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Proteome profiling of exosomes derived from plasma of heifers with divergent genetic merit for fertility

Yong Qin Koh,* Hassendrini N. Peiris,* Kanchan Vaswani,* Fatema B. Almughlliq,* Susanne Meier,† Chris R. Burke,† John R. Roche,†‡ Charlotte B. Reed,† Buddhika J. Arachchige,* Sarah Reed,* and Murray D. Mitchell*[1]

*University of Queensland Centre for Clinical Research, Faculty of Medicine, The University of Queensland, Brisbane, Queensland 4029, Australia
†DairyNZ Ltd., Private Bag 3221, Hamilton 3240, New Zealand
‡School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

ABSTRACT

The current study evaluated exosomes isolated from plasma of heifers bred to have high or low fertility through developing extreme diversity in fertility breeding values, however, key animal traits (e.g., body weight, milk production, and percentage of North American genetics) remained similar between the 2 groups. The exosomes were isolated by a combined ultracentrifugation and size exclusion chromatography approach and characterized by their size distribution (nanoparticle tracking analysis), morphology (transmission electron microscopy), and presence of exosomal markers (immunoblotting). In addition, a targeted mass spectrometry approach was used to confirm the presence of 2 exosomal markers, tumor susceptibility gene 101 and flotillin 1. The number of exosomes from plasma of high fertility heifers was greater compared with low fertility heifers. Interestingly, the exosomal proteomic profile, evaluated using mass spectrometry, identified 89 and 116 proteins in the high and low fertility heifers respectively, of which 4 and 31 were unique, respectively. These include proteins associated with specific biological processes and molecular functions of fertility. Most notably, the tetratricopeptide repeat protein 41-related, glycodelin, and kelch-like protein 8 were identified in plasma exosomes unique to the low fertility heifers. These proteins are suggested to play a role in reproduction; however, the role of these proteins in dairy cow reproduction remains to be elucidated. Their identification underscores the potential for proteins within exosomes to provide information on the fertility status and physiological condition of the cow. This may potentially lead to the development of prognostic tools and interventions to improving dairy cow fertility.

Key words: bovine, plasma, fertility, reproduction, exosomes

INTRODUCTION

Dairy cows with good fertility and reproductive performance are vital for the profitability and sustainability of dairy farms, especially in seasonal-calving pasture-based systems (Burke and Verkerk, 2010). As a consequence of the increased metabolic pressure for milk production in past decades, fertility of the dairy cow has decreased (Roche et al., 2011). Furthermore, an inflammation through immune (Formigoni and Trevisi, 2003; Piccinini et al., 2004) or infectious (McDougall et al., 2007) challenge can have long-term consequences for the fertility of dairy cows. This peripartum inflammatory state likely contributes to the decline in fertility and reproductive success by disrupting endocrine signaling, negatively affecting uterine homeostasis, and invoking perturbations in ovarian function and oocyte development (Karsch et al., 2002; Bromfield et al., 2015).

It has recently been established that exosomes can mediate inter- and intracellular signaling under normal and pathological conditions through the transfer of bioactive molecules (e.g., proteins and microRNA) that can modulate the biological function of the recipient cells (e.g., immune response, cellular adhesion, development, proliferation, and metabolism; Corrado et al., 2013; McGough and Vincent, 2016; Crookenden et al., 2017). Several studies have identified the involvement of exosomes in providing autocrine (i.e., local signals between the same cell type, such as cancer cells; Weaver, 2017), paracrine (i.e., local signals between different cell types, such as between epithelial cancer cells and stromal cells; Heneberg, 2016), and endocrine (i.e., distant signals between many types of cells usually

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[1]Corresponding author: murray.mitchell@uq.edu.au

carried in bodily fluids, such as blood; Zduriencikova et al., 2015) signaling. Exosomes are membranous nanovesicles of endocytic origin that are defined by characteristics such as size, density, and morphology as well as the presence of exosomal markers [e.g., tumor susceptibility gene (**TSG**) 101 and flotillin 1; Lötvall et al., 2014]. Concomitant changes in the concentration or the content of exosomes may provide informative details of the overall health condition of the animal, including the possible physiological status underlying fertility.

To improve our knowledge in key areas of dairy cow reproductive physiology, a long-term project was initiated using cows with similar key animal traits (e.g., BW, milk production, and percentage of North American genetics) but with extreme diversity in their EBV for fertility. It was envisaged that the underlying physiological basis of low-fertility heifers could be elucidated through the investigation of the content (e.g., proteins) encapsulated by exosomes isolated from the plasma of 2 cohorts of heifers that were identified to be of either low or high fertility. We hypothesized that heifers selected on the basis of differential fertility EBV would have exosomal signatures in plasma representative of their fertility genotype.

MATERIALS AND METHODS

Animals and Management

Heifers used in this study were part of a larger experiment (n = 60 Holstein-Friesian heifers) from a recently established fertility animal model described by Meier et al. (2017). From this larger group, 24 heifers were identified as being either high fertility (n = 12) or low fertility (n = 12) based on the extreme diversity in their EBV for fertility but being similar in genetic character for other key traits (e.g., BW, milk production, and percentage of North American genetics). The EBV for fertility is measured using 8 predictor traits. The predictor traits used to estimate the fertility breeding values include whether cows are mated within 21 d of the planned start of mating in first-, second-, and third-parity cows (expressed as a binomial trait); whether cows calved in the first 42 d after the planned start of calving in second-, third-, and fourth-parity cows (as a binomial trait); and milk volume in a cow's first lactation and BCS in a cow's first lactation at 60 DIM. The details of the genetic merit of the 24 heifers are presented in Table 1. All heifers used in the current study were postpubertal and had expressed a minimum of 1 spontaneous estrus (Meier et al., 2017). To minimize interventions and to align with other studies to better understand the underlying drivers of fertility in dairy cattle, the timing of sampling was aligned with an estrus event stimulated with prostaglandins as described by Reed et al. (2017). The animals and their management as well as the procedures for sample collection were managed by DairyNZ Ltd. (Hamilton, New Zealand) with approval by the Ruakura Animal Ethics Committee (AEC 13574 and 13934).

Blood Collection

Blood was drawn from a jugular vein into EDTA Vacutainer tubes (Greiner Bio-One, Kremsmunster, Austria). The tubes were then centrifuged at 1,120 × *g* for 12 min at 4°C, and the plasma aspirated was stored at −80°C.

Exosome Isolation from EDTA Plasma

Exosomes were isolated by coupling ultracentrifugation and size exclusion chromatography as described by Almughlliq et al. (2017). In brief, plasma (10 mL) of high- (n = 12) and low- (n = 12) fertility heifers was diluted with an equal volume of PBS (Gibco, Life Technologies Australia Pty Ltd., Mulgrave, Victoria, Australia) and centrifuged at 2,000 × *g* for 30 min at 4°C, 12,000 × *g* for 30 min at 4°C, and at 100,000 × *g* for 75 min at 4°C. It was then filtered through a 0.22-μm polyethersulfone membrane filter (Corning Inc., Corning, NY) and then ultracentrifuged at 100,000 × *g* for 120 min. The pellet was reconstituted in 500 μL of PBS and layered on a qEV size exclusion column (Izon Science, Christchurch, New Zealand). Fractions of 500 μL were collected in separate tubes (n = 16) per the manufacturer's instructions. The 16 fractions were concentrated using a vacuum concentrator (Eppendorf Concentrator Plus, Eppendorf, Hamburg, Germany) for 1.5 h at room temperature and stored at −80°C until further analyses.

Nanoparticle Tracking Analysis

The nanoparticle tracking analysis was conducted using a NanoSight (Salisbury, UK) NS500 instrument (NanoSight NTA 3.0 Nanoparticle Tracking and Analysis Release Version Build 0064) as previously described (Kobayashi et al., 2014; Salomon et al., 2014). This method was used to determine the size distribution and total exosome particle number of the EDTA plasma.

Western Blot Analysis

The proteins were extracted from the isolated exosomes using radioimmunoprecipitation assay buffer (Sigma-Aldrich, Castle Hill, New South Wales, Austra-

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