

The frontiers of biomedical science and its application to animal science in addressing the major challenges facing Australasian dairy farming

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Abstract. Extraordinary advances are occurring in biomedical science that may revolutionise how we approach health and disease. Many have applications in the dairy industry. We have described one particular area of extracellular vesicles that have already proven to be of interest in diagnostics and prognostics for fertility and assessment of 'transition' cows (i.e. evaluation of the problems related to the risk of clinical diseases in dairy cows, such as mastitis and milk fever, during transition period). The addition of measurements of circulating RNA and DNA may prove of value in identifying dairy cows with higher risks of clinical diseases and potentially poor fertility. We describe the exciting opportunity provided by the possibility of generating exosomes to order as therapeutic agents to potentially enhance fertility. The even more radical concept of using exosomes to deliver a CRISPR-linked gene editing function is presented. Undoubtedly, the use of biomedical advances to assist the dairy industry is an obvious and practical approach that has significant merit.

Additional keywords: dairy cow, exosomes, fertility.

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Introduction

Dairy cow health and fertility have declined over successive decades with genetic selection for increased milk production (Garnsworthy *et al.* 2008; Roche *et al.* 2011), while, at the same time, consumers have begun to focus more consistently on animal welfare and the desire to ensure that production animals have 'a life worth living' (Adamczyk 2018). In most industries, therefore, genetic-selection priorities have changed from productivity focussed to a better balance of production and functional traits, and management practices are being more intensely scrutinised for their effect on both the animal's functional and affective states (Mellor 2016). Historically, many of the functional traits have been difficult to measure. However, with the development of new technologies for understanding the health and function of different tissues, there is a real opportunity to advance genetic selection for animal health and well-being and to individualise animal care through understanding the effect of farm management on each animal.

The use of non-invasive or minimally invasive methods for diagnosing or predicting the onset of disease or inflammation in real-time or categorising metabolic state patient-side is revolutionising human medicine (Mal 2016). New rapid and precise technologies for genomic, transcriptomic and metabolomic testing are constantly being developed and improved on. In medical practice, they offer clear advantages in patient comfort and well-being, due to either the immediacy of results (i.e. patient side and in real time) and the limited resources required that are declining in price exponentially, or by avoiding investigatory procedures that require periods of post-operative convalescence (Andión *et al.* 2014; Jo Delaney 2018).

These technologies and approaches can be transferred into animal science and veterinary clinical practice, enabling detailed tissue phenotyping without biopsies, and to better understand the effects of farm management on an animal's functional and, possibly, affective states. Although blood sampling is at the core of these methods, there is also the potential use of milk

in the case of dairy cows (Fricke *et al.* 2016). The use of saliva and urine, although possible, is more problematic in cattle.

At least two approaches to minimally invasive deep-tissue phenotyping have been advanced in recent years. One has been the use of circulating (free) DNA and RNA (Lo 2016; Lo and Lam 2016). In this, translation into clinical practice is occurring (Sun *et al.* 2017, 2018). In parallel, but perhaps slightly behind, is research into the use of exosomes as both diagnostic and therapeutic drug-delivery mechanisms. In the space available, we will focus in the potential uses of exosomes in dairy cow research.

Exosomes: the new frontier in patient care

Exosomes are small (40–120 nm), stable, lipid-bilayer nanovesicles that are formed by the inward budding of multivesicular bodies and are released into extracellular compartments, and identified in biological fluids (e.g. in milk, blood, urine and saliva; Mitchell *et al.* 2015; Koh *et al.* 2017). Through the analysis of tissue-specific markers on the exosomes, the tissue of origin can be determined (Salomon *et al.* 2014). They contain a diverse array of signalling molecules, including messenger RNA (mRNA), micro-RNA (miRNA), proteins, lipids and membrane receptors (Hata *et al.* 2010; Record *et al.* 2014; Gangoda *et al.* 2015; Mitchell *et al.* 2015), and they interact with target cells via multiple pathways. They can either directly activate target cell-membrane receptors, modify the extracellular milieu of the target cell, or fuse with the cell membrane and release their molecular cargo into the target cell (Cronin *et al.* 2012).

The cargo of circulating exosomes is indicative of the health status of a specific tissue, granting the capacity for use of exosomes as a tool for disease diagnosis without invasive and expensive biopsy techniques. The use of exosomes as diagnostic agents is particularly prevalent in the early detection of cancers (Melo *et al.* 2015; Kalluri 2016), although they offer considerable promise in the detection of other diseases also because differences in their cargo are being linked to disease states (Mitchell *et al.* 2015; Ngo *et al.* 2018). For example, exosomes have a role in the pathogenesis of neurodegenerative disease such as Alzheimer's and Parkinson's disease and exosomal content from tissues differs when cancerous tumours are present (Taylor and Gercel-Taylor 2008; Vella *et al.* 2016). Excitingly, exosomes have the potential to be 'loaded' with pharmaceutical agents or intracellular mediators including miRNAs (Lakhal and Wood 2011; Luan *et al.* 2017), which means that they could be prognostic, diagnostic or therapeutic agents.

The importance of exosomes in health and disease in humans has incited research into exosomes in dairy cattle and may lead to their application as a tool for diagnosing bovine health status (a summary of selected bovine exosome publications is provided in Table S1, available as supplementary material for this paper). With limited space to explore the role of these technologies in the animal sciences, we will focus on the role of exosomes as potential diagnostic and prognostic methods for fertility and transition cow health in dairy cows, as well as the use of exosomes for therapeutics. We will also incorporate, possibly, the most exciting advance in biomedical science in recent years, namely the CRISPR–Cas9 system for gene editing

(Hille *et al.* 2018), with research in exosomes (Kim *et al.* 2017; Lin *et al.* 2018).

The decline in dairy cow fertility

The decline in dairy cow fertility has been attributed to the intensive genetic selection that has focussed primarily on milk-production traits. Until recently, very few selection indices have included functional traits, such as the health and reproduction of the dairy cows (Miglior *et al.* 2005; Walsh *et al.* 2011; Berry *et al.* 2016). As a result, milk-production capacity has increased dramatically, but fertility has steadily declined (Garnsworthy *et al.* 2008; Roche *et al.* 2011). Consequently, it has been estimated that 50% of the improved profitability from genetic selection for milk production has been lost, at least in grazing systems, due to the decreased productivity associated with reduced fertility (Evans *et al.* 2006). Poor reproductive efficiency in dairy herds results in longer inter-calving intervals, lower voluntary culling and greater replacement rates, increased cow maintenance costs, and slower genetic progress (Royal *et al.* 2000). Indications of poor fertility include the frequent occurrence of abnormal oestrus cycles, poor conception rates to first and successive inseminations and loss of pregnancies (Royal *et al.* 2000).

Fertilisation rates in single-ovulating dairy cows remain relatively high at above 80% and, as such, are not thought to be the issue associated with the production-related loss of fertility (Diskin *et al.* 2006; Sartori *et al.* 2010; Walsh *et al.* 2011). However, early embryonic loss is of concern, as greater rates of embryonic loss/fetal mortality and resulting lower calving rates occur to a greater extent in high-producing dairy cows than in moderate-producing dairy cows (Diskin *et al.* 2006; Diskin and Morris 2008; Walsh *et al.* 2011). The inability of the embryo to signal its existence (e.g. failure to initiate maternal recognition of pregnancy is one factor associated with early embryonic loss; Walker *et al.* 2010). A key component in this signalling is interferon tau, which is required to inhibit the release of anti-luteolytic prostaglandin F_{2α} (PGF_{2α}), and thus prevent the degradation of the corpus luteum and enable the continued release of progesterone, which is required for the maintenance of pregnancy (Diskin *et al.* 2006; Diskin and Morris 2008; Walsh *et al.* 2011).

Dairy cow health and the transition into lactation

A successful transition into lactation requires several metabolic, nutritional, physiological and immunological changes to occur in concert (i.e. homeorhesis). These changes make the transition period the time of greatest risk of metabolic and infectious disease for dairy cows, with an estimated third to a half of all cows succumbing to disease (LeBlanc 2010). In fact, in grazing systems, the risk of death during the first 3 weeks post-calving is three to six-fold greater than that at any other stage of lactation (Compton 2018).

Animals unable to meet the metabolic demands associated with the onset of lactation will experience metabolic disorders such as milk fever, ketosis, fatty liver, enteritis and displaced abomasum (Bigras-Poulin *et al.* 1990; Ingvarsen 2006). Likewise, bacteria-induced inflammatory disorders, such as mastitis and uterine infection, are most prevalent in the first

few weeks post-calving (Sheldon and Dobson 2004; Østergaard *et al.* 2005) and can have profound effects not only on the animal's health, but on the odds of successfully breeding subsequently (LeBlanc 2010). The severity of these inflammatory disorders and the ability to resolve their infections vary greatly among cows, suggesting that some cows are more at risk to the disease and the failure to adequately resolve (Formigoni and Trevisi 2003; Piccinini *et al.* 2004; McDougall *et al.* 2007; Fair 2015). For example, persistence of bacterial contamination in the uterus can result in inflammation, histological lesions of the endometrium, delayed involution and lower conception rates (i.e. negatively affect embryo survival; Sheldon *et al.* 2006; Herath *et al.* 2009).

There is a critical need to identify dairy cows with high fertility and those more vulnerable or resistant to disease. The identification of these animals using biomarkers could lead to early interventions and higher survival rates, resulting in reproductive success. Exosomes offer a potential route for the discovery of such markers.

Role of exosome in dairy cow fertility and health

Recently, we investigated the exosomal content of dairy cows differing in their metabolic health status during the onset of lactation (Crookenden *et al.* 2016b), their uterine health status (Almughlliq *et al.* 2018) and their fertility status (Mitchell *et al.* 2016; Koh *et al.* 2018). The exosomes isolated in each population of dairy cows demonstrated protein cargo profiles different from those of their control counterparts, indicating that the proteomic profile of an exosome is influenced by the 'environment' in which it is packaged. Still to be uncovered in these exosomes are the other signalling molecules which they carry, such as miRNA. The differences in exosomal cargo could be of utility in the development of biomarker panels for identification of health/disease status in dairy cows or, potentially, remedial therapy to improve health or the odds of a successful pregnancy.

The miRNA content of bovine milk exosomes differs depending on the stage of lactation (e.g. colostrum in contrast to mid-lactation milk; Hata *et al.* 2010). Several of the miRNAs and proteins identified in milk exosomes are those that have important roles in maintaining mammary gland health and initiating the immune response (Hata *et al.* 2010; Reinhardt *et al.* 2012; Koh *et al.* 2017). This implies that milk exosomes may have a role in protecting the mammary gland from infection and responding to bacterial pathogens. Indeed, exosomes isolated from bovine milk have unique cargo during mastitis when compared with milk from non-infected animals. For example, milk exosome miRNA profiles differ before, and 48 h after, infection of the mammary gland with *Staphylococcus aureus*, which may lead to the identification of biomarkers of subclinical mastitis (Sun *et al.* 2015). Sun *et al.* (2015) identified 14 miRNAs that were significantly differentially expressed between the control and infected animals and suggested that exosomal miRNAs miR-223 and miR-142-5p may be used as biomarkers of early mammary-gland infection. Furthermore, this gives insight into the host defence mechanism that could be utilised for development of novel treatments of bacterial infection without the need for antibiotics.

MicroRNA-223 was identified only in milk exosomes from infected animals and miR-142-5p was upregulated over 250-fold in exosomes from *S. aureus*-infected animals compared with control animals (Sun *et al.* 2015). In concordance with this, Cai *et al.* (2018) also identified significant upregulation of miR-223 and miR-142-5p in milk exosomes of cows with experimentally induced *S. aureus* infection compared with control animals. This further highlighted these miRNAs as clinically important in mastitis and as potential targets for mastitis diagnosis (Cai *et al.* 2018).

The importance of exosomes in communication among cells, including those of the immune system, is well established (Théry *et al.* 2009). Interestingly, miR-223 is an important regulator of innate immune function and is involved in granulopoiesis, the haematopoietic process of granulocyte maturation (Fukao *et al.* 2007). Neutrophils are one such granulocyte and produce exosomes that have an important role in the inflammatory response (Gasser and Schifferli 2004). Neutrophil function is dampened over the calving period with the transition into lactation, which is evident by gene-expression changes that are likely to increase the risk of infectious disease (Detilleux *et al.* 1995; Crookenden *et al.* 2016a). Exosomes are involved in both immune stimulation and tolerance, depending on the cell origin (Raposo *et al.* 1996), and several studies have proposed the potential use of exosomes in immunotherapy (Pêche *et al.* 2003; Aline *et al.* 2004; Morse *et al.* 2005). Therefore, it is likely that exosomes could be used as a solution to improve immune function, which would be particularly useful during the challenging transition period and during the establishment of embryo within the uterus in early pregnancy.

Roles for exosomes in communication during the development of gametes (Sullivan *et al.* 2005), culture of embryo *in vitro* (Qu *et al.* 2017) and between the embryo and mother (Cleys *et al.* 2014; Saadeldin *et al.* 2015) have also been described. Our recent investigations have focussed on the effects of exosomes isolated from cows with differing functional characteristics (i.e. divergent genetic merit for fertility, metabolically divergent and the presence or absence of subclinical endometritis on target-cell functions; e.g. cell proliferation and gene expression; Almughlliq *et al.* 2017; Crookenden *et al.* 2017). Our published findings have shown that endometrial gene expression is altered by exosomes from metabolically divergent animals and those with subclinical infection. Our, as yet, unpublished findings have also indicated a possible role for exosomes in the recovery of the uterus postpartum for subsequent reproduction (F. B. Almughlliq, Y. Q. Koh, H. N. Peiris, K. Vaswani, S. Meier, C. R. Burke, J. R. Roche, M. A. Crookenden, B. J. Arachchige, S. Reed and M. D. Mitchell, unpubl. data). Moreover, exosomes from the dairy cows genetically selected for inferior reproductive performance significantly altered the production of prostaglandins by endometrial cells towards a pattern negatively associated with the maintenance of pregnancy (Fig. 1; lower ratio of prostaglandin E₂ (PGE₂) to PGF_{2α}). These findings suggest that low-fertility exosomes carry compounds that prevent the necessary suppression of the anti-luteolytic PGF_{2α} (anti-luteolytic factor) and, therefore, potentially prevent the establishment/maintenance

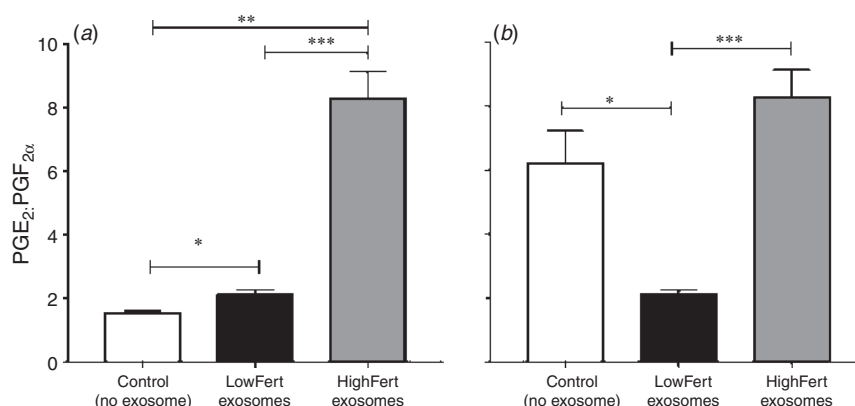


Fig. 1. Ratio of prostaglandin E₂ (PGE₂) to prostaglandin F_{2α} (PGF_{2α}) production by (a) bovine endometrial epithelial cells (bEEL) and (b) bovine endometrial stromal cells (bCSC) following co-incubation with exosomes isolated from the plasma of dairy cows designated low fertility (LowFert Exosomes) or high fertility (HighFert exosomes). Non-parametric Kruskal–Wallis one-way ANOVA $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$. Data are means \pm s.e.m. ($n = 12$).

of pregnancy. The mechanism by which these low-fertility exosomes prevent this suppression is still unclear and needs further investigation. However, collectively, our research results have indicated that exosomes are able to package, protect and deliver content that is capable of altering the functions of peripheral tissues in dairy cows.

Exosomes, therefore, may provide a solution for (1) identifying cows that differ in their fertility function and/or their risk of developing disease during the transition period, enabling the identification of those animals that would benefit from early intervention, and (2) disease or subfertility treatment, utilising them as molecular delivery systems to deliver cargo to specific cell types. If we can achieve these solutions, we will be able to ameliorate diseases that create major challenges for dairy farmers and animal welfare.

Exosomes as a therapeutic drug-delivery vehicle

In human medicine, several molecules such as proteins, miRNA, mRNA, small interfering RNA (siRNA), and various chemical drugs and antivirals have been loaded into exosomes with the objective of using the exosomes as a drug-delivery vehicle. For example, Haney *et al.* (2015) introduced the protein catalase into exosomes, while miRNAs have also been encapsulated (Momen-Heravi *et al.* 2014).

An exosome may be an ideal candidate vehicle for delivering therapeutics. They have many of the desirable features of an ideal drug-delivery system, such as a long circulating half-life, the intrinsic ability to target tissues and biocompatibility (Ha *et al.* 2016), meaning that they are less likely to suffer immune rejection by the recipient's body (Ha *et al.* 2016). For example, minimal adverse effects have been reported with the cross-species treatments (Zhu *et al.* 2017). Furthermore, encapsulating drugs or anti-inflammatory compounds in exosomes appears to increase the effectiveness of the delivered material. For example, the inflammatory activity of the phenolic compound curcumin is enhanced when it is encapsulated in exosomes (Sun *et al.* 2010).

Recently, the anti-cancer drug paclitaxel displayed a 50-fold increase in efficacy when encapsulated within an exosome compared with when it was introduced directly (Kim *et al.* 2016). Exosomes encapsulating molecules may, therefore, serve as a more potent therapeutic delivery mechanism.

Milk exosomes, in particular, are ideal vehicles as they can deliver content across species with bovine milk exosomes known to be taken up by human phagocytes (Pitari *et al.* 2000; Izumi *et al.* 2015) and minimal adverse immune or inflammatory responses have been reported (Munagala *et al.* 2016). In the dairy industry, a major advantage of using milk to generate exosomes is that large volumes of milk are readily available. Collections can be made more frequently than with other fluids such as plasma. Large numbers of high-purity exosomes (fewer contaminating particles) can also be obtained from milk than from some other fluids such as saliva and urine (Koh *et al.* 2017; Vaswani *et al.* 2017).

Using exosomes as a vehicle is also advantageous as they can be introduced into the body via several routes. Some routes of administration include intranasal spray, intravenous injection and orally via injecting (i.e. by drinking fortified milk, among others). Zhuang *et al.* (2011) used curcumin encapsulated exosomes from the nasal region to the brain. Another research group has studied the effects of administering exosomes both intradermally and subcutaneously (Hao *et al.* 2006).

The development of targeted drug-delivery system for within the brain has been hindered by the inability of therapeutic molecules, with proven *in vitro* efficacy, to cross the blood brain barrier. A study by Alvarez-Erviti *et al.* (2011) highlighted the potential of exosomes to cross the blood–brain barrier. Here, *GAPDH* siRNA was loaded via electroporation into RVG exosomes, and delivered by intravenous injection into the tails of C57BL/6 mice (Alvarez-Erviti *et al.* 2011). Following which, a gene-specific knockdown as mediated by the exosomes was observed in the striatum, mid-brain and cortex of these mice (Alvarez-Erviti *et al.* 2011). Zhuang *et al.* (2011) used exosome-encapsulated curcumin for brain-related complications. Several other studies have reported

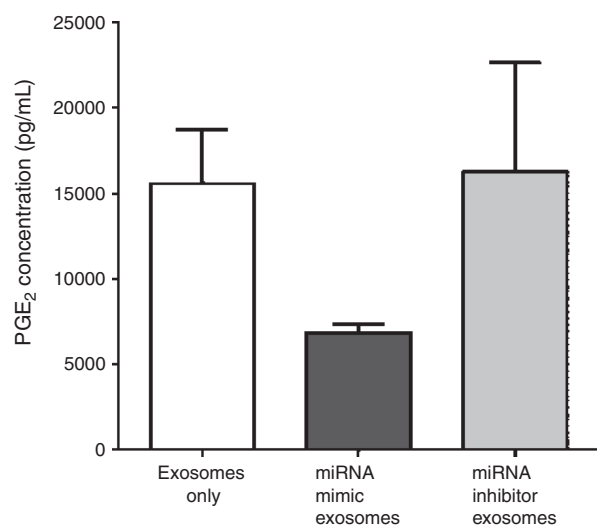


Fig. 2. Treatment of bovine endometrial epithelial cells (bEEL) with exosomes only, exosomes loaded with miRNA mimic (miR-143) and exosomes loaded with miRNA 143 inhibitor by incubation. Prostaglandin E₂ (PGE₂) production is decreased by half after co-incubating cells with miRNA mimic (miR-143)-loaded exosomes.

similar abilities for exosomes to cross the blood–brain barrier and affect brain functions (Zhuang *et al.* 2011; Ridder *et al.* 2014; Haney *et al.* 2015).

Currently there are several methods of exosomal loading (i.e. incorporation of molecules into an intact exosome vesicle). These involve sonication, incubation, electroporation, blue light and chemical-based methodologies (Sun *et al.* 2010; Kim *et al.* 2016; Yim *et al.* 2016; Qu *et al.* 2017). Luan *et al.* (2017) suggested that passive loading via incubation may be less disruptive to exosomes; however, electroporation has proven successful in several studies and seems to be more widespread in its use (Alvarez-Erviti *et al.* 2011; Luan *et al.* 2017). To enable detection of loaded exosomes after delivery in *in vivo* models, fluorescent dyes labelling the exosome membrane are employed (Zhuang *et al.* 2011). DiO, DiR and PKH lipophilic dyes are commonly used in both *in vitro* and *in vivo* studies, as they have an affinity for the exosome membranes (Zhuang *et al.* 2011; Ohno *et al.* 2013; Tian *et al.* 2014).

We recently evaluated the possibility of loading miRNA into milk exosomes (Fig. 2). The miRNA chosen was miR143 due to its ability to inhibit the action of cyclooxygenase (COX-2; also known as prostaglandin-endoperoxide synthase 2, prostaglandin H synthase; Ochs *et al.* 2011). COX-2 is an enzyme that catalyses the conversion of arachidonic acid to prostaglandins (e.g. PGE₂ and PGF_{2α}), which are crucial for the initiation and maintenance of pregnancy. Therefore, it serves as a potential miRNA of choice for loading into exosomes as a therapeutic agent for drug delivery, to improve reproductive function. The incubation methodology evaluated for the loading of miR143 and its inhibitor (specifically inhibit miR143 function) was that of 1 h at 37°C, as described by (Johnsen *et al.* 2014). The miR143 and miR143 inhibitor-loaded exosomes were co-incubated with bovine endometrial epithelial cells. Media was collected after 24 h of incubation and PGE₂ production measured. The production of PGE₂ by bovine endometrial

epithelial cells decreased when treated with loaded miR143 milk exosomes. This inhibitory effect on PG production is in line with the expected result, as miR143 has been documented to suppress Cox2 (Ochs *et al.* 2011). Still to be evaluated is the gene and protein expression of COX-2 in these cells. These results are promising and, with further development, the technique could be a potential candidate as a therapeutic agent to improve reproductive function.

The limitations currently arising in exosome applications are due to the infancy of this research field. As yet, the mechanisms by which exosomes target other cells (i.e. selectively deliver their content) are largely unknown. Another limitation is identifying the cell site of origin. By better understanding these two issues, both the sensitivity in diagnostics and therapeutics can be improved. Recent publications have shown an improvement in the ‘signal to noise ratio’ of knowing the cell site of origin, with examples being the use of cell-surface marker Glypican-1 for exosomes of cancer-cell origin, placental alkaline phosphatase for exosome from placental origin and Interferon tau for exosomes of bovine conceptus origin (Sarker *et al.* 2014; Melo *et al.* 2015; Nakamura *et al.* 2016). Several publications have also described ways of modifying the surface of an exosome in an attempt to target their delivery. These include both covalent and non-covalent strategies for the incorporation of target molecules on the surface of pre-isolated exosomes (Smyth *et al.* 2014; Wang *et al.* 2014; Nakase and Futaki 2015; Hood 2016; Armstrong *et al.* 2017; Luan *et al.* 2017) as well as using transfection to generate exosomes with targets on their surface. Through further development of methodologies to identify cells of origin and incorporate target structures, the potential of exosomes in diagnostics and therapeutics will greatly improve.

Conclusions and implications

The discovery of long-distance, inter-cellular nanoparticle messengers has provided a potential diagnostic and therapeutic technology. There is increasing evidence indicating that tissues differentially populate exosomal cargo, depending on their health status and functional state, providing a potential diagnostic platform for diseases that, historically, could have been detected only via invasive procedures, such as operations or biopsies. Furthermore, understanding the role of these molecular cargo components could provide new therapeutic drugs, but, more importantly, the exosome itself provides a vehicle for drug delivery that is targeted, long-lived, and, importantly, not prone to negative host reactions. As the physical technology that allows the measurement of exosomal cargo develops, such that it is easier, faster and less expensive to measure the contents of the exosomal cargo, it is plausible that in the next decade cow-side tests for the most common or important diseases and therapeutic solutions will be developed.

Conflicts of interest

The authors declare no conflicts of interest.

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