Effect of circulating exosomes from transition cows on Madin-Darby bovine kidney cell function

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ABSTRACT

The greatest risk of metabolic and infectious disease in dairy cows is during the transition from pregnancy to lactating (i.e., the transition period). The objective of this experiment was to determine the effects of extracellular vesicles (microvesicles involved in cell-to-cell signaling) isolated from transition cows on target cell function. We previously identified differences in the protein profiles of exosomes isolated from cows divergent in metabolic health status. Therefore, we hypothesized that these exosomes would affect target tissues differently. To investigate this, 2 groups of cows ($n = 5/\text{group}$) were selected based on the concentration of β -hydroxybutyrate and fatty acids in plasma and triacylglycerol concentration in liver at wk 1 and 2 postcalving. Cows with high concentrations of β -hydroxybutyrate, fatty acids, and triacylglycerol were considered at increased risk of clinical disease during the transition period (high-risk group; $n = 5$) and were compared with cows that had low concentrations of the selected health indicators (low-risk group; $n = 5$). At 2 time points during the transition period (postcalving at wk 1 and 4), blood was sampled and plasma exosomes were isolated from the high-risk and low-risk cows. The exosomes were applied at concentrations of 10 and 1 $\mu\text{g}/\text{mL}$ to 5×10^3 Madin-Darby bovine kidney cells grown to 50% confluence in 96-well plates. Results indicate a numerical increase in cell proliferation when exosomes from high-risk cows were applied compared with those from low-risk cows. Consistent with an effect on cell proliferation, quantitative reverse transcriptase

PCR indicated a trend for upregulation of 3 proinflammatory genes (granulocyte colony-stimulating factor, ciliary neurotrophic factor, and CD27 ligand) with the application of high-risk exosomes, which are involved in cellular growth and survival. Proteomic analysis indicated 2 proteins in the low-risk group that were not identified in the high-risk group (endoplasmic and catalase), which may also be indicative of the metabolic state of origin. It is likely that the metabolic state of the transition cow affects cellular function through exosomal messaging; however, more in-depth research into cross-talk between exosomes and target cells is required to determine whether exosomes influence Madin-Darby bovine kidney cells in this manner.

Key words: transition cow, metabolic disease, extracellular vesicle

INTRODUCTION

Exosomes are extracellular vesicles that contain proteins, mRNA, and microRNA (**miRNA**) and are transported in blood as long-distance, intercellular messengers. They are biomarkers of metabolic or disease state (Distler et al., 2006; Taylor and Gercel-Taylor, 2008; Crookenden et al., 2016a), and their effect on target tissues is influenced by the physiological state of the secreting cell (Théry et al., 2009; Mathivanan et al., 2010). For example, tumor-derived exosomes contain a different protein cargo than exosomes secreted from nonmalignant cells (Andre et al., 2002). Furthermore, proteomic investigation of bovine exosomes has suggested that these nanoparticles are important for lactation physiology and mammary immune function and as indicators of metabolic health (Reinhardt et al., 2013; Crookenden et al., 2016a).

The transition to lactation in dairy cows is associated with metabolic and physiological stress as well as

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altered immune function (Drackley, 1999; Sordillo and Raphael, 2013; Crookenden et al., 2016b). During this period there is a high incidence of inflammatory disorders, such as mastitis and endometritis, which negatively affect milk production and reproductive function (LeBlanc et al., 2002a,b; Halasa et al., 2007). However, the degree of physiological and immunological stress in early lactation varies among cows (Heiser et al., 2015; Crookenden et al., 2016b). We have previously identified differences in the protein cargo of exosomes from dairy cows divergent in fertility (Mitchell et al., 2016) and metabolic health status over the transition period (Crookenden et al., 2016a). We further hypothesized that differences in exosomal cargo evident in cows divergent in metabolic health have integral roles in regulating the physiology of important tissues (e.g., liver) via intercellular cross-talk.

To investigate this hypothesis, exosomes were isolated from the plasma of cows with divergent indicators of metabolic health during the transition period. To assess the action of these exosomes on target cells, they were cocultured *in vitro* with Madin-Darby bovine kidney (MDBK) cells, which have been established as a model system for aspects of bovine liver cell metabolism (Bionaz et al., 2008; Thering et al., 2009; White et al., 2012; Zhou et al., 2015).

MATERIALS AND METHODS

Animals

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations (RAEC#12799) in accordance with the New Zealand Animal Welfare Act (Ministry of Primary Industries, 1999). The parent experiment was undertaken at Scott Farm (Hamilton, New Zealand; 37°46'S 175°18'E) between July and October 2013.

Cows previously identified as having either a high risk ($n = 5$) or a low risk ($n = 5$) of metabolic dysfunction based on blood plasma fatty acids (FA; mmol/L), BHB (mmol/L), and liver triacylglycerol (% liver wet weight) during the 2 wk immediately after calving were used for this study. The classification of these cows has been described previously (Crookenden et al., 2016a). Briefly, the high-risk cows had greater ($P < 0.01$) values for the 3 variables compared with cows in the low-risk category during wk 1 ($d 4 \pm 2$) and wk 2 ($d 11 \pm 2$) postcalving. Values for high-risk cows versus low-risk cows were 1.80 versus 0.66 ± 0.32 , 1.00 versus 0.45 ± 0.25 , and 9.0 versus 1.8 ± 1.59 for FA, BHB, and triacylglycerol, respectively, during wk 1 postcalving and 1.20 versus 0.30 ± 0.33 , 0.86 versus 0.48 ± 0.11 , and 10.3 versus 1.4 ± 1.59 during wk 2 postcalving (mean $\pm$ SE of the

difference). Average milk yield ($P < 0.05$), percentage fat ($P = 0.2$), and percentage protein ($P < 0.05$) during wk 1 and 2 of lactation were 23.6 kg, 5.0%, and 3.9%, respectively, for the high-risk group and 18.4 kg, 4.2%, and 4.2%, respectively, for the low-risk group.

Blood samples for exosome isolation (wk 1 and 4) were collected by coccygeal venipuncture into evacuated blood tubes containing EDTA anticoagulant. Blood was immediately placed on ice and centrifuged at $1,500 \times g$ for 12 min at 4°C, and the plasma was aspirated and stored at -20°C until assayed.

Exosome Isolation

The workflow used for the isolation of exosomes is presented in Figure 1. Exosomes were isolated from plasma (2 mL) at wk 1 and 4 by ultracentrifugation as previously described (Salomon et al., 2014; Sarker et al., 2014). Briefly, plasma was diluted 1:1 with PBS (pH 7.4) and centrifuged at $12,000 \times g$ for 30 min at 4°C (Sorvall high-speed microcentrifuge, fixed rotor angle = 900; Thermo Fisher Scientific Inc., Asheville, NC). The resulting supernatant (2 mL) was filtered (0.2 μ m), 8 mL of PBS (pH 7.4) was added, and the solution was centrifuged at $100,000 \times g$ for 120 min at 4°C (Sorvall T-8100 fixed-angle ultracentrifuge rotor; Thermo Fisher Scientific Inc.). The supernatant was then carefully removed, 300 μ L of PBS was added, and the pellet (extracellular vesicle) was resuspended. A discontinuous OptiPrep gradient (Sigma-Aldrich, Castle Hill, NSW, Australia) was used to isolate exosomes from the total extracellular vesicle. The gradient was made by diluting a stock solution of OptiPrep (60% wt/vol) with 0.25 M sucrose-10 mM Tris (pH 7.5) to make 40% (wt/vol), 20% (wt/vol), 10% (wt/vol), and 5% (wt/vol) solutions that were carefully layered, and the sample was placed on top. Tubes were centrifuged in an ultracentrifuge swinging bucket rotor (SW40 Ti Rotor, Beckman Coulter, Australia Pty. Ltd., Lane Cove, NSW, Australia) at $100,000 \times g$ for 20 h at 4°C. Fractions from each sample gradient (12×1 mL) were removed and washed in ultracentrifuge tubes (Sorvall; Thermo Fisher Scientific Inc.) by adding 9 mL of PBS and centrifuging at $100,000 \times g$ for 2 h at 4°C. The resulting supernatant was discarded, and the exosome pellets from each of the 12 fractions were resuspended in 50 μ L of PBS and stored at -20°C until further analysis.

Validation of Exosome Isolation

The density of each fraction was measured in a control OptiPrep gradient tube by determining the absorbance at 244 nm (Spectrostar Nano microplate

reader, BMG Labtech, Mornington, Victoria, Australia). The density of each fraction was calculated from absorbance readings, and exosomes were expected at 1.12 to 1.19 g/mL (Schröder et al., 1997; Tauro et al., 2012). Exosomes were characterized by size distribution using a NanoSight NS500 instrument (NanoSight NTA version 2.3 Nanoparticle Tracking and Analysis Release Version Build 0033, NanoSight Ltd., Wiltshire,

UK) as previously described in Salomon et al. (2014) and Sarker et al. (2014; Supplementary Figure S1; <https://doi.org/10.3168/jds.2016-12152>). Western blot was used to verify the presence of endocytic markers TSG101 (1:500; sc-6037, Santa Cruz Biotechnology, Dallas, TX) and CD63 (1:1,000; EXOAB-CD63A-1, System Biosciences, Palo Alto, CA; Théry et al., 2006; Koh et al., 2016).

Figure 1. Workflow for the isolation of exosomes from bovine plasma in high-risk and low-risk cows. OptiPrep: Sigma-Aldrich, Castle Hill, NSW, Australia.

Exosome Coculture

Epithelial (MDBK) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously (Bionaz et al., 2008; Thering et al., 2009). Five thousand cells per well were plated on 96-well plates and grown at 37°C and 5% CO₂ for 24 h in 100 µL/well of Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific Inc.) supplemented with 5% fetal bovine serum (FBS; Bovogen, Interpath Services Pty Ltd., Heidelberg West, Victoria, Australia) and 1% antibiotic, antimycotic solution (10,000 U of penicillin, 10,000 g of streptomycin, and 25 g of amphotericin B/mL; Gibco, Thermo Fisher Scientific) to achieve 50% confluency before treatment exposure. Upon 50% confluency, cells were incubated

for 40 h in DMEM supplemented with 5% exosome-depleted FBS plus exosome treatments or controls. Exosome-depleted FBS was prepared using ultracentrifugation (Sorvall, Thermo Fisher Scientific) at 10,000 × *g* for 20 h at 4°C. All treatments (10 or 1 µg/mL of exosomal protein from fractions 3 to 6 and 7 to 9) were applied in triplicate in 3 preparations of MDBK cells. Negative controls included DMEM supplemented with PBS (10 µL), FBS (10 µL), and exosome-depleted FBS (10 µL). Cell proliferation and morphology were analyzed using the IncuCyte Live-Cell Imaging system (Essen BioScience, Ann Arbor, MI), which uses high-density phase contrast real-time cell imaging as a proliferation parameter (Salomon et al., 2013; Koh et al., 2016). Images of cell proliferation were acquired every 2 h for the duration of the experiment (Figures 2 and 3).

After 40 h of exosome treatment, the cell culture media were collected, and technical triplicates were pooled and stored at −20°C for ELISA analysis. The cells and exosome coculture from each well were collected using 200 µL of RLT lysis buffer (Qiagen, Hilden, Germany) with 1% β-mercaptoethanol per well. Technical triplicates were pooled and stored at −80°C for subsequent RNA and protein extraction.

RNA Extraction

Cell lysates were homogenized by passing through a 25-gauge needle attached to a 1-mL sterile syringe at least 15 times. Total RNA was extracted from cell homogenates using a Qiagen RNeasy Mini kit according to the manufacturer's instructions. An on-column DNA digest was used to remove genomic DNA (Qiagen). The RNA was eluted in 20 µL of DNase-/RNase-free water by completing 2 elutions of 10 µL. The quantity and purity of RNA was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and stored at −80°C until further analysis.

cDNA Synthesis and Gene Expression Analysis

Total RNA (400 ng) was reverse transcribed using the RT² HT First Strand Kit (Qiagen). Reverse-transcriptase negative controls were also generated by excluding the enzyme, and cDNA samples were stored at −20°C. The bovine RT² Profiler PCR Array was used to quantify the gene expression of chemokines and cytokines by reverse-transcriptase quantitative PCR (PABT-150ZC-24; Qiagen). The PCR arrays were cycled using the following parameters: 1 × (95°C for 10 min) followed by 40 × (95°C for 15 s and 60°C for 1 min) cycles using the ABI StepOne machine (Applied Biosystems, Foster City, CA). The endogenous control

Figure 2. IncuCyte (Essen BioScience, Ann Arbor, MI) proliferation data of Madin-Darby bovine kidney cocultures with exosome fractions 3 to 6. (A) Exosomes isolated at wk 1 from low-risk cows at 1 µg/mL concentration (open circles) and 10 µg/mL concentration (filled circles). (B) Exosomes isolated at wk 1 from high-risk cows at 1 µg/mL concentration (open squares) and 10 µg/mL concentration (filled squares). Results are presented as means at each time point ± SEM; *n* = 5. There was no interaction between dose, time, and risk group (*P* > 0.05). However, the effect of dose was most evident at 24 to 40 h (*P* < 0.05) in the low-risk group. The dose of exosomes isolated at wk 4 did not affect proliferation (*P* > 0.05; data not shown).

genes included on the array were β -actin (*ACTB*), TATA box binding protein (*TBP*), *GAPDH*, and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). Gene expression was normalized to the endogenous control gene *HPRT1* using the cycle threshold ($\Delta\Delta C_t$) method because this gene was the most stably expressed across all treatments. Gene expression results were generated for wk 1 coculture samples. The data for cocultures of both fractions (3–6 and 7–9) were combined for gene expression analysis to determine the difference between high-risk and low-risk exosome cocultures.

Protein Extraction

Total protein from cells was extracted using acetone precipitation from the protein fraction that flows through the column during the RNA binding step of

the RNA extraction. Four volumes (4.8 mL) of ice-cold acetone was added to the flow-through in 15-mL Corning tubes and incubated overnight at -20°C . Samples were centrifuged for 10 min at $13,000 \times g$, air dried, and resuspended in 100 μL of 50 mM ammonium bicarbonate (pH 8.5). Protein was stored at -20°C pending mass spectrometry analysis.

MS Analysis

Coculture samples chosen for liquid chromatography (LC)/MS were based on IncuCyte and gene expression results; MDBK cocultured with exosomes isolated from wk 1 plasma using fractions 3 to 6 were chosen for proteomic analysis. These coculture samples were considered the most physiologically relevant because the cows were at their most metabolically divergent at

Figure 3. IncuCyte (Essen BioScience, Ann Arbor, MI) proliferation data of Madin-Darby bovine kidney cocultures with exosome fractions 3 to 6. (A) Exosomes isolated at wk 1 (10 $\mu\text{g}/\text{mL}$ concentration). (B) Exosomes isolated at wk 4 (10 $\mu\text{g}/\text{mL}$ concentration). (C) Exosomes isolated at wk 1 (1 $\mu\text{g}/\text{mL}$ concentration). (D) Exosomes isolated at wk 4 (1 $\mu\text{g}/\text{mL}$ concentration). Results are presented as means at each time point $\pm$ SEM; n = 5.

this time point. Total protein from these samples was sonicated for 15 min and reduced with dithiothreitol for 1 h. Proteins were then alkylated in 10 mM iodoacetic acid for 1 h in the dark. The samples were diluted to 1:10 with 50 mM ammonium bicarbonate and digested with trypsin (20 µg) at 37°C for 18 h. The samples were then desalted by solid phase extraction using a STAGE tip protocol (stop and go extraction tips for matrix-assisted laser desorption and ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics; Rappsilber et al., 2003). The eluted peptides were dried by centrifugal evaporation to remove acetonitrile and redissolved in Solvent A (0.1% formic acid in water). The resulting peptide mixture was analyzed by LC-MS/MS on a 5600 Triple TOF mass spectrometer (AB Sciex, Framingham, MA) equipped with an Eksigent Nanoflow binary gradient HPLC system (Dublin, CA) and a nanospray III ion source. The MS/MS spectra were collected using information dependent acquisition with a survey scan ($m/z = 350$ – $1,500$) followed by 12 data-dependent product ion scans of the 12 most intense precursor ions. All mass spectra were analyzed using the MASCOT search engine (www.matrixscience.com) and ProteinPilot (AB Sciex) against the Swissprot database with the species set as bovine (<http://www.ebi.ac.uk/uniprot/download-center>). Positive identifications were ascribed where MASCOT scores were >30 . False discovery rate was estimated using a reversed sequence database.

Peptide information from ProteinPilot (version 4.5, AB Sciex) was used to calculate the relative abundance of proteins within the data set. Data were quality trimmed by removing peptides with an unused score of <0.5 , a contribution score of 0, and a confidence score of <50 . Mass accuracy (ppm error) was calculated using the equation

$$\text{ppm error} = (\text{dMass}/\text{Prec MW}) \times 1,000,000,$$

where dMass = the observed value from mass spectrometer and Prec MW = the theoretical value calculated from periodical table. Peptides were retained if ppm error was between -20 and 20 . The relative abundance of proteins within a sample was calculated by summing the peptide intensity (PrecursorSignal) for a given protein and dividing by the total peptide intensity of that sample:

$$\begin{aligned} \text{relative abundance (\%)} = \\ \frac{(\text{sum of PrecursorSignal of a given protein})}{\text{sum of total PrecursorSignal}} \times 100. \end{aligned}$$

Statistics

To test the effect of risk category and dose of exosomes on MDBK cell proliferation during the course of the coculture experiment, both an area under the curve analysis for cellular kinetics and a linear mixed model analysis were used (SAS 9.3; SAS Institute, Cary, NC). The linear mixed model included the fixed effects of risk category, dose, and time of acquisition and their interactions and the random effect of animals. The covariance error structure for repeated measures over time points within animals in each risk category was determined using Akaike information criterion. A first-order autoregressive model was found to be the most appropriate error structure. The distribution of proliferation data was tested for normality using Shapiro–Wilk, Kolmogorov–Smirnov, and Anderson–Darling tests, and the residuals of data were found to be normally distributed. An unpaired t -test was used to determine the effect of risk (low-risk and high-risk coculture samples) on expression of genes as quantified by reverse-transcriptase quantitative PCR. The arithmetic mean and standard deviation of relative protein abundance was calculated for the control, low-risk, and high-risk coculture samples. If a peptide signal was not detected, a numerical placeholder of 0.01% was entered. Relative protein abundance was analyzed using a 2-sample t -test (PROC MIXED, SAS 9.3), and significance was declared if $P < 0.05$ (Supplementary Table S1; <https://doi.org/10.3168/jds.2016-12152>).

RESULTS AND DISCUSSION

The aim of this experiment was to investigate whether exosomes isolated from cows different in their metabolic state during the peripartum period affected the proliferation and function of MDBK cells. Proliferation data of MDBK cells treated with exosomes are presented in Figures 2 and 3. Gene expression data and LC-MS/MS proteomics results are presented in Figures 4, 5, and 6 and Supplementary Table S1 (<https://doi.org/10.3168/jds.2016-12152>). The results indicate that protein changes may be associated with cow risk category; 40 and 15 unique proteins were identified in the low-risk and high-risk samples, respectively (Figure 5). There tended to be an effect of exosome concentration on proliferation in the low-risk cows at wk 1 in which cells treated with 1 µg of exosomes exhibited faster growth at 24 to 40 h compared with cells treated with 10 µg of exosomes (Figure 2). There was no effect ($P > 0.05$) of risk category on MDBK cell proliferation (Figure 3), cytokine gene expression (Figure 4), or protein abundance (Figure 6). However, sufficient differences

Figure 4. Gene expression results of high-risk and low-risk coculture models at wk 1 [data from cocultures of both pooled fractions (3–6 and 7–9) combined]. (A) Granulocyte colony-stimulating factor (*CSF3*; $P = 0.10$). (B) Ciliary neurotrophic factor (*CNTF*; $P = 0.09$). (C) CD27 ligand (*CD70*; $P = 0.14$). Gene expression is normalized to endogenous control hypoxanthine phosphoribosyltransferase 1 using the cycle threshold ($\Delta\Delta C_t$) method. Results are presented as means $\pm$ SEM; $n = 5$.

indicate an effect of exosome source on the cellular physiology of MDBK cells (Figure 5). Further research with greater numbers of cows is required to determine the effect of metabolic state on exosome messaging of target tissues.

IncuCyte Results

The high-risk cows were selected based on indicators of liver dysfunction (excessive FA mobilization and a failure to fully oxidize FA, which led to increased BHB production and the accumulation of FA in liver). Such a metabolic profile would be expected to generate systemic and tissue-specific inflammation (Ohtsuka et al., 2001; Sordillo and Mavangira, 2014). A previous study investigating the protein cargo of the exosomes used in the current study indicated differences between these treatment groups supporting such an effect (Crookenden et al., 2016a).

Initial analyses support the hypothesis that the metabolic state of the cow may differentially alter cell proliferation. The MDBK cells treated with low-risk exosomes at wk 1 (Figure 2) tended to demonstrate lower proliferation ($P > 0.05$) when exosomes were applied at the largest dose (10 μ g compared with 1 μ g). Furthermore, a numerical ($P > 0.05$) increase in cell proliferation occurred in the high-risk cows compared with the low-risk cows when applied at 10- μ g concen-

Figure 5. Venn diagram of unique proteins identified in control, high-risk, and low-risk groups using liquid chromatography–tandem MS demonstrating proteins shared or unique to each group. Proteins were counted if they were identified in ≥ 1 sample in each group.

tration (Figure 3). The lower rate of proliferation with the application of more low-risk exosomes (Figure 2) is consistent with Aucher et al. (2013), who demonstrated that exosome-derived miRNA is capable of inhibiting target cell proliferation. The low-risk exosomes were isolated from cows with a reduced inflammatory phenotype and may carry miRNA specific to this. Recent research suggests that exosomal miRNA could be used to treat metabolic disease (Thomou et al., 2017). Labeling of exosomal contents to track transfer of exosomal contents into target cells should be considered in future experiments (Lai et al., 2015). Although limited by the low numbers of animals in the current study, results indicate that the peripartum metabolic state of the dairy cow likely affects the influence that exosomes have on cell function.

Furthermore, the numerical differences in proliferation between the high-risk and low-risk cocultures are consistent with published studies that indicate that proinflammatory conditions increase cellular proliferation. The conditions of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis are characterized by hepatic inflammation and FA accumulation in the liver (Hardy et al., 2016)—characteristics similar to those reported in high-risk cows in the experiment described here and in Crookenden et al. (2016a). Our previous study reported that the exosomal protein cargo was influenced by the metabolic state of the high-risk cows, which was proinflammatory in nature (Crookenden et al., 2016a). Consistent with this, exosomes isolated from a primary hepatocyte culture model of nonalcoholic steatohepatitis were proinflammatory and could induce inhibition of cell apoptosis (Hirsova et al., 2016). Therefore, it was hypothesized that exosomes isolated from divergent risk groups would result in differences in MDBK cell proliferation. It is possible that the exosomes isolated from the high-risk and low-risk cows are not programmed to target MDBK cells due to their kidney origin or that the nonclinical fatty liver disease in early-lactation dairy cows may not alter proliferation of kidney cells. Additionally, the exosomes isolated from blood represent a composite of release from all tissues that may serve to dilute any liver-specific changes in exosome released for the high-risk and low-risk cows tested here. Nevertheless, the results presented indicate no significant effect of the metabolic state of the exosome donor cows on the proliferation of MDBK cells despite a numerical difference in cellular proliferation in favor of the high-risk treatment.

Gene Expression Results

The cytokine and chemokine PCR array identified 32 genes that meet cycle threshold cut-offs (<30) for

gene expression quantification. Of these, no significant differences in gene expression were found between high-risk and low-risk exosome coculture samples. However, 3 genes tended to be increased in the high-risk coculture samples compared with the low-risk samples (Figure 4). These were the genes encoding granulocyte colony-stimulating factor (*CSF3*; $P < 0.10$), ciliary neurotrophic factor (*CNTF*; $P < 0.10$), and CD27 ligand (*CD70*; $P = 0.14$), all of which were upregulated in the high-risk cows relative to the low-risk cows.

Granulocyte colony-stimulating factor is an important survival and proliferation factor of neutrophils (Basu et al., 2002) that can act on neuronal cells as a neurotrophic factor to induce neurogenesis and inhibit apoptosis (Schneider et al., 2005). Interestingly, this is the same function as the protein product of the gene *CNTF*, the expression of which also tended to be increased in high-risk coculture samples. The similar functions of these 2 genes in cellular proliferation support numerical differences presented in Figure 3. MDBK cells are an epithelial-like cell line. The 3 genes that tended to be upregulated in the high-risk coculture samples are all expressed in the thymic epithelium (Haynes et al., 2000; Coquet et al., 2013). The epithelial cells of the thymus express several cytokines for T-lymphocyte development and confer immunotolerance to self-antigens. Other epithelial cells have a capacity for immunomodulation as they provide the first barrier against foreign invaders (Rescigno et al., 2008; Malvisi et al., 2016); therefore, MDBK cells may have some capacity for immunomodulation. Conversely, these genes may be indicative of the exosomal tissue of origin and could reflect mRNA from the thymus. This is a limitation of the coculture model, and one that requires further investigation. The tendency for differences in gene expression between the high-risk and low-risk cultures potentially indicates altered immune function and support for the numerical differences in proliferation (Figure 3) by exosomes from cows in the high-risk category for metabolic dysfunction; however, further investigation is required to confirm this.

Proteomics Results

The results of the protein analysis are presented in Table 1, Figures 5 and 6, and Supplementary Table S1 (<https://doi.org/10.3168/jds.2016-12152>). Unique proteins were identified between cells treated with plasma exosomes isolated from high-risk and low-risk cows. These differences may be indicative of the metabolic state of the cows from which they were derived; however, extensive changes were not evident. Two of the unique proteins identified in the low-risk group (≥ 2 cows) and not identified in the high-risk group were endoplasmic

Figure 6. Heat map of proteins identified in high-risk and low-risk coculture samples by liquid chromatography–tandem MS. Relative abundance of proteins is the sum of peptide signals for a given protein as a percentage of the total peptide signal for that sample. Proteins presented are those identified in $\geq 50\%$ of cows in both risk groups. The order of proteins is based on abundance of protein within the control sample, with the most abundant at the top. Results are presented as a ratio relative to the protein abundance in the control sample whereby a ratio of 0 indicates the same abundance as the control, >0 (blue) indicates a greater abundance compared with the control, and <0 (orange-red) indicates a lower abundance compared with the control. The P -value for the effect of risk, analyzed using a 2-sample t -test (PROC MIXED, SAS 9.3, SAS Institute Inc., Cary, NC), is included next to the heat map.

Table 1. Unique proteins and representative accession number of proteins identified in control, high-risk, and low-risk groups using liquid chromatography–tandem MS¹

Accession no.	Protein name
Common to all groups	
A4ZZF8	α -Actinin
A5PJ79	Transketolase
A6H6Y0	HNRNPD protein
B0JYL8	Cofilin
B0JYN3	L-Lactate dehydrogenase
B0JYN6	α -2-HS-glycoprotein
B0JYQ0	ALB protein
E1BH22	Histone H2A
E1BHA5	40S ribosomal protein S19
E1BHJ0	Profilin
E9RHW1	Heat shock 27-kDa protein
F1MB08	α -Enolase
F1MLB8	ATP synthase subunit α
F1MPU0	Clathrin heavy chain
F1MUZ9	60-kDa heat shock protein, mitochondrial
F1N6C0	Calmodulin
G3MZ21	Hemoglobin fetal subunit β
G3N2P2	Histone H3.3C
G3X6N3	Serotransferrin
G3X807	Histone H4
G8JKZ8	Thioredoxin
I6YIV1	Annexin A2
I7CT57	Vitamin D binding protein
O18787	Elongation factor 1 α
P01966	Hemoglobin subunit α
P02253	Histone H1.2
P10096	Glyceraldehyde-3-phosphate dehydrogenase
P19120	Heat shock cognate 71 kDa protein
P21752	Thymosin β -10
P48616	Vimentin
P60712	Actin, cytoplasmic
P62935	Peptidyl-prolyl cis-trans isomerase A
P63103	14-3-3 protein
P81947	Tubulin α -1B chain
Q0QEM9	ATP synthase subunit β
Q0QEV5	Ribosomal protein L18
Q0VCX2	78-kDa glucose-regulated protein
Q2KJF1	α -1B-glycoprotein
Q2VYC3	Eukaryotic translation elongation factor 1 β
Q3SYT9	Poly(RC) binding protein 2
Q3SZN8	Ribonuclease/angiogenin inhibitor
Q3T087	60S ribosomal protein
Q3T0P6	Phosphoglycerate kinase
Q3ZC44	Heterogeneous nuclear ribonucleoprotein A/B
Q3ZC87	Pyruvate kinase
Q3ZCH0	Stress-70 protein, mitochondrial
Q58D62	Fetuin-B
Q5E956	Triosephosphate isomerase
Q5E9F5	Transgelin-2
Q5GN72	α -1-acid glycoprotein
Q6PVY3	Macrophage migration inhibitory factor
Q862H7	Protein S100
Q862Q2	Similar to ribosomal protein S3
Q8MII4	Metallothionein
Q9BG14	Peroxisome oxidoreductase
Common to exosome treatment	
A6QPM9	PTMS protein
E1B9K1	Polyubiquitin-C
E1BCL5	60S acidic ribosomal protein P1
E1BF20	HNRNPH1
F1MCT8	SYNCRIP
F1MMK9	Protein AMBP
F1MXP8	Prosaposin
F1N169	FLNA
F1N3A1	Thrombospondin-1

Continued

Table 1 (Continued). Unique proteins and representative accession number of proteins identified in control, high-risk, and low-risk groups using liquid chromatography–tandem MS¹

Accession no.	Protein name
F1N647	Fatty acid synthase
G3X6S5	SH3 domain-binding glutamic acid-rich-like protein
O46375	Transthyretin
P00442	Superoxide dismutase [Cu-Zn]
P13696	Phosphatidylethanolamine-binding protein
P34955	α -1-antiproteinase
P60661	Myosin light polypeptide 6
P61603	10-kDa heat shock protein, mitochondrial
P62866	Ubiquitin-like/S30 ribosomal fusion protein
P68002	Voltage-dependent anion-selective channel protein
Q2HJH9	Programmed cell death protein
Q3B7M5	LIM and SH3 domain protein
Q3SYU2	Elongation factor
Q3SZ57	α -Fetoprotein
Q3SZ65	Eukaryotic initiation factor 4A-II
Q3T0Q4	Nucleoside diphosphate kinase B
Q3T0Q6	Cellular nucleic acid-binding protein
Q3T165	Prohibitin
Q3ZBA4	RNA binding motif (RNP1, RRM) protein
Q3ZBH0	T-complex protein 1 subunit β
Q3ZBS7	VTN
Q3ZBT1	Transitional endoplasmic reticulum ATPase
Q3ZBZ8	Stress-induced-phosphoprotein
Q5E9E2	Myosin regulatory light polypeptide
Q5EAD2	D-3-phosphoglycerate dehydrogenase
Q7SIH1	α -2-Macroglobulin
Q862P4	Similar to elongation factor-1-gamma
Q865V6	Macrophage-capping protein
V6F9A2	Apolipoprotein A-I preproprotein
Common to low risk and control	
A5D7E8	Protein disulfide-isomerase
G8JL04	Thymosin β 4 X-linked
Q95M18 ²	Endoplasmin
Unique to low risk	
A0JNP8	LOC516032 protein
A4IFI0	IGLL1 protein
A5PJE3	Fibrinogen α chain
A6QLL8	Fructose-bisphosphate aldolase
A6QNL5	PDIA6 protein
A8E641	DPYSL5 protein
B0JYP6	IGK protein
E1B925	ARID1A
E1BNF9	SLAMF9
E1BQ37	SFPQ
F1MAV0	Fibrinogen β chain
F1MDH3	TLN1
F1MY44	HNRNPM
F1N301	RPL22
F1N6D5	116-kDa U5 small nuclear ribonucleoprotein
G3X7A5	Complement C3
G3X8D6	Iodothyronine deiodinase
P00432 ²	Catalase
P07589	Fibronectin
P12234	Phosphate carrier protein, mitochondrial
P16116	Aldose reductase
P28801	Glutathione S-transferase P
P35705	Thioredoxin-dependent peroxide reductase, mitochondrial
P81644	Apolipoprotein A-II
Q0IIL9	Receptor protein-tyrosine kinase
Q0QF29	Malate dehydrogenase
Q1RMN8	Immunoglobulin light chain, lambda gene cluster
Q2HJH4	HNRPDL protein
Q2KJH6	Serpin H1
Q3MHY1	Cysteine and glycine-rich protein
Q3SZ20	Serine hydroxymethyltransferase, mitochondrial
Q3SZ62	Phosphoglycerate mutase

Continued

Table 1 (Continued). Unique proteins and representative accession number of proteins identified in control, high-risk, and low-risk groups using liquid chromatography–tandem MS¹

Accession no.	Protein name
Q3SZZ9	FGG protein
Q3T0B6	Complement component 1 Q subcomponent-binding protein
Q3T0E0	Copper transport protein ATOX1
Q3T101	IGL@ protein
Q3ZBF7	Prostaglandin E synthase
Q5E9B7	Chloride intracellular channel protein
Q8SQH5	ADP/ATP translocase
V6F832	Alpha-crystallin B chain
Unique to high risk	
Q5E962	Aldo-keto reductase family 1, member B1
S5G966 ²	Ribosomal protein L23a Y-linked protein
P48427	Tubulin-specific chaperone A
P63048 ²	Ubiquitin-60S ribosomal protein L40
A1L5B7 ²	SERPINE1 mRNA binding protein
Q2TBR3	SET nuclear oncogene
P62992	Ubiquitin-40S ribosomal protein S27a
G3MXG3	CCDC88A
P63243	Guanine nucleotide-binding protein
B9VPZ5	Lactoferrin
P61283	Barrier-to-autointegration factor
Q861P7	HLA-B-associated transcript
G5E5F7	MAGEB4
F1MTV9	Nucleophosmin
A6H767	Nucleosome assembly protein 1-like
Unique to control	
E1BKE1	FRYL
G3N131	G3N131

¹Proteins were counted if they were identified in ≥1 sample in each group.
²Protein was identified in ≥50% of cows in a particular risk group and 0% in the other risk group.

(gp96) and catalase. Endoplasmin is a chaperone protein with a key role in regulating folding of immune proteins and is therefore critical to immune function (Schild and Rammensee, 2000). This result supports previously discussed changes in gene expression related to immune regulation. Furthermore, recombinase-mediated loss of endoplasmin in hepatocytes leads to decreased cellular activity and compromises liver function (Rachidi et al., 2015), highlighting an important role in metabolic health. Catalase activity has been described in microvesicles, where it acts as a scavenger of reactive oxygen species and protects against oxidative damage (Hulsmans and Holvoet, 2013). These results may indicate enhanced cellular function in MDBK cells treated with exosomes from low-risk cows. However, no substantial proteomic changes can be related to the divergent metabolic states from where the exosomes originated.

The proteomic data presented here are consistent with previous studies investigating exosomes. Relative abundance between risk groups did not reveal significant changes associated with metabolic risk category. Lactate dehydrogenase was the only protein greater ($P < 0.05$; Supplementary Table S1; <https://doi.org/10.3168/jds.2016-12152>) in low-risk cocultures compared with high-risk cocultures (calculated using

relative protein abundance). Lactate dehydrogenase catalyzes the conversion of pyruvate to lactate with the regeneration of NADH to NAD⁺, which is essential in hypoxic and anaerobic conditions, and as a result is up-regulated in such conditions (Alegre et al., 2015). Several other proteins involved in cellular metabolism that are frequently identified in exosomes were identified by proteomic analysis in the current study—for example, histone proteins (H2B, H4, and H3.3), annexin proteins (A2), cofilin 1, heat shock proteins (60 kDa, 70 kDa), 14-3-3 proteins (sigma, zeta/delta), phosphoglycerate kinase, and ATP synthase (Mathivanan et al., 2010; Welton et al., 2010). The proteins identified in the current study are consistent with exosome-related changes in target cell metabolism; however, there is no clear evidence that the changes are related to a metabolic state of exosomal origin.

Limitations of the Study Design

We acknowledge that the number of animals included in each risk category is low; therefore, the results need to be considered with caution. The number of animals in each risk group (n = 5) was limited by the number of animals in the parent experiment (n = 45). Only 10 cows had significantly divergent metabolic profiles at

both wk 1 and 2 postcalving. Furthermore, exosomal protein isolated from the plasma samples was limited. Finally, only 2 concentrations of exosomes were used as a dose comparison (1 and 10 $\mu\text{g/mL}$). Based on the results of the dose-response proliferation curves (Figure 2), it is possible that comparisons including more dose rates would reveal further changes.

The low concentration of protein from the exosome and MDBK cell coculture likely contributed to the variation in peptides revealed by LC-MS/MS. Improved methods for identification of low-abundance peptides may have revealed changes in protein expression of physiological importance (Kedia et al., 2015). This is a novel preliminary study; the presented results could be validated or extended with a bigger starting cohort of cows. However, we have generated the maximum data from the limited sample numbers available.

CONCLUSIONS

The current study investigated the effect of plasma exosomes from 2 groups of metabolically divergent cows on MDBK cell function. We conclude that only limited, nonsignificant changes in gene expression and protein expression were evident when MDBK cells were exposed to exosomes derived from cows with high or low risk of metabolic dysfunction. The functional changes were inconclusive in determining the risk category of origin. However, this study provides a foundation for future studies investigating cross-talk between exosomes and target cells in cows with subclinical health dysfunction during the transition period.

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