpit material); Table S2. mRNAs in BVEs from RM, SBM+, and SBM- that contained a Kozak sequence.

Conflicts of interest

All authors declare no conflicts of interest.

Ethical statement

All procedures involving animals were conducted in accordance with the University of Nebraska-Lincoln's Institutional Animal Care and Use Committee and approved by the Institutional Animal Care Program (September 28, 2021, protocol 2152). All individuals provided written consent before participation.

Author's contribution

Conceptualization and design: Shu J, Braga CP, Cui J, Adamec J and Zempleni J; methodology: Shu J, Braga CP, Cui J and Adamec J; software and validation: Cui J, Adamec J and Zempleni J; formal analysis: Cui J, Adamec J and Zempleni J; investigation and data curation: Shu J, Braga CP, Cui J and Adamec J; resources: Cui J, Adamec J and Zempleni J; writing and original draft preparation: Shu J, Braga CP, Cui J and Adamec J; writing, review and editing: Zempleni J; supervision: Cui J, Adamec J and Zempleni J; project administration: Zempleni J; funding acquisition: Zempleni J. All authors have read and agreed to the published version of the manuscript.

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