The intracellular source, composition and regulatory functions of nanosized vesicles from bovine milk-serum

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A hypothesis that the source of milk-serum derived vesicles (MSDVs) is the Golgi apparatus (GA) was examined. Using dynamic light scattering and electron microscopy, it was shown that MSDVs are composed of globular structures with hydrodynamic sizes of 70 ± 15 nm. More than 60% of the total protein content of MSDVs was associated with the MSDV lumen and 30% was associated with the MSDV membrane. Casein was the major protein found in the MSDV lumen. The conclusive markers of the GA, lactose synthase components (α-lactalbumin and galactosyltransferase) and activity (synthesis of lactose from glucose and UTP-galactose), the presence of casein in micellar form in the MSDV lumen, and a high luminal content of citric acid, were demonstrated in the lumen of MSDVs. Though MSDVs composed only 0.7% of the milk mass, they accounted for a high proportion of the total milk content of reactive (15% Cu and 18% Fe) and toxic minerals (60% Cd and 65% Pb), which strongly suggests that MSDVs serve as an avenue to protect mammary epithelial cells from the toxic effects of these minerals by storing them intraluminally and secreting them into milk. The presence of micellar casein in the MSDV lumen, along with the presence of metal transporters in their membranes, is responsible for this impressive capacity for storing reactive and toxic minerals. Exposing a single mammary gland to lipopolysaccharide challenge induced changes in regulatory proteins stored in the lumen of MSDVs (tissue plasminogen activators, plasminogen and plasmin) and in the activity of xanthine oxidase and alkaline phosphatase. Thus, we have demonstrated that MSDVs are under the regulation of the nucleus and respond to extracellular signals.

Introduction

The most unique attribute that defines the vertebrate class Mammalia is the production of milk. Milk is the primary nutrient for the neonate, and has many additional roles, such as support of the newborn immune system. Mammary gland (MG) epithelial cells (MECs) are among the most active tissues in the animal kingdom. This trait is especially important in mammals selected for high milk production, most notably in modern dairy cows. Currently, dairy cows selected for high milk yield can produce 30–60 liters of milk per day; this milk typically contains ~10% dry matter composed of 30 to 40 g L⁻¹ fat and protein, ~50 g L⁻¹ lactose and 15 to 20 g L⁻¹ minerals. Among the components secreted in milk, membranoprolipoproteins represent the most complex compounds, both chemically and structurally.¹,²

Two types of milk components are secreted while being surrounded by lipoprotein membranes.³ Milk fat is secreted by a mechanism unique to the MG: membrane-enclosed cytoplasmic lipid droplets composed mainly of triglycerides are synthesized at the endoplasmic reticulum (ER) and are secreted into the MG lumen as 1 to 5 μm-sized particles by enveloping with the MEC apical membrane.³ The typical number of these milk fat globule membranes (MFGMs) in bovine milk is ~10¹⁰ mL⁻¹.⁴

In addition, the milk of various mammals contains nanosized vesicles in the range of 50 to 70 nm that resides within the milk serum (whey).¹ In cows, the number of these milk serum derived vesicles (MSDVs) was estimated to be ~10¹⁵ mL⁻¹, which accounts for 40–60% of the total content of ~1.5 g kg⁻¹ milk lipoproteins.⁵,⁶ Thus, the rate of MSDV secretion is ~5 logarithmic orders greater than the secretion of MFGMs.

MSDVs of bovine origin were first isolated in the early 1950s and were described as MEC-derived xanthine oxidase-rich microsomes.⁶,⁷ MSDVs of bovine origin were further characterized in the 1970s and were described as either MEC-derived plasma membrane particles⁸,⁹ or vesicles secondly derived from these particles.¹⁰,¹¹
from the MFGM following fat secretion into milk. However, experimental evidence enables us to conclude that the concept that MSDVs are derived from MFGMs is highly dubious. Meanwhile, in parallel and unrelated studies carried out during the early 1970s, human milk samples were shown to contain nanosized particles that exhibit many of the features characteristic of retroviruses. In particular, these human lipoprotein membrane-surrounded retrovirus-like particles, with a density of 1.16–1.19 g mL$^{-1}$, were found to contain a single-stranded 60 and 70S RNA, which was physically associated with a reverse transcriptase. However, cDNA that was prepared from these particles hybridized exclusively with human genomic DNA, indicating a human origin for the particles and disproving the assumption that retroviruses cause breast cancer. Interest in MSDVs remained dormant for about 3 decades. Recently, MSDVs were isolated from human milk and were described as exosomes with immune activity, which renewed interest in MSDVs. In addition to possessing immune activity, it was discovered that MSDVs found in human, porcine and bovine milk contain microRNA (miRNA), which could be transferred to other cells and be functional in their new locations.

Currently, it is well established that exosomes are derived from virtually all kinds of cells and thus are present in various biological fluids, including blood, urine, seminal fluid and milk, and that exosomes possess important biological roles, particularly immunological and regulatory, due to their ability to enable intercellular communication. The secretion of exosomes from cells is related to the fusion of multivesicular bodies (MVBs) surrounded by the plasma membrane, which release their content (endosomes) to the extracellular space. The source of the endosomes entrapped within MVBs is the lumen that is recycled from plasma membranes. Thus, the secretion of exosomes enables the secretion of proteins that are located within their double-stranded lipoprotein membranes. The lumen was not considered before 2007 to be a significant contributor to the function of exosomes, as it entraps only a tiny fraction of the cell cytosol. However, in 2007, it was demonstrated that the lumen of exosomes serves as a carrier of mRNA and miRNA stored in that lumen; since then, it has become increasingly clear that intraluminal genetic material plays an essential role in the function of exosomes in intercellular communication.

Nevertheless, in contrast to the prevailing view that MSDVs are exosomes, based on the presence of biochemical markers in their lipoproteins, it has been suggested that MSDVs originate in the Golgi apparatus (GA). Moreover, to the best of our knowledge, no explanation or evidence that could clarify the following question has yet been provided: how is genomic material incorporated into vesicles circulating between the plasma membrane and MVBs? The assumption that vesicles derived from the GA are the source of genomic material that is found in extracellular fluids provides a more plausible answer to this question because of the direct link between the GA, the ER ribosomes and the cell nucleus. Furthermore, vesicles derived from the GA may be endowed with additional functional capacities, such as those related to the unique characteristics of proteins stored in their lumen. To the best of our knowledge, no explanation or evidence that could clarify the following question has yet been provided: how is genomic material incorporated into vesicles circulating between the plasma membrane and MVBs? The assumption that vesicles derived from the GA are the source of genomic material that is found in extracellular fluids provides a more plausible answer to this question because of the direct link between the GA, the ER ribosomes and the cell nucleus. Furthermore, vesicles derived from the GA may be endowed with additional functional capacities, such as those related to the unique characteristics of MSDVs.

The aim of the present study is to provide direct evidence proving that the source of MSDVs is the GA of the MEC and to highlight their main features and physiological roles. In order to prove these points, we show that MSDVs resemble non-swollen secretory vesicles (SVs), which are known to be derived from the GA. The fact that MSDVs can store casein in micellar form and the demonstration that MSDVs contain the components of lactose synthase, and thus can synthesize lactose from glucose and UDP-galactose in their lumen, were taken as major evidence, which prove this hypothesis. The effect of lipopolysaccharide challenge of a single mammary gland was used as an experimental means to show that the intra-luminal and membrane composition of MSDVs is under nuclear regulation of the cells which secrete these vesicles.

**Experimental methods**

**General procedures**

All reagents, except when otherwise mentioned, were obtained from Sigma Chemical Co. (Rehovot, Israel). The protein concentrations of different samples were determined by the Bradford method.

For the analytical procedures described in this paper, high quality raw milk with superior hygienic quality (somatic cell count $\leq$ 470 000 cell per mL and with no bacterial isolates) obtained during the noon milking (out of three daily milkings) from Israeli-Holstein cows was used. Milk samples (~500 mL) were taken over a period of 5 years from ~50 cows that were between 36 and 271 days in lactation and belonged to the experimental herd of the Agricultural Research Organization, as previously described. Soon after sampling, the milk was defatted under cold conditions and the skim milk was stored at $-20^\circ$C for further analyses. The fat and lactose content, the total protein and its distribution among casein and whey, and the somatic cell counts were determined in individual milk samples as described previously.

**Isolation of MSDVs**

The milk samples were initially centrifuged at 5000g for 30 min at 4 $^\circ$C to remove milk fat globules (MFGs) as well as somatic cells. The skim milk (in 50 mL tubes) was then subjected to ultracentrifugation (Beckman Coulter Instruments, Fullerton, CA, USA) at 4 $^\circ$C for 1 h at 100 000g. Residual MFGs were removed from the top of the vials, and the serum, deprived of vesicles, was collected and stored at $-20^\circ$C. The fluffy material containing the MSDVs was layered above the firm and white casein sediment; this material was separated and diluted in 50 mL of 10 mM Tris buffer, pH = 7.4, containing 300 mM mannitol and 1 mM KCl and centrifuged at 4 $^\circ$C for 1 h at 100 000g. The final sediment was diluted $\times$4 in the above solution, snap frozen in liquid nitrogen and stored at $-80^\circ$C for further analyses. The casein sediment was collected and stored at $-20^\circ$C.
Hydrodynamic size of MSDVs

The distribution and the average hydrodynamic diameter of the MSDV nanoparticles in aqueous solution were measured by dynamic light scattering on a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Measurements were carried out at a scattering angle of 173° at 633 nm. The average hydrodynamic diameter (±5 nm) was calculated based on the intensity distribution using the Stokes–Einstein relation, assuming that particles have spherical shapes and using a built-in algorithm. The average sizes were obtained from three measurements (10 runs per measurement) for each sample using the built-in algorithm for number-weighted statistics.

Electron microscopy

The MSDVs isolated as described above were negatively stained. A 10 µL drop was added to a 400 mesh formvar/carbon grid and incubated for 10 min at room temperature. Ten µL of 1% phosphotungstic acid in double distilled H2O was added and rapidly blotted from the grid, which was then examined using a Philips CM10 (Netherlands) transmission electron microscope (TEM) operated at 80 kV.

SDS-PAGE

The MSDVs isolated as described above and the MFGMs prepared as described previously were used for SDS-PAGE chromatography. The SDS-PAGE procedures were carried out on MSDV samples which were pretreated as follows: the membranes of the MSDVs were disrupted using radioimmuno-precipitation assay buffer (RIPA) buffer as described previously. The disrupted vesicles were ultracentrifugated in 5 mL vials at 4 °C for 1 h at 100 000g. The sediment was washed twice with distilled water and then dissolved in 50 mM Tris buffer, pH 8 for SDS-PAGE.

The molecular weights of the separated proteins were estimated by comparing the protein mobility against a set of standards, viz.: myosin, 200 000; β-galactosidase, 116 000; phosphorylase b, 92 500; bovine serum albumin, 66 500; ovalbumin, 45 000; carbonic anhydrase, 31 000; soyabean trypsin inhibitor, 21 500; and lysozyme, 14 400.

Immunoelectron microscopy

The MSDV samples isolated as described above were processed for detection of the presence and distribution of αs1-casein and milk fat globule-epithelial growth factor 8 protein (MFG8) between the membrane and intraluminal space by immunoelectron microscopy procedures adopted from a previously published procedure. The samples were fixed in 1% glutaraldehyde, post-fixed in 0.5% glutaraldehyde and then washed with 0.1 M phosphate buffer solution (PBS) pH 7.4; each time, they were maintained for 2 h at 4 °C. The samples were rinsed with shaking in PBS pH 7.4 for 30 min and dehydrated in a series of graded ethanol solutions (25, 50 and 75%; 30 min per step), and finally placed in 100% ethanol for 1 h. The samples were infiltrated with LR White resin (CAS number 94188-59-7), first for 30 min in resin : ethanol (2 : 1, v/v), and finally overnight at 48 °C in resin alone, with two changes. The samples were embedded in fresh LR White resin in BEEM capsules and polymerized at 60 °C for 24 h (electron microscopy procedures manual: http://sharedresources.fhrc.org/sites/default/EMProceduresManual.pdf). The grids were placed on droplets of 0.05 M Tris-buffered saline (TBS), pH 7.6, containing 1/30 normal goat antisem for 10 min to block non-specific binding sites. The samples were incubated overnight at 4 °C with αs1-casein (Santa Cruz, monoclonal mouse IgG1, Cat # sc-373711, CA, USA), and MFG8 (Santa Cruz, rabbit polyclonal IgG, Cat # sc-33546, CA, USA) antibodies with concentrations in the range of 1 to 50 mg mL−1, and in the presence of normal goat antisem. The grids were then washed with TBS and treated with secondary anti-rabbit antibody coated with colloidal 5 nm gold particles (EY Lab. Inc., San Mateo, CA, USA), diluted 1 : 20, 1 : 50, 1 : 100 in TBS. The samples were placed in 2% glutaraldehyde for 5 min, then 1% OsO4 for 15 min, and were stained with 3% uranyl acetate for 1 h at 40 °C and rinsed in water. The samples were then observed with a Philips CM10 TEM operated at 80 kV.

Western blot

Immunoblottings were performed on isolated MSDVs with a Bio-Rad system (ChemiDoc™ Touch Gel and Western Blot Imaging System, Berkeley, CA, USA) according to the manufacturer’s instructions. After the transfer of proteins from the 12% polyacrylamide gel to the PVDF membrane, the membrane was blocked for 1 h with 3% (w/v) skimmed milk in TBST (a mixture of Tris-buffered saline and Tween 20). The antibodies used in this study were toll-like receptor 2 (TLR2) (Santa Cruz, rabbit IgG Cat # sc-10738), toll-like receptor 4 (TLR4) (Santa Cruz, rabbit IgG Cat # sc-10741, CA, USA) and MFG8 (Santa Cruz, rabbit polyclonal IgG, Cat # sc-33546, CA, USA).

De novo synthesis of lactose in the MSDV lumen

β,1-4-Galactosyltransferase (GalT) activity was determined in MSDVs isolated as described above (in the Isolation of MSDVs subsection) and disrupted with RIPA buffer and in milk serum by a previously described procedure. Lactose synthesis was measured in MSDVs isolated as described in the Isolation of MSDVs section. The basal concentrations of glucose and lactose in MSDV disrupted with RIPA buffer were used as a blank (i.e., the glucose and lactose values after incubation with the experimental solutions were subtracted from the glucose and lactose concentrations at the beginning of the experiment). The experimental solution was composed of 250 µL of vesicle solution that contained ~10 mg mL−1 protein, 20 µL of 10 mM glucose dissolved in the buffer salt mixture, 20 µL of 10 mM UDP-galactose dissolved in the buffer salt mixture and 210 µL of buffer salt solution composed of 150 mM mannitol in Tris HCl 10 mM, pH 7.4 that contained 4 mM of KCl, 10 mM of NaCl and 3 mM of MnCl2. The final concentrations in the incubation solution were: glucose and UDP-galactose – 0.4 mM, KCl – 27 mM, NaCl – 67 mM and MnCl2 – 15 mM. In addition, controls were made by adding...
phlorizin to the salt solution to reach a final concentration of 3 mM in the final incubation solution.

The vesicles (6 replications for each incubation period) were incubated under the above conditions for 30, 60, 90 and 120 min. The reactions were stopped by immersing the vesicles in an ice bath. The vesicles were then separated by centrifugation at 15,000 rpm in an Eppendorf centrifuge (5424R, Hamburg, Germany) and washed 3 times with 10 mM Tris HCl, pH = 7.4; RIPA buffer was added to a volume of 0.5 mL and the samples were analyzed for glucose and lactose. The citric acid concentration was measured in non-treated vesicles. The concentrations of glucose, lactose and citric acid were related to the protein compositions of the MSDV membranes, i.e., of non-disrupted samples. The concentrations of the following metabolites were determined by previously described procedures: glucose, lactose and citric acid.

RNA content in the lumen of MSDVs

Total RNA was isolated from 300 μL of MSDVs (equivalent to ~30 mL milk) after disruption with RIPA buffer, using Trizol (Invitrogen, Carlsbad, CA, USA). The RNA content was quantified by using an e-spect UV-vis spectrometer (Malcom, Tokyo, Japan) and confirmed by using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Analysis of elements in MSDV and milk serum and determination of their mass distribution in whole milk

Element concentration analysis was carried out on skim milk, whey (skim milk from which the MSDVs and casein were removed by ultracentrifugation), on MSDVs after disruption of their membranes with RIPA buffer and on the supernatant of disrupted MSDVs following ultracentrifugation (as described above for the SDS-PAGE analysis). The concentrations of the elements were measured in these preparations by inductively coupled plasma-atomic emission spectrometry (Spectro, Kleve, Germany) as previously described. The distribution of the elements between MSDVs, MSDVs following ultracentrifugation, and whole milk (ignoring the content of minerals in the fat globules) was calculated on the basis of the mass of the MSDVs in milk as described previously.

Effect of intramammary challenge with lipopolysaccharide (LPS) on the activities of tissue-plasminogen activator, plasminogen and plasmin in protein precipitated from MSDVs and on alkaline phosphatase (Alp) and acid phosphatase (Acp) activity in MSDVs and milk serum

The protocols of this study were approved by the Institutional Animal Care Committee of the Agricultural Research Organization, which is the legitimate body for such authorizations in Israel.

The layout details of the study as well as information on milk yield, milk composition and changes in milk pertaining to immune response and imposition of nositative stress in response to an LPS challenge have been previously described. Briefly, 12 Israeli Holstein heifers with low somatic cell counts (47,000 ± 5000 cells per mL) and no bacterial detection that produced 33.2 ± 3.1 kg milk per d were divided into two groups of six cows. In the control group, two mammary quarters, one front and one rear were infused with 10 mL of a sterile non-pyrogenic saline solution (Teva Pharmaceutical Industries Ltd, Tel Aviv, Israel). The other two counter quarters served as controls. In the experimental cows, two mammary quarters, one front and one rear, were infused with 10 μg of LPS (Escherichia coli, O55B5; Sigma Chemical Co.) dissolved in 10 mL of sterile non-pyrogenic saline, while the two counter quarters served as controls. The intramammary infusion was injected using a special applicator following careful sterile cleaning of the teat surface. During the 4 days of the study (~24, 0, +24, +48 and +72 h, where 0 h refers to the day of infusion), every quarter of each cow was separately milked into an individual container, and milk samples were taken after the milk yield was recorded. The milk was discarded 7 days following the infusion. In this publication, we present the effect of the above-described LPS challenge on the activity of tissue-plasminogen activator (t-PA), plasminogen (Plg) and plasmin (PL) in casein micelles isolated by ultracentrifugation from skim milk and protein precipitated from MSDVs (as described in the Isolation of MSDVs and SDS-PAGE subsections). Casein micelle pellets isolated from milk by ultracentrifugation and the proteinaceous pellets derived from disrupted MSDVs were dissolved and diluted in 0.05 modified Tris buffer composed of 0.05 M Tris, 0.1 M NaCl, and 0.01% Tween 80, pH 7.6 to reach a final concentration of ~1 mg mL⁻¹. The activities of PL, Plg and t-PA were determined as described previously and related to protein concentration. In addition, the effects of the LPS challenge on the activities of alkaline phosphatase (Alp) and acid phosphatase (Acp) in milk serum containing and deprived of MSDVs were determined as previously described.

Statistical analysis

The results of the chemical analysis are presented as mean ± SD. The results in the graphs of the glucose uptake-lactose synthesize experiment and in the LPS challenge experiment are presented as mean ± SEM.

The results of the LPS challenge experiment were analyzed by a statistical model described previously. Briefly, the model used was:

\[ Y_{ijkm} = \mu + C_i + T_j + T_k + Q_{m}(T_jC_i) + e_{ijkm} \]

where \( Y_{ijkm} \) is the variable within cow, treatment, quarter and day, \( C_i \) is the cow class effect, \( T_j \) is the treatment class effect, \( T_k \) is the day class effect, \( Q_{m}(T_jC_i) \) is the treatment-day interaction effect, \( Q_{m}(T_jC_i) \) is the quarter within cow treatment error term for the treatment effect and \( e_{ijkm} \) is the residual error. LPS treatment did not affect the responses in the mammary glands of the running control cows (cows whose glands were not treated with LPS), therefore, these results are not reported.

Results

Size, appearance and composition

The hydrodynamic sizes of the MSDVs as determined by dynamic light scattering were 70 ± 15 nm, consistent with TEM
micrographs showing that the MSDVs are composed of nearly round vesicles in the range of 50 to 100 nm (Fig. 1A). In general, the size of the MSDVs is in agreement with previous results for vesicles isolated from the serum of bovine milk$^{20,25}$ and human milk.$^{17}$ The major proteins composing MFGMs are well characterized.$^{39}$ Comparison of the protein composition in MFGMs and MSDVs by Coomassie stained SDS-PAGE gel electrophoresis (Fig. 2A) shows that the major proteins in both membrane sources are the same. However, even at this low resolution, it becomes obvious that the concentrations of xanthine oxidase ($\sim$147 kDa) and butyrophilin ($\sim$59 kDa) are higher in MFGMs than in MSDVs, consistent with previous results.$^{20,40}$ On the other hand, the casein (CN) subtypes, $\kappa$-CN, $\alpha_{s1}$-CN, $\alpha_{s2}$-CN and $\beta$-CN (19–24 kDa), and the major whey proteins, $\alpha$-lactalbumin ($\sim$14 kDa) and $\beta$-lactoglobulin ($\sim$18 kDa), are clearly seen in the gel for MSDVs with disrupted vesicles, but are absent or barely seen in the MFGM chromatogram (Fig. 2A).

The ultracentrifugation of the disrupted MSDVs resulted in an extensive reduction of caseins in the specimens (Fig. 2A). The disappearance of caseins was associated with the reduction of additional proteins (Fig. 2A). A white sediment which resembled micellar casein was observed at the bottom of the ultracentrifuge tubes with disrupted MSDVs. No loss of caseins or formation of precipitates was seen when intact MSDVs were ultracentrifuged. SDS-PAGE chromatograms of the proteins in the sediment show the presence of the 4 casein subtypes and of the proteins that disappeared from the disrupted samples (Fig. 2A). Similarly, TEM micrographs of gold immunostaining with antibodies toward $\alpha_{s1}$-CN showed that the lumen of MSDVs is densely filled with $\alpha_{s1}$-CN (Fig. 1B and C). It is relevant to note that the presence of casein sediment and the presence of soluble $\alpha$-lactalbumin and $\beta$-lactoglobulin was demonstrated in SVs isolated from rat MECs.$^{29,41}$ These findings serve as the basis for the commonly accepted concept that milk proteins are secreted into milk via SVs.$^{42}$ On the other hand, TEM

![Fig. 1](image_url)  
**Fig. 1**  
Visualization of MSDV by TEM (A), presence of $\alpha_{s1}$-casein in the MSDV lumen by immunoelectron microscopy (B and C), presence of MFGE8 (D) and TLR4 (E) in the MSDV membrane, and presence of MFGE8, TLR2 and TLR4 in the MSDV membrane by western blot (F). Notes: (A) shows a TEM micrograph of the globular bodies of MSDVs, which are consistent with the hydrodynamic size of MSDVs as determined by dynamic light scattering. In (B–E), high magnification immunoelectron microscopy micrographs focusing on single vesicles are presented. The gold nanoparticles used in this procedure perforate the membrane, resulting in the staining of $\alpha_{s1}$-casein within and outside the vesicles because of the leakage of casein to the area surrounding the vesicles (B and C). On the other hand, the strong association of MFGE8 (D) and TLR4 (E) with the lipoprotein membrane surrounding MSDVs resulted in a clear marking of the membrane without evidence of leakage or intra-vesicular markings. Consistently, the western blot of proteins from the membranes of intact MSDVs shows the presence of membrane proteins: MFGE8, TLR2 and TLR4 (F).
micrographs of pre-embedded colloidal gold immunostained MSDVs with antibodies towards MFGE8 (Fig. 1D) and TLR4 (Fig. 1E) obviously demonstrate that these proteins are located within the MSDV membrane, consistent with previous findings.\textsuperscript{20,43} The presence of MFGE8 and TLR2 in MSDVs was demonstrated by western blotting (Fig. 1F), consistent with their investigation in MSDVs by mass spectroscopy.\textsuperscript{20}

A comparison of the SDS-PAGE chromatograms of the proteins in non-disrupted MSDVs with MSDVs whose membranes were disrupted with RIPA buffer demonstrated that the markings of caseins in the disrupted vesicles were stained more intensively than in intact MSDVs (Fig. 2B). This result is consistent with measurements of protein distribution among the MSDV membrane and MSDV lumen (Fig. 3). On the other hand, the staining of proteins that are strongly associated with membranes (XO and butyrophilin) was similar in the disrupted and non-disrupted vesicles (Fig. 2B).

Previously, it has been shown\textsuperscript{*} that the average yield of MSDVs is 6.3 mL L\textsuperscript{-1} (SD ± 1.7) or 7.3 g kg\textsuperscript{-1} (±1.8). The specific gravity of MSDVs in that study was 1.12 (±0.03), which is fairly consistent with the value of 1.10 to 1.15 obtained by ultracentrifugation in a sucrose gradient\textsuperscript{48} or of vesicles derived from human milk and classified as exosomes.\textsuperscript{87} The protein content of isolated MSDV and MFGM membranous substances was previously found to be ~25 mg g\textsuperscript{-1}, which represents ~0.65% of the total proteins in milk.\textsuperscript{*} In the present experiment, the typical total MSDV protein content in the buffer solution after disrupting the membranes was ~10 mg mL\textsuperscript{-1} (Fig. 3). The protein content in MSDVs in which the membranes remained intact accounted for ~40% of the total content, whereas the content of the protein precipitated by ultracentrifugation of membrane-disrupted MSDVs accounted for an additional ~30% (Fig. 3). This means that ~30% of the total protein content in MSDVs was not accounted for by the above-described procedure.

Xanthine oxidoreductase (XOR) is a major protein in MSDVs\textsuperscript{4} (Fig. 1A); it is present in the membrane in two forms, xanthine oxidase and xanthine dehydrogenase. Xanthine dehydrogenase was found to be firmly associated with the membranes of MSDVs on the luminal side. In the next section, we present evidence for the presence of lactose synthase, which is composed of \(\alpha\)-lactalbumin and GalT in MSDVs. The lactose synthase complex is associated with the lumen of the GA\textsuperscript{39,44} and GalT is known to be strongly associated with membranes.\textsuperscript{45}

Thus, the protein content in MSDVs which was not explained by our fractionation scheme may be accounted for by proteins that are firmly associated with MSDV membranes on the luminal side and by proteins, such as \(\beta\)-lactoglobulin, which are present in the lumen of MSDVs in a “true” solution and, therefore, do not precipitate by ultracentrifugation, as well as by some other proteins which are lost during the procedure. Previously, it has been shown by us and others that the membranes of MSDVs are a rich source of enzymes, including xanthine dehydrogenase, xanthine oxidase, acid phosphatase, alkaline phosphatase, nucleotide pyrophosphatase, \(\gamma\)-glutamyltranspeptidase, sulphhydryl oxidase and Mg\textsuperscript{2+}-ATPase.\textsuperscript{2,49} Below, we show that the components of lactose synthase are located within the vesicles and that the activity of xanthine oxidase and alkaline...
phosphatase in the membranes of MSDVs is under regulation. Taken all together, our studies show that most proteins (>60%) in MSDVs are not associated with the plasma membrane, as is supposed to be the case with exosomes.

**De novo lactose synthesis**

A single previous report demonstrated the presence of GalT in milk. In the present experiment, GalT activity in whey was found to be 28.2 (±5.1) nmol h⁻¹ L⁻¹ milk (Fig. 4), whereas GalT activity in the lumen of MSDVs was 156.1 (±12.2) nmol h⁻¹ L⁻¹ milk (Fig. 4). As the content of MSDVs in a liter of milk is ~6.3 mL, it appears that GalT activity in the lumen of MSDVs is ~87 fold more concentrated than in whey. This result is consistent with the tendency of GalT to be firmly bound to membranes and with the overall resemblance between the internal compositions of MSDVs and SVs; in particular, both membrane sources share the content of lactose synthase.

α-Lactalbumin and GalT compose the lactose synthase enzyme, which converts glucose and UDP-galactose into lactose with liberation of free UDP. Incubation of MSDVs with glucose and UDP-galactose has been associated with a time-dependent...
Metabolite content in MSDVs

In addition to lactose (Fig. 4; 260 ± 15 μmole g⁻¹ protein), the lumen of MSDVs contains a high concentration of citric acid (Fig. 4; 515 ± 21 μmole g⁻¹ protein). When the content of these metabolites is converted into intraluminal concentration (lactose in the range of 6 mM and citric acid in the range of 12 mM), taking into account that the typical protein composition in MSDVs is 24 mg mL⁻¹, it becomes clear that the concentration of these metabolites is much higher than what can be expected to be found in the MEC cytosol. The concentration of citrate in the MSDV lumen was about equal to that of milk, but that of lactose was much lower than the typical concentration in milk (~145 mM). Consistently, the concentration of lactose in MSDVs is considerably lower than in bovine SVs at 1188 μmole g⁻¹ protein. The RNA content in MSDVs isolated from 50 mL milk was 7.2 ± 1.2 μg (Fig. 4), which agrees with previous findings of RNA in nanosized vesicles isolated from bovine milk.

Elemental content in MSDVs

In general, the concentrations of macro- and micro-elements in whole milk, whey and milk micellar casein fractions in the present experiment (Table 1) are consistent with the typical composition of those elements in bovine milk. The concentrations of heavy toxic metals in milk can vary over a large range, and those found in the present study for cadmium and lead are consistent with relatively low levels of those metals in milk; also, the levels were smaller than the newly established Codex standard, 20 μg kg⁻¹ for Pb and 500 μg kg⁻¹ for Cd. The Ca concentration in MSDVs mirrored that of whole milk and was mostly associated with MSDV proteins (Table 1). The ratio between the Ca content within MSDVs and the total Ca content in milk (0.72%) was similar to the proportion of MSDVs in milk. As in the case of Ca, the intravesicular concentrations of Mg, P, and Zn were similar to those of milk, and the proportions of Mg, P, and Zn out of the total milk content and their associations with...
intravesicular proteins were similar to that of Ca. The intravesicular concentrations of Na and K were similar to their concentrations in milk; thus, the contribution of Na and K to the total milk content was similar to the ratio between the MSDVs and the milk mass. However, as in whole milk, intravesicular Na and K were not precipitated with proteins by ultracentrifugation, indicating that they are truly soluble in MSDVs.

All the minerals mentioned below were mostly associated with intravesicular proteins as indicated by their disappearance upon protein precipitation by ultracentrifugation. The intravesicular concentrations of trace elements, Cu, Fe, Mn, Se and Si, were much higher than in milk, accounting for 2% (Mn), 3% (Se), 4% (Si), 15% (Cu) and 18% (Fe) of the respective total milk mineral content (Table 1). The concentrations of toxic minerals were much higher in the intravesicular compartments than in milk, accounting for 8% (Ba), 60% (Cd) and 65% (Pb) of the total milk content. In the cases of Cd and Pb, their concentrations in whey fell to below detectable levels; therefore, calculating their concentrations in casein micelles was not possible. It is well established that Ca, Cu, Fe, Na, and Zn enter cells and intracellular membrane compartments through dedicated transporters and are important in the secretion of trace elements into milk.

When the intravesicular concentrations of Ca, Cu, Fe, Na, and Zn were used as independent variables and those of the other intravesicular minerals were used as dependent variables, the following significant correlations \( (n = 12, \text{ and at least } P < 0.05) \) were found: Ca was significantly interrelated with Ba, Mg, and Zn; Cu was significantly interrelated with Fe, Mn, Pb, Fe, K, Na, and Pb; Na was significantly interrelated with Ca, Fe, Mg, and Pb; and Zn was significantly interrelated with K, Mg, Na, Sc, Si, and Cd.

Effect of intramammary challenge with lipopolysaccharide on the plasmin system, xanthine oxidase, alkaline phosphatase and acid phosphatase activities

The plasmin system, which is composed of plasminogen activator (PA), non-activated zymogene, plasminogen and the serine protease plasmin, is a ubiquitous enzymatic system expressed in mammalian milk. The degradation of casein and the liberation of active components from casein by the plasmin system have been shown to play a regulatory role in the day-to-day regulation of milk secretion and the induction of involution upon the initiation of milk stasis. Recently, it has been shown that t-PA is the PA that degrades casein and that t-PA, Plg and PL are embedded in casein micelles in milk and are most likely secreted from MECs attached to the casein micelles.

![Graphs](rsc Advances, 4, 2015, 161-175, 2015)
In a previous experiment, it has been shown that 25% of xanthine oxidase (XO) activity is present on the outer surface of MSDVs, whereas 46.7% occurs in solution and the remaining on the outer surface of MFGMs. Similarly, the activities of Alp and Acp in milk are distributed between the outer surface of MSDVs and MFGMs; also, they are present in a truly soluble form in whey. The activities of Alp and Acp in MSDVs are about twice their activity in whey.

In the present experiment, we looked at the effect of an LPS challenge to single mammary glands of cows on the activity of the plasmin system in the lumen of MSDVs and casein micelles in milk; we also examined the effects on the challenge on the activities of XO, Alp and Acp in whey, MSDV membranes, and whey deprived of MSDVs (Fig. 6 and 7). LPS treatment of single glands did not induce a response of the plasmin system in MSDVs or milk micelles or a response of XO, Alp and Acp in the control glands, indicating that responses in enzyme activities were specific to the effect of LPS on the treated glands. The LPS challenge of experimental glands induced a transient ~2-fold increase in t-PA activity at 24 h post-treatment in proteins stored in the MSDV lumen and milk casein, as well as a corresponding ~1.7-fold decrease in PG activity and an ~11-fold increase in PL activity (Fig. 5A–C). These changes in the activities of Plg and PL were reflected by a decrease in the PG/PL ratio from ~27 to ~1.5 at 24 h post-treatment in MSDVs and casein micelles in milk. The changes in the t-PA, Plg and PL activities in milk casein were similar to those in whole milk under similar conditions. Thus, the results of this experiment are consistent with the suggestion that the components of the plasmin system are secreted into milk while being embedded with the casein micelles.

LPS treatment did not increase the activity of Acp (a lysosomal enzyme) in whey (Fig. 7A), MSDVs or whey deprived of MSDV (data not shown), indicating that the treatment did not induce changes in the secretion of this enzyme in comparison to pre-treatment and control glands. LPS treatment induced a significant transient increase in the activities of XO and Alp in whey, MSDVs and whey deprived of MSDVs (Fig. 7B and C), which reached a peak at 24 h post-treatment, indicating that LPS induced a dramatic increase in the secretion of these enzymes into the milk. However, when comparing the activities of XO and Alp in MSDVs and in whey deprived of MSDVs, it

![Fig. 7](image-url)
becomes clear that most of the increases in the activities of these enzymes in whey are related to the increases in their secretion into milk via the MSDVs. In comparison to the response of the plasmin system components and XO activity to the LPS challenge, the Alp activity responses remained high at 48 h post-treatment; at 72 h post-treatment they were still significantly higher than the pre-treatment values, whereas the values of the plasmin system components and XO activity returned to pre-treatment levels at 72 h post-treatment (Fig. 7B and C).

Discussion

Electron microscopy micrographs of fusions between MVB and the plasma membrane and the consequent release of vesicles have so far been the strongest evidence for the endosomal origin of nanosized extracellular vesicles, commonly defined as exosomes. Alternatively, also using electron microscopy, it has been shown that intraluminal vesicles of MVBs can be delivered for degradation into lysosomes (see Fig. 8 for illustration). Providing direct evidence of the cellular source of endosomes incorporated into MVBs would require the use of streaming imagery at electron microscopy resolution. Although, to the best of our knowledge, there is no direct proof, it is commonly believed that the source of exosomes is the plasma membranes of cells. This assumption is based on the fact that exosomes have a similar membrane orientation to the plasma membrane as well as their protein composition is similar, which is composed mostly of proteins of the plasma membrane. However, because the GA releases numerous amounts of SVs, which fuse with the plasma membrane and thus determine the plasma membrane composition, the same argument may apply to vesicles released by the GA. Indeed, at least on theoretical grounds, it has been illustrated in schematic figures in several reviews that vesicles derived from the GA may serve as a source of intraluminal endosomes in MVBs. The present experiment was carried out to prove that nanosized vesicles, which are secreted by MEC and are isolated from the milk serum of bovine milk, are derived from the GA. We provide 3 lines of evidence, each of which provides conclusive evidence for that assumption. Casein is the main protein in bovine milk, constituting 2/3 of the total protein content of milk (~20 g L⁻¹). After synthesis of the 4 casein sub-types in the ER, a casein macromolecule is assembled in the GA into micelles, which are stored in vesicles. The casein micelles are composed of ~40 monomers of casein sub-types with molecular weights of ~1 500 000 Daltons. We show here that the MVB lumen is densely filled with casein in its micellar form, indicating that the source is the GA. On the other hand, the possibility that their source is the plasma membrane is nil because this

![Fig. 8](image-url)  
An illustration of the voyage made by Golgi apparatus (GA)-derived vesicles to the apical plasma membrane in MECs. On the right side of the scheme, an illustration of the movement and osmotic swelling of secretory vesicles (SVs) from the GA to the apical plasma membrane and the release of casein micelles following fusion of SVs with the membrane. On the left side of the scheme, an illustration of the conventional view of how exosomes are derived from internalization of plasma membrane vesicles, the passing of these vesicles into early and late multivesicular bodies (MVBs) and finally the release of exosomes from late vesicular bodies into the extracellular space following fusion of MVB with the plasma membrane. The central part of the scheme describes the formation and secretion of golgisomes. Unique to golgisomes is that they are under direct regulation of the nucleus and that they respond to external signals, as illustrated by the line between the plasma membrane receptor of lipopolysaccharide (LPS), TLR4 and the nucleus. The interrelationship between the nucleus–ER–ribosome and the GA is also illustrated.
membrane lacks storage capacity. The synthesis of lactose is a unique feature of the GA in mammals. Thus, the demonstration of lactose synthase components in the lumen of MSDVs and the demonstration of the actual synthesis of lactose when MSDVs were incubated with lactose precursors provide additional definitive evidence for the Golgi apparatus origin of MSDVs. The third line of evidence is based on the demonstration that an extracellular cue (LPS) dramatically affected the intraluminal and membrane protein composition of MSDVs. This response can be explained as an effluent response, cell nucleus–ER–GA, to the well-defined afferent effect of LPS signaling that affects the cell nucleus. Below, we discuss that evidence in detail as well as the data that demonstrate that the secretion of MSDVs plays an indispensable physiological role in MEC function.

The evidence that MSDVs are derived from the GA
It is well established that lactose is synthesized in the GA of mammals. Lactose is synthesized in the GA by the incorporation of Glu with UDP-Gal by lactose synthase to yield lactose and UDP. Here we show that lactose synthase components, α-lactalbumin and galactosyltransferase, are present in the lumen of MSDVs. In previous studies, it was shown that under appropriate incubation conditions, lactose can be synthesized from Glu and UDP-Gal in whole mammary cells (mammary acini), from GA-derived fractions and from SV-rich fractions. In the present experiment, we demonstrated that lactose is synthesized in the lumen of MSDVs following their incubation in a medium that contains Glu and UDP-Gal. Consistent with the above-mentioned in vitro experiments, we show that the uptake of Glu can be inhibited by phlorizin, an inhibitor of glucose transport, and that the inhibition of Glu uptake inhibited lactose synthesis.

SVs from several epithelial tissues, including the pancreas, liver and parotid gland, have been shown to be tightly packed with proteins and other organic compounds. Because the intravesicular constituents of those vesicles are composed of high-molecular-weight compounds which are osmotically inactive, they are relatively small and un-swollen, and thus stable during homogenization and centrifugal isolation. Here we show that MSDVs are small (in the nano-range), are packed with proteins and can be stably isolated by ultracentrifugation from milk. In contrast to the features of the above-mentioned types of SVs and MSDVs, secretory vesicles in lactating mammary epithelial cells are swollen and distended, thus reaching the micron range, which makes them exceptionally fragile and unstable under ultracentrifugation. What makes MG SVs different from SVs in other tissues is that MG SVs are packaged with a high concentration of lactose which is similar to the concentration of lactose in milk. The size of SVs immediately after being released from the GA is similar to that of MSDVs (ref. 29 vs. present results; i.e., in the sub-micron range). On their way to the apical membrane, the SVs of MECs become swollen and distended, with diameters in the range of 0.2 to 1.2 μm. It has been hypothesized that osmotic swelling of MG SVs on their way from the GA to the apical plasma membrane accounts for the secretion of the liquid phase (milk minus the lipid globules) of milk. The presence of a high concentration of lactose, an osmotically active molecule which cannot permeate membranes, packed in the MG SVs accounts for their swelling. The lactose concentration in MSDVs is much higher than can be expected to be found in the cytoplasm. However, the lactose concentration in MSDVs is considerably lower than the lactose concentration in milk or in SVs, which explains why these vesicles do not swell and their size remains on the nanosized scale. Nevertheless, as in MG SVs, MSDVs contain lactose synthase, casein in micellar form, citrate and minerals. The minerals inside MSDVs are distributed according to the expected effect of micellar casein on mineral distribution between the micelles and the bathing solution. Thus, the present results unambiguously indicate that the cellular source of MSDVs is the GA and strongly suggest that MSDVs are a non-swollen subtype of SV that are released intact into the milk (Fig. 8). The size of typical casein micelles in milk is ~150–250 nm, which is greater than the size of MSDVs. However, casein micelles have a sponge-like structure, which may explain their ability to be squeezed inside the MSDV lumen and their swelling inside SVs along with an increase in hydration.

MSDVs have an important physiological role
Whereas the content of casein and whey proteins inside the MSDV lumen does not significantly contribute to the content of casein in milk, our results strongly suggest that it has an essential role in the regulation of the secretion of elements into milk. Casein micelles are composed of casein subtypes, αs1-CN, αs2-CN, β-CN and κ-CN, which contain phosphoserine and carboxyl residues that serve as binding sites for different minerals. In addition to the presence of casein in micellar form, we located proteins such as α-lactalbumin in the MSDV lumen, which can bind minerals. Consistent with the mineral binding capacity of those proteins, we show that except for monovalent ions, most of the minerals inside the MSDVs are associated with proteins that precipitated following ultracentrifugation.

Modern dairy cows secrete daily between 0.7 to 1.4 kg of minerals in their milk; these minerals originate from the blood. This means that MECs accumulate many minerals that cannot be metabolized or stored, and some of these minerals can become toxic to MECs. Fe and Cu are minerals that are essential in small amounts, but can easily reach toxic levels due to their tendency to react with peroxides and produce free radicals. The ability to accumulate elements such as Fe and Cu in the lumen of MSDVs allows MECs to avoid the accumulation of toxic levels of these elements in cells, in order to prevent the formation of free radicals in milk and, thus, to prevent the deterioration of milk quality during storage in the glands while preserving the availability of essential micro-elements such as Fe, Cu, Zn, Se to offspring. In the case of highly toxic elements such as Ba, Cd and Pb, this function becomes critically important for MEC function, which may explain their very high proportion in MSDVs. The dilution of toxic elements in milk may make their presence in
milk less critical for offspring. In any case, from an evolutionary point of view, preserving the life of the mother with the capacity to give birth repeatedly is more important than the life of her offspring, which carry only half of her genes.

The GA is a rich source of metal ion transporters, including several types of Ca\(^{2+}/\)Mn\(^{2+}\) pumps,\(^{74}\) type IIa Na\(^+/\)Pi-cotransporters,\(^{75}\) and Cu,\(^{76}\) Fe\(^{77}\) and Zn\(^{78}\) transporters. Consistently, Ca\(^{2+}\)-dependent ATP hydrolysis has been observed in the Golgi vesicles of lactating rat mammary glands\(^{79}\) and the MSDVs of bovine milk;\(^{80}\) also, a type IIa Na\(^+/\)Pi-cotransporter was localized in MSDVs isolated from goat’s milk.\(^{79}\) The Zn transporter ZIP13 can also transport Fe,\(^{77}\) and NRAMP2 (natural resistance-associated macrophage protein 2)/DMT1 (divalent metal transporter 1) is a metal transporter conserved from prokaryotes to higher eukaryotes which exhibits an unusual broad substrate range, including Fe, Zn, Mn, Cu, Cd, Co, Ni, and Pb.\(^{80}\) This overlap in metal transport may explain the high correlation between the minerals stored within MSDVs.

In conclusion, the capacity of MSDVs to store minerals in proportions that significantly exceed their mass proportion in milk is high enough to justify the considerable allocation of resources devoted to the secretion of MSDVs. The high proportion of mineral binding inside the lumen of the vesicles may be explained by two major factors: (i) the presence of various types of metal transporters in MSDV membranes, which allows the concentration of metals in the lumen, and (ii) the presence of intraluminal mineral-binding proteins, mainly in the form of casein micelles, which chelate the transported minerals and prevent their leakage from the vesicles.

The interaction between TLR4 and LPS activates a well-defined signal transduction pathway to the cell nucleus, the nuclear factor-kappa B (NF-kB) pathway,\(^{81}\) which in turn causes the secretion of components of the innate immune system, such as critical proinflammatory cytokines,\(^{82}\) NO\(^{83}\) and the plasmin system,\(^{77}\) which is part of the milk-borne negative feedback system that regulates milk secretion.\(^{75,81}\) The synthesis of proteins destined to compartments of the secretory pathway takes place in the ribosomes according to information transported by t-RNA from the nucleus.\(^{84}\) After completion of synthesis and folding, the proteins proceed further to their final destinations through the Golgi complex. In polarized cells, such as MECs, the main compartment of protein sorting is the GA, where apical and basolateral cargoes are segregated and targeted to their specific destinations.\(^{85}\) Hence, the increase in the activity of the plasmin system components in the lumen of vesicles in which the mammary gland was challenged with LPS provides direct evidence that this was an effector response to the afferent effect of LPS on the cell nucleus. The changes in the intraluminal activity of the plasmin system reflect the role of this system in ruling casein degradation,\(^{29}\) and are consistent with additional findings on the similarity between the internal composition of MSDVs and SVs. On the other hand, the changes in XO and Alp in the MSDV membranes most likely reflect the contribution of MSDVs to the innate immune response, as these enzymes are free to exert their effects while being attached to MSDVs.\(^{3,57,83}\)

Previous studies ascribed an immunological role to MSDV-like structures (exosomes) which were isolated from milk.\(^{7,18,21}\) In the present experiment, we found that the MSDV membrane contains immunoglobulin G, butyrophilin, MFGE8 and receptors for endotoxins (TLR2 and TLR4). All these components have immunological functions,\(^{29}\) which sustain this possibility and suggest that MSDVs may have a particular function as a platform of scavenger receptors and as a modulator of immune functions.

Defining MSDVs as golgisomes: what makes them different from exosomes?

In milk, MSDVs are not exposed to relevant concentrations of glucose and UDP-galactose such as those used to induce lactose synthesis in the vesicles in this study. Thus, the lactose synthase activity in MSDVs is most likely a redundant phenomenon that reflects their GA origin. However, the secretion of such large numbers of complicated structures is most likely associated with essential physiological functions, as indeed is supported by the evidence discussed above. To the best of our knowledge, this is the first study that provides evidence that the composition of vesicles secreted from cells to the extracellular environment is under genomic regulation and can be modified in response to environmental signals such as LPS. Consequently, we suggest terming MSDVs as golgisomes (Gsomes), to reflect their similarity in size to exosomes and their GA origin, hence, similarly to SVs (Fig. 8). The presence of RNA material in Gosomes is consistent with their GA origin and supports the notion that their internal composition is subject to regulation by the cell nucleus. Research on exosomes from various cell types has revealed a striking interrelationship between the biological qualities of the vesicles and their cell origins; for instance, exosomes derived from cancer cells increase tumorigenicity, and vesicles derived from leukocytes possess immunological functions.\(^{86}\) This interrelationship between cell type and the biological function of vesicles would have made less sense if the source of these vesicles was the plasma membrane, but can be a direct outcome if the source is the GA in the form of Gosomes. Thus, many of the vesicles currently defined as exosomes might in fact be Gosomes, as is shown here to be the case for vesicles secreted by MECs.

The association of Gosomes with the GA, along with their abundance and the simplicity of their isolation from milk, highlights their potential to serve as a model for exploring the presence and function of GA-derived vesicles and GA-associated transporters, such as metal ion transporters. The presence of toxic elements in milk and milk products is of major public concern because it may pose health problems, especially for infants.\(^{51}\) The present results indicate that analyzing the mineral content stored in Gosomes may serve as a sensitive indicator for the exposure of dairy cows to toxic elements, and hence, as a sensitive gauge for milk safety.

Conclusions

Compelling evidence that milk serum derived vesicles (MSDVs) originate from the GA and are secreted intact into milk is presented. We show that the intraluminal protein composition of
those nanosized vesicles is characteristically different from what is expected to be found in exosomes and more closely resembles that of the secretory vesicles of epithelial cells. We also show that proteins associated with the outer surface of the MSDV membrane perform important physiological functions in milk as components of the innate immune system. Moreover, casein stored in the lumen of MSDVs enables them to accumulate reactive (Fe, Cu) and toxic metals (Cd, Pb) and, thus, prevent their deleterious effects on mammary epithelial cells. We show that the membranous and intraluminal composition of MSDV are under nucleus regulation and respond to the challenge induced by glandular exposure to LPS. Based on the novel and unique properties of MSDVs, we suggest terming those nanosized vesicles is characteristically different from what is expected to be found in exosomes and more closely resembles that of the secretory vesicles of epithelial cells. We also show that proteins associated with the outer surface of the MSDV membrane perform important physiological functions in milk as components of the innate immune system. Moreover, casein stored in the lumen of MSDVs enables them to accumulate reactive (Fe, Cu) and toxic metals (Cd, Pb) and, thus, prevent their deleterious effects on mammary epithelial cells. We show that the membranous and intraluminal composition of MSDV are under nucleus regulation and respond to the challenge induced by glandular exposure to LPS. Based on the novel and unique properties of MSDVs, we suggest terming

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