Invited review: MicroRNAs in bovine colostrum—Focus on their origin and potential health benefits for the calf

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ABSTRACT

Colostrum is the first milk produced by a cow after she gives birth. Compared with mature milk, it has a high concentration of immunoglobulin G. Calves are born without circulating antibodies, thus ingestion of colostrum is necessary to protect the calf against pathogens in the first challenging weeks of life. In addition to the life-saving supply of antibodies, colostrum contains minerals, vitamins, growth factors, and immune cells. Recently, microRNAs (miRNAs) were added to that list. MicroRNAs are short, non-coding RNA molecules that can regulate gene expression at the post-transcriptional level. They are thought to act as key regulators of diverse biological and developmental processes. Colostrum contains higher amounts of miRNAs than mature milk; immune- and development-related miRNAs are prominent. Their expression pattern in milk is likely to be influenced by maternal nutrition and environment. The fat content of the maternal diet appears to have a major effect on expression of miRNAs in milk and in the neonate. The immunological state of the mammary gland seems to affect miRNA expression as well. In cows diagnosed with subclinical mastitis, alterations in the expression of miRNAs in milk have been observed. It is believed that miRNAs in colostrum and milk are signaling molecules passed from mother to newborn. They are packaged in extracellular vesicles, which makes them resistant to the harsh conditions in the gastrointestinal tract. Therefore, they can reach the small intestine, where they are absorbed and transferred into the bloodstream. MicroRNAs are important for the development of the intestines. For example, miRNAs stimulate cell viability, proliferation, and stem cell activity of the intestinal epithelium. Furthermore, miRNAs seem to act as key players in the development of the complete immune system. They can, among other

things, regulate B- and T-cell differentiation and affect interleukin production of macrophages. The abundance of miRNAs in colostrum and milk and the possibility for their absorption in the intestines of the neonate supports the hypothesis that these tiny molecules are important for the development of the newborn. The probable relation of diet to the expression of miRNAs by the mother creates a possible avenue to optimize expression of miRNAs and improve neonatal maturation. **Key words:** colostrum, microRNA, neonatal development, dairy cow

INTRODUCTION

Calves are born agammaglobulinemic, meaning that they lack circulating antibodies. Their acquired immune system is completely present at birth but is not yet operational (Chase et al., 2008). In the first weeks of life, the neonate's ability to fight off pathogens is therefore fully dependent upon ingesting colostrum. Colostrum is the first milk that a female mammal produces after giving birth. In cows, colostrum contains a large portion of IgG, which is absorbed in the calf's intestines and transported to the bloodstream to protect the calf from pathogens (Chase et al., 2008). This transfer of passive immunity is crucial for the calf's survival in the first challenging weeks of life. Absorption efficiency of IgG is highest during the first hours of life and ends approximately 24 h after birth ("gut closure"; Lora et al., 2018). Calves unable to absorb enough antibodies from colostrum have a higher risk of morbidity or death at a young age (1.91 and 2.12 times more likely, respectively; Raboisson et al., 2016).

Modern farmers are becoming more aware of the importance of fostering the health of newborn and young cattle to breed high-yielding dairy cows. Early life development can even determine the lifetime performance as a lactating dairy cow (Soberon et al., 2012).

In addition to transmitting antibodies, colostrum provides many other important nutrients and bioactive components, such as fat, protein, hormones, growth

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factors, vitamins, and minerals (Kehoe et al., 2007). Recently, microRNAs (**miRNAs**) have been added to that list (Chen et al., 2010).

MicroRNAs are short, noncoding RNA molecules of approximately 21 to 25 nucleotides (nt; Lau et al., 2001). In 1993, Lee et al. discovered that the *lin-4* gene in *Caenorhabditis elegans* encoded a small RNA molecule that seemed to regulate translation of the *lin-14* mRNA (Lee et al., 1993). It took almost 7 yr until the next gene that encodes a short RNA transcript, *let-7*, was discovered, again in the nematode *C. elegans* (Reinhart et al., 2000). One year later, these novel short RNA molecules were categorized in a new class and thereafter referred to as miRNAs (Lagos-Quintana et al., 2001). Today, more than 38,000 precursor miRNAs, expressing almost 49,000 mature miRNAs have been discovered in 271 species (miRBase, release 22; Kozomara and Griffiths-Jones, 2011).

This review focuses on the bovine species and discusses the miRNA expression pattern in colostrum with possible effects on the calf after miRNA ingestion. Management factors that can alter miRNA expression patterns in colostrum are also presented.

MICRORNA EXPRESSION IN COLOSTRUM AND MILK: AN EVOLUTIONARY ADVANTAGE FOR MAMMALS?

The mammalian species arose more than 200 million years ago and can be distinguished from other species by the development of a mammary gland that produces milk as part of the reproductive strategy (Lefèvre et al., 2010). Besides its nutritional value, milk has a distinct function in delivering biochemical signals to the neonate. These maternal regulators (e.g., miRNAs), direct the newborn's growth and development (Power and Schulkin, 2013).

MicroRNAs are, in general, highly conserved between different species. For example, the *let-7* family is detected in arthropods, nematodes, fish, birds, and mammals (Lee et al., 2007). In other words, at some point throughout evolution, post-transcriptional regulation by miRNAs became indispensable. However, similarities in miRNA sequences between different species does not mean they share the same function. In arthropods, *let-7* plays a role in the transition from larval to adult stage, whereas in mammals, the *let-7* family contains more members and are involved in neural differentiation, limb development, and oncogenesis in the lung (Lee et al., 2007). As the structural complexity of organisms increases, so too does the number of novel miRNAs detected. It is thought that this increase in miRNAs is the basis for the formation of new tissues and organs throughout evolution (Lee et al., 2007). This implies

that a more complex expression of miRNAs contributed to the evolution of a mammary gland and lactation. MicroRNAs therefore have 2 functions: (1) the miRNAs expressed in mammary gland tissue are related to the functionality of the mammary gland itself; and (2) recent discoveries of circulating miRNAs in mammals in several body fluids, including colostrum and milk (Chen et al., 2010; Weber et al., 2010), indicate that these miRNAs play a key role in the communication between mother and offspring. This could mean that they are valuable for neonatal development.

MICRORNA EXPRESSION PATTERNS IN THE MAMMARY GLAND

The miRNA first discovered in *C. elegans*, *lin-4*, plays a key role in the development from larval to adult stage, and its expression is stage-specific. At the end of the first larval stage, expression is activated. The *lin-4* gene is a negative regulator of the LIN-14 protein: an increase in *lin-4* induces a decrease in LIN-14 protein, which seems to be a crucial step in the development from larvae to adult (Lee et al., 1993). When we apply this knowledge to development of lactation in the cow, we can hypothesize that miRNAs could contribute to the regulation of this process. Moreover, by understanding miRNA expression patterns in the mammary gland during lactation, we might gain insight into the origin, regulation, and function of miRNAs expressed in colostrum and milk.

In the study of Li et al. (2012b), the miRNA expression profile of mammary gland tissue originating from a cow in lactation (2 mo postpartum) was compared with mammary gland tissue of a nonlactating, nonpregnant cow. Of the 544 miRNAs detected in both libraries, only 56 were differentially expressed (**DE**) between the lactation and nonlactation period (Table 1). For example, the expression of miR-141, which downregulates the translation of signal transducer and activator of transcription 5 (STAT5) protein, is reduced in the mammary gland during lactation (Li et al., 2012b). The STAT5 protein is crucial for alveolar development in the mammary gland and subsequently for lactogenesis (Liu et al., 1997). Therefore, it is likely that miR-141 plays a role in the regulation of lactation. In another study, 13 miRNAs associated with cellular proliferation, lipid metabolism, and innate immunity were selected to perform a real-time reverse-transcription PCR on bovine mammary gland tissue from different lactational stages (dry period, fresh period, and early lactation; Wang et al., 2012). All but one of the miRNAs (miR-31) seemed to be downregulated in the dry period compared with during lactation (Table 1; Wang et al., 2012). Because of the small subset of miRNAs analyzed, these results

Table 1. Comparison of differentially expressed (DE) microRNAs (miRNAs) in mammary gland tissue during different lactation stages

Sample period	Species	Detection method ¹	DE miRNAs/ total detected	Upregulated miRNAs ² in mammary gland tissue	Reference
Dry period vs. lactation	Bovine	RT-qPCR	13/13	In lactation: miR-10a, miR-15b, miR-16, miR-21, miR-31, miR-33b, miR-145, miR-146b, miR-155, miR-181a, miR-205, miR-221, and miR-223	Wang et al. (2012)
Nonlactation vs. lactation	Bovine	NGS	56/544	In non-lactation: miR-10a, miR-146b, miR-205, miR-221 In lactation: miR-33b	Li et al. (2012b)
Transition vs. peak lactation	Caprine	NGS	131/568	In transition period: miR-15b, miR-21, miR-155, miR-221, miR-223	Hou et al. (2017)
Dry period vs. lactation	Caprine	NGS	169/294	In dry period: miR-15b, miR-16, miR-21, miR-31, miR-145, miR-181a, miR-205, miR-221, and miR-223	Li et al. (2012a)

¹RT-qPCR = reverse transcription quantitative PCR; NGS = next-generation sequencing.

²Solely DE miRNAs from the 13 miRNAs selected by Wang et al. (2012) are represented.

cannot confirm an overall downregulation of miRNAs in the dry period. Lactation-related differential expression of miRNAs seems to be present in other ruminants as well. In the dairy goat, 169 miRNAs were differentially expressed, with 165 downregulated and 4 upregulated miRNAs during peak lactation (75 d postpartum) compared with the dry period (Li et al., 2012a). A total of 131 miRNAs were identified as differentially expressed with 45 upregulated and 86 downregulated miRNAs in the transition (2 d postpartum) versus peak lactation (90 d postpartum; Hou et al., 2017). The transition is the period between colostrum and mature milk production and lasts for approximately 5 d. In this period, colostrum gradually changes into milk as the protein (mainly immunoglobulins) and fat contents decrease (Sánchez-Macías et al., 2014).

Several miRNAs that show a higher expression in dairy goat mammary glands during mid lactation (120 d postpartum) compared with early lactation (30 d postpartum) were linked to milk fat synthesis in the mammary gland (Lin et al., 2013). Moreover, expression of these particular miRNAs seemed to be upregulated with a higher concentration of prolactin, a hormone that regulates lactation (Lin et al., 2013). Overall, it is plausible that miRNAs play a part in regulating lactation and that their expression is related to different stages of lactation. However, it remains unclear how miRNA expression in the mammary gland is regulated and which mechanism underlies the differential temporal expression. Study results show some important contradictions. For example, miR-10a, miR-146b, miR-205, and miR-221 were upregulated in lactational mammary gland tissue in the study of Wang et al. (2012), whereas Li et al. (2012b) reported upregulation of these miRNAs

in nonlactational mammary gland tissue (Table 1). The reason for this is unclear; a difference in the animal's management's history (e.g., environment, nutrition) or differences in RNA isolation and detection methods used have been suggested (Izumi et al., 2012). Another explanation could be interspecies dissimilarities (Silveri et al., 2006; Wang et al., 2012). This could explain the difference in miRNA expression between the caprine and bovine mammary gland, which is plausible because even miRNA expression in the mammary gland between 2 cattle breeds differs. Comparison of miRNA expression in the mammary gland between Holstein-Friesian and Limousin heifers showed differential expression of 54 miRNAs, and the majority of these miRNAs were upregulated in Limousin heifers (Wicik et al., 2016). The miRNAs that differed most between the 2 breeds seemed to inhibit genes related to metabolic processes and signaling pathways that are important in stem cell activity and the development of the mammary gland (Wicik et al., 2016). This was explained by the more advanced development of the mammary gland of dairy cows compared with beef cows (Wicik et al., 2016), with the result that these genes are less inhibited. However, the sample size in that study was low—only 4 animals per group were sampled. Moreover, samples were collected at the slaughterhouse, which means that no background information (e.g., management, nutrition, treatment, lactation stage) of the animals was available. Further studies are needed to confirm DE of miRNAs between different cattle breeds and the implications of this DE.

We can conclude that miRNAs play an important role in the development of the mammary gland and that miRNA expression is stage-dependent. How-

ever, functionality of the miRNAs and the underlying mechanism between miRNA expression and mammary gland development and lactation stages must be further investigated.

MICRORNA EXPRESSION PATTERNS IN COLOSTRUM AND MILK

Differential Expression Between Colostrum and Milk

Over the past few years, miRNAs present in bovine colostrum and mature milk samples have been identified using next-generation sequencing (Chen et al., 2010) and microarray analysis (Izumi et al., 2012; see Table 2). Chen et al. (2010) detected 230 and 213 known miRNAs in colostrum and milk respectively, with 198 miRNAs present in both. Colostrum and milk were collected from 80 cows at 7 d and 9 mo postpartum, respectively. Izumi et al. (2012) detected 100 known miRNAs in colostrum and 53 in milk, with 51 miRNAs common to both. They sampled 5 cows within 3 d postpartum for colostrum and 5 cows from 8 d to 8 mo in lactation for mature milk. One explanation for the differing results between these 2 studies could be the different sampling volumes. Chen et al. (2010) extracted RNA from 100 mL of pooled colostrum and mature milk, whereas Izumi et al. (2012) used only 2.5 to 6.5 mL of individual colostrum and milk samples to extract RNA. Furthermore, Chen et al. (2010) performed an additional PAGE purification of small RNA molecules, enabling them to detect very small quantities of miRNAs (Izumi et al., 2012). Another important difference is the sampling time of colostrum. The most striking difference between colostrum and milk is the concentration of antibodies, especially IgG₁ (Barrington et al., 2001). There is some disagreement in literature about the duration of colostrum secretion by the cow. Some study results state that colostrum production stops immediately after giving birth, whereas others claim that it lasts for 7 d (McGrath et al., 2016). The generally agreed upon definition is that colostrum is the milk produced in the first 3 d after parturition (McGrath et al., 2016). Whether a difference in miRNA expression could exist in this first 7 d of lactation has not been investigated. Also important to note is that the sample groups used in the study of Izumi et al. (2012) lacked standardization, as colostrum samples were derived almost exclusively from heifers, whereas, for mature milk, exclusively multiparous cows were sampled. Whether this could influence the results is unclear because, so far, an effect of parity on miRNA expression has not been studied.

Several studies demonstrated a higher miRNA concentration in bovine milk at the beginning of the lacta-

tion (1 to 7 d) compared with mid lactation (2 to 4 mo; Hata et al., 2010; Izumi et al., 2012) or late lactation (6 to 9 mo; Chen et al., 2010; Izumi et al., 2012; Sun et al., 2013). In porcine breast milk exosomes, miRNA expression profiles clustered together for colostrum samples (taken at 0 and 3 d of lactation) and for mature milk (taken at 7, 14, 21, and 28 d of lactation; Gu et al., 2012). This difference in expression between colostrum and milk is intriguing, although the reason for it remains unclear. We can hypothesize that it is related to the needs of the neonate, but this requires further study. Whether secretion of miRNAs in milk and colostrum is of importance to the cow is not clear. MicroRNAs are thought to regulate the immune system of the cow, which is summarized in the review of Lawless et al. (2014b). In human and mouse research, it has been shown that miRNAs are key players in regulating the immune response; however, similar research has yet to be conducted in bovine research (Lawless et al., 2014b). Furthermore, deep research is needed to find a link between miRNAs secreted in colostrum and milk and the medical history or immunity of the cow herself.

Origin of miRNAs Expressed in Colostrum and Milk

Because of the different miRNA expression profiles between plasma and milk in several mammalian species, the contribution of the maternal circulation to miRNAs expressed in milk is presumably limited (Kosaka et al., 2010; Izumi et al., 2014; Modepalli et al., 2014; Alsaweed et al., 2016). The presence in milk-derived microvesicles of several miRNAs that are expressed in mammary gland tissue has been confirmed (Hata et al., 2010). The study of Li et al. (2012a) showed that the top 20 most abundant miRNAs in the dairy goat mammary gland and the top 20 most abundant miRNAs in raw milk had 13 common members (let-7a, let-7b, let-7c, let-7f, let-7g, miR-21, miR-230a, miR-2103, miR-2107, miR-2143, miR-2148a, miR-2320, and miR-2423-5p). When the miRNA expression profiles of different bovine milk fractions (fat, whey, and cells) were compared with that of mammary gland tissue, the miRNome of the milk fat fraction was most similar to that of the mammary gland, followed by whey. This is in contrast with the cell fraction, which had the fewest similarities (Li et al., 2016). Therefore, endogenous synthesis in the lactating mammary epithelium is thought to be the main source of miRNAs in milk (Li et al., 2012a; Modepalli et al., 2014; Alsaweed et al., 2016). Most of the extracellular miRNAs appear to be released passively, although there is some evidence of selective release of miRNAs by cells (Pigati et al., 2010; Li et al., 2012a). This process is likely cell-type specific, yet the

Table 2. Summary of total number of microRNAs (miRNAs) detected in milk, colostrum, and the mammary gland

Number	Matrix	Sample amount	Species	Technique ¹	Extraction method ²	Remarks	Reference
281	Skim milk	50–100 mL	Human	Microarray analysis	mirVana miRNA isolation kit (Ambion)		Kosaka et al. (2010)
429	Skim milk	300 µL	Human	Quantitative real-time PCR	miRNeasy kit (Qiagen)		Weber et al. (2010)
386	Skimmed colostrum	300 µL	Human	Quantitative real-time PCR	miRNeasy kit (Qiagen)		Weber et al. (2010)
395	Mammary gland d 1 postpartum	Unknown	Murine (rat)	NGS	Trizol reagent (Invitrogen)		Zhang et al. (2014)
400	Mammary gland d 7 postpartum	Unknown	Murine (rat)	NGS	Trizol reagent (Invitrogen)		Zhang et al. (2014)
292	Milk cells	24–78 mL	Human	Microarray analysis	miRNeasy mini kit (Qiagen)		Alsaweed et al. (2016)
242	Milk fat	24–78 mL	Human	Microarray analysis	miRCURY RNA Isolation-Biofluids kit (Ambion)		Alsaweed et al. (2016)
921	Mammary gland	Unknown	Bovine	Microarray analysis	miRNeasy mini kit (Qiagen)	56 miRNAs differentially expressed between lactation and non-lactation mammary glands	Li et al. (2012b)
491	Milk exosomes	Unknown	Porcine	NGS (Solexa sequencing)	Trizol reagent (Invitrogen)		Chen et al. (2014)
100	Colostrum whey	2.5–5.5 mL	Bovine	Microarray analysis	miRNeasy mini kit (Qiagen)		Izumi et al. (2012)
53	Mature milk whey	3.5–6.5 mL	Bovine	Microarray analysis	miRNeasy mini kit (Qiagen)		Izumi et al. (2012)
230	Colostrum	100 mL	Bovine	NGS (Solexa sequencing)	Trizol LS reagent (Invitrogen)		Chen et al. (2010)
213	Mature milk	100 mL	Bovine	NGS (Solexa sequencing)	Trizol LS reagent (Invitrogen)		Chen et al. (2010)
297	Milk isolated monocytes	Total volume of 1 milking	Bovine	NGS (Solexa sequencing)	mirVana RNA isolation kit (Ambion)	Milk from Holstein Friesians infected in vivo with <i>Streptococcus uberis</i>	Lawless et al. (2014a)
303	Mammary gland peak lactation	Unknown	Caprine	NGS (Solexa sequencing)	Trizol reagent (Invitrogen)		Li et al. (2012a)
337	Mammary gland dry period	Unknown	Caprine	NGS (Solexa sequencing)	Trizol reagent (Invitrogen)		Li et al. (2012a)

¹NGS = next generation sequencing; Solexa sequencing (Illumina Inc., San Diego, CA).

²Ambion, Austin, TX; Qiagen, Valencia CA; Invitrogen, Carlsbad, CA.

mechanism behind selective release of miRNAs remains unclear (Pigati et al., 2010).

MiRNAs in Colostrum and Milk Are Packaged in Exosomes

Milk-derived miRNAs, in contrast to mRNA and cellular miRNAs, are stable under harsh conditions: they are resistant to RNase treatment, an acidic environment, (Hata et al., 2010; Kosaka et al., 2010; Gu et al., 2012; Izumi et al., 2012; Modepalli et al., 2014), and freeze-thaw cycles (Kosaka et al., 2010; Gu et al., 2012; Muth et al., 2018). This suggests that miRNAs can survive the environment of the neonate's gastrointestinal tract (GIT) and can, as such, be absorbed in the intestines (Kosaka et al., 2010; Gu et al., 2012; Izumi et al., 2012). Kirchner et al. (2016) studied the effect of heat treatment of bovine milk on the survival of miRNAs expressed in milk. Ultra-high heat treatment (preheating: 23 s at 95°C; direct steam injection: 5 s at 142°C), extended shelf-life treatment (preheating: 20 s at 90°C; direct steam injection: 5 s at 127°C) and boiling (preheating: >300 s at >80°C; boiling: 30 s at 100°C) led to substantial degradation of miRNAs. Pasteurization (20 s at 72°C), on the other hand, showed no reduction in miRNA levels (Kirchner et al., 2016). The above-mentioned techniques are used for treatment of bovine milk for human consumption. Pasteurization is also used by farmers to lower the bacterial load of colostrum. It is advised to heat colostrum for 30 to 60 min at 60°C. This technique uses a lower temperature than commercial milk treatment to avoid degradation of colostral IgG (Elizondo-Salazar et al., 2010). In this regard, it is possible that pasteurization of colostrum does not affect miRNA concentration. However, the effect of the duration of heating colostrum on the miRNA concentration is not fully clear yet and should be further investigated. In the study of Benmoussa et al. (2016), commercially available dairy milk was used to test the survival rate of miRNAs in the GIT. To mimic the digestion of a healthy adult, they used the TNO computer-controlled in vitro gastrointestinal model, TIM-1, which is composed of 4 connected compartments that mimic the environment of the stomach, duodenum, jejunum, and ileum, respectively. Benmoussa et al. (2016) showed that a high amount of milk-derived miRNAs withstood these conditions and that miRNAs reached the small intestine (the main location for absorption of nutrients). Therefore, it is likely that miRNAs may be functional in the GIT of neonates after ingestion of milk (Izumi et al., 2014). A potential explanation for the high survival rate of miRNAs under these conditions is their storage in cell-derived membrane vesicles such as microvesicles or exosomes (see Figure 1; Hata

et al., 2010; Kosaka et al., 2010; Gu et al., 2012; Izumi et al., 2012; Modepalli et al., 2014; Wolf et al., 2015; Alsaweed et al., 2016; Benmoussa et al., 2016; Rani et al., 2017).

In the literature, the terminology of these cell-derived membrane vesicles can lead to confusion because different nomenclature is used (Mathivanan et al., 2010). The term “microvesicles” is often used to refer to a mixed population of vesicles, including exosomes, shedding microvesicles, and apoptotic bodies (Mathivanan et al., 2010), although in many reports, “microvesicle” is specially used to describe a vesicle shed directly from the plasma membrane (Figure 1; van Dommelen et al., 2012). Exosomes are nanosized (40–100 nm diameter) vesicles with a phospholipid bilayer that originate from the endosomal system and are released to the extracellular environment through multivesicular bodies after budding with the plasma membrane (van Dommelen et al., 2012). Chen et al. (2014) demonstrated that exosomes isolated from porcine milk contained 176 known and 315 novel mature miRNAs. This phenomenon was also seen in bovine colostrum and milk (Hata et al., 2010). In addition, in human as well as in bovine milk, miRNAs can also be transported within milk fat globules (Munch et al., 2013; Alsaweed et al., 2016; Li et al., 2016). Milk fat globules are synthesized and released by mammary epithelial cells and contain mainly triacylglycerols (Heid and Keenan, 2005). Because the secretion of milk fat globules marks a complete surrounding of the plasma membrane before the globule is split off from the cell, these globules could be seen as a specific form of shedding vesicles (see Figure 1; Heid and Keenan, 2005). In the process of milk fat globule secretion, miRNAs present in the cytoplasm of mammary epithelial cells can be enclosed by these fat globules before being released into the milk (Li et al., 2016). Furthermore, an association between miRNAs and large protein complexes is also suggested (Modepalli et al., 2014). Extracellular miRNAs are found to be associated with argonaute RISC catalytic component 2 (**AGO2**), a protein that plays a central role in the RNA-induced silencing complex (RISC) in plasma and cell culture medium (Turchinovich et al., 2011). It is thought that these AGO2/miRNA complexes are by-products of cell death and are highly stable in the extracellular space (Turchinovich et al., 2011).

Influences on Expression Pattern in Milk and Colostrum

Could external factors such as nutrition, environment, or health of the dam alter the miRNA expression in milk? After noting a large variation in miRNA expression between individuals, Kosaka et al. (2010) has

suggested nutritional and environmental influences on the expression patterns of miRNA in milk.

Nutrition. As we already know, nutrition has an important influence on milk composition in dairy cows, with milk fat composition being very sensitive to dietary changes (Sutton, 1989). Since Boussingault and Lebel discovered that nutrition affects milk composition (Playfair, 1841), a great deal of research in ration formulation has been conducted. After the discovery of miRNAs in colostrum and milk, some researchers are shifting their focus toward the effect of nutrition on miRNA composition in milk, but the connections remain tenuous. Research in women and mice has already clearly revealed the effect of the diet on miRNA

expression in milk and in offspring (Zhang et al., 2009; Munch et al., 2013; Benatti et al., 2014).

It seems that a high-fat diet can influence the expression pattern of miRNA in human milk. Women who consume a diet rich in fat (30% carbohydrate, 55% fat, and 15% protein) showed increased expression of miR-67 and miR-27 in their milk fat globules compared with women on an high-carbohydrate diet (60% carbohydrate, 25% fat, and 15% protein; Munch et al., 2013). Mice that received a diet rich in fat (5.24 kcal/g), showed 25 DE miRNAs compared with mice fed a control diet (3.85 kcal/g; Chen et al., 2017). When cows were fed a diet supplemented with 5% safflower or linseed oil, 22 and 14 miRNAs (7 in common), respec-

Figure 1. Common mechanisms for extracellular appearance of microRNAs (miRNAs): they can be stored in shedding microvesicles (1), exosomes (2) or associated with Argonaute 2 (AGO2) protein (3). © 2013 Hessvik, Sandvik and Llorente. Adapted from Hessvik et al. (2013). This figure was created using Servier Medical Art templates (<https://smart.servier.com>), licensed under a Creative Commons Attribution 3.0 Unported License.

tively, were differentially expressed in the mammary gland (Li et al., 2015). The 7 DE miRNAs common to both treatments were associated with gene targets related to lipid metabolism (Li et al., 2015). Whether these DE miRNAs could affect the offspring's metabolism is not clear. Besides an alteration in miRNA expression, supplementation with safflower or linseed oil caused a reduction in milk fat (Li et al., 2015). There seems to be a connection between maternal nutrition during lactation and miRNA expression in the milk, especially for fat content of the diet. Although the crude protein level of the maternal diet during late gestation affects IgG absorption in calves (Quigley and Drewry, 1998), the effect of maternal protein supply on miRNA expression in colostrum has yet to be determined.

Furthermore, it seems that maternal diet during pregnancy and lactation can affect miRNA expression in the offspring (Zhang et al., 2009). When female mice were fed a high-fat diet (22.6% fat, 23% protein and 48.6% carbohydrate) during pregnancy and lactation, an increase of 10 and a decrease of 23 miRNAs were detected in liver tissue of the offspring compared with that in dams fed a standard chow diet (10% fat, 68.8% carbohydrate, and 18% protein; Zhang et al., 2009). Benatti et al. (2014) used the same approach and reported that feeding female mice with a high-fat diet during gestation and lactation led to up- and down-regulation of miR-370 and miR-122, respectively, in the liver of offspring. The reduction in miR-122 and increase in miR-370 in liver were associated with changes in the oxidative pathway (Benatti et al., 2014). It is possible that these differences in expression already find their origin in the uterus and are maintained during suckling. This is only an assumption, however, as the effect on miRNA expression was not studied if pups from standard chow mothers would suckle mothers on the high-fat diet and vice versa. Further research is warranted to reveal whether a similar effect of maternal dietary changes occurs in dairy calves. As with expression patterns in the milk, no link has been found so far between protein content of the maternal diet during gestation and miRNA expression in the offspring (Liu et al., 2011; Cong et al., 2012; Jacometo et al., 2018).

Infection. In extensive production systems with highly productive dairy cows, mastitis is the most common production-related condition (Seegers et al., 2003). This disease can have a distinct effect on the physiology of the mammary gland and the production results of the cow (Seegers et al., 2003). Therefore, it is likely that this disease could affect the expression of miRNA in the mammary gland and consequently in both colostrum and milk.

When the bovine mammary gland was challenged with a *Staphylococcus aureus* infection, 6 miRNAs were

significantly differentially expressed in milk exosomes, miR-142-5p, miR-223, miR-183, and miR-99a-5p were upregulated, and miR-2285g-3p and miR-101 were downregulated (Sun et al., 2015). In vitro experiments with cultured bovine mammary epithelial cells revealed changes in miRNA expression after inoculation with *Streptococcus uberis*. MicroRNA expression was measured at 4 time points (1, 2, 4, and 6 h after infection) and 21 miRNAs seemed to be differentially expressed. Fifteen miRNAs were upregulated and 7 miRNAs were downregulated, with one miRNA (miR-29e) being upregulated 2 h after infection and downregulated 6 h after infection (Lawless et al., 2013). Furthermore, monocytes isolated from milk showed differential expression of 26 miRNAs after in vivo inoculation of the mammary gland with *Strep. uberis* (Lawless et al., 2014a). It is hypothesized that differentially expressed miRNAs after bacterial infection could regulate immune response in the mammary gland (Lawless et al., 2013, 2014a; Sun et al., 2015). Moreover, it is thought that miRNAs that target genes associated with the immune response are downregulated after a bacterial infection, meaning that suppression of these genes by miRNAs is less pronounced (Lawless et al., 2014a). This research in miRNA expression during bacterial IMI has great potential to find noninvasive biomarkers for early detection of subclinical mastitis (Lawless et al., 2014a; Sun et al., 2015). MicroRNAs have become very popular in human medicine as biomarkers for early detection of several diseases, such as breast cancer for example (Etheridge et al., 2011). Given the high prevalence of mastitis in dairy cattle, it would be interesting to dig deeper into its effect on milk miRNA expression and, more importantly, to develop better diagnostic tools in the early detection of this disease. Moreover, it is possible that altered miRNA expression due to mastitis affects neonatal development. Although highly discouraged, many farmers still administer waste milk from cows with mastitis to their calves (Vasseur et al., 2010). In this regard, better knowledge on implications for the calf of feeding mastitic milk and the DE of miRNAs due to mastitis is needed.

MiRNAs in Colostrum and Milk: Potential Implications for the Neonate

Neonates clearly ingest miRNAs via colostrum, but do those miRNAs remain biologically active after delivery in the gut? It could be that they either act (only) locally in the GIT or that they are absorbed in the intestines and delivered to other organs where they affect gene expression. However, these hypotheses require further study. Additionally, it is believed that insufficient uptake of dietary miRNAs cannot be compen-

sated for by endogenous synthesis of miRNAs (Baier et al., 2014). When mice were fed miRNA-depleted milk, the plasma concentration of miR-29b decreased by 61% compared with mice fed miRNA-containing milk (Baier et al., 2014); miR-29b is associated with osteoblastogenesis—specifically, it targets inhibitors of osteoblast differentiation (Li et al., 2009). In addition to a decrease in miR-29b, a lower expression of Krüppel-like factor 8 (*KLF8*) mRNA was found in the liver of miRNA-depleted mice (Baier et al., 2014); *KLF8* is a DNA-binding transcriptional regulator that contributes in different biological processes (Funnell et al., 2013). Whether *KLF8* is regulated by miR-29b and what the biological relevance of this decrease might be were not reported. However, based on the predictions of TargetScanMouse (release 7.2; http://www.targetscan.org/mmu_72/), the repression of *KLF8* by miR-29b seems rather low with a context++ score of -0.05 (Agarwal et al., 2015). This score is calculated as the sum of the contribution of 14 features for each of the 4 site types. Site type (e.g., 8mer) and the minimum distance of the site from the stop codon are 2 of the 14 features (Agarwal et al., 2015). The value range goes from 1 to -1 , and the more negative the score, the greater the repression (Riffo-Campos et al., 2016).

Transportation of miRNAs to the Neonate.

The uptake of dietary delivered miRNAs has received increasing attention over the past few years (Hata et al., 2010; Zhang et al., 2012; Snow et al., 2013; Baier et al., 2014; Modepalli et al., 2014; Hirschi et al., 2015; Wolf et al., 2015; Rani et al., 2017). Although the presence of plant- and animal-specific miRNAs delivered through food to serum of human (Zhang et al., 2012) and animals (Zhang et al., 2012; Yang et al., 2015a,b) has been confirmed, it is thought that in certain diets, the concentration of absorbed miRNAs is often too low to exert a biological effect on the recipient (Snow et al., 2013). This could be due to degradation of the miRNAs in the GIT or because of a low supply via the diet (Snow et al., 2013). However, several questions remain—are these miRNAs even important nutrients? How is their absorption regulated? And which concentration is needed to be biologically active?

Mechanism Behind the Absorption of miRNAs in the GIT. We know that miRNAs are absorbed in the GIT and transferred to the bloodstream. What is the mechanism behind this absorption? As mentioned above, miRNAs in colostrum and milk are stored in exosomes. It is hypothesized that exosomes and microvesicles carry specific surface proteins that attach to the intestinal epithelium (Modepalli et al., 2014; Wolf et al., 2015). This suggests that the absorption of miRNAs through the intestinal wall is carried out by endocytosis, whereby exosomes and microvesicles

release their contents into the epithelial cells (Wolf et al., 2015). The uptake of miRNAs has been seen in different types of cells. When murine embryonic fibroblasts (NIH-3T3 cells) were incubated with milk-derived microvesicles, evidence of miRNA transfer was observed (Hata et al., 2010). This phenomenon was also seen when cultured macrophages were incubated with milk- and colostrum-derived microvesicles (Sun et al., 2013). In addition, in humans, an increase in miRNA concentration was seen in human peripheral blood mononuclear cells (PBMCs) after ingestion of cow milk (Baier et al., 2014). Moreover, the membrane structure of the microvesicles and exosomes seems to be crucial in the miRNA transfer to other cells (Sun et al., 2013). The overall number of miRNAs present in colostrum and milk are contained in cell-derived vesicles. This suggests that these miRNAs are meant to be absorbed in the GIT of the recipient. If true, this strengthens the hypothesis that they are an important and biologically meaningful nutrient for the neonate.

Concentration-Dependent Absorption of miRNAs. In contrast to the findings of Snow et al. (2013), where the absorption of miRNAs delivered through 3 different diets (vegetarian, soy-enriched, and casein and lard diet) was minimal, miRNAs delivered through milk consumption appear to be absorbed in meaningful amounts in the intestines. The study of Arntz et al. (2015) revealed an uptake of bovine milk-derived extracellular vesicles (**BMEV**) in the ileum in in vitro experiments (Arntz et al., 2015). In an in vivo experiment, the absorption of 2 miRNAs, miR-29b and miR-200c, in humans after ingestion of bovine milk was investigated (Baier et al., 2014). Before consumption, the cow milk contained 148 ± 42 and 680 ± 151 pmol/L of miR-29b and miR-200c, respectively. Plasma concentrations of miR-29b showed a linear dose-response relation with the amount of milk consumed (0.25, 0.5, and 1 L); plasma concentrations of miR-200c were also highest for the highest consumption amount, although this dose-response relation was not linear. The maximum serum concentration of miR-29b occurred 3.4 to 4.2 h after the ingestion of milk. Furthermore, the return to baseline concentrations of these miRNAs took more time with higher milk consumption, 9 h for 0.25- and 0.5-L doses and 24 h when 1 L of milk was consumed (Baier et al., 2014). These postprandial increases of miRNA levels in human serum could also be seen after the ingestion of chicken eggs (Zempleni et al., 2015). Besides being concentration-dependent, the absorption of food-delivered miRNAs seems to be miRNA-specific as well.

Newborns as well as adults are able to absorb miRNAs from their diet. In neonates, whose first diet consists mainly of maternal milk, an increase in serum

miRNA concentrations was observed after ingestion of colostrum (Gu et al., 2012) and milk (Gu et al., 2012; Modepalli et al., 2014). In the study of Chen et al. (2016), an intracellular elevation of miRNAs was seen after the incubation of neonatal piglet-derived intestinal enterocytes (IPEC-J2) with milk exosomes. When miRNA absorption in piglets was compared following consumption of colostrum versus mature milk, 9 of 13 immune-related miRNAs showed significantly higher expression in the serum of piglets given only colostrum (Gu et al., 2012). Because these immune-related miRNAs are more highly expressed in colostrum than in mature milk (Gu et al., 2012), the higher absorption of these miRNAs in colostrum-fed piglets is likely concentration related.

Furthermore, uptake of miRNAs seems to be facilitated by prolonged and high dosage exposure (Hirschi et al., 2015). It is suggested that some dietary components could stimulate the uptake of miRNAs (Hirschi et al., 2015). For example, mice fed honeysuckle (*Lonicera japonica*) versus chow supplemented with a synthetic plant miRNA, miR-168a, showed higher serum levels of this miRNA (Yang et al., 2015b). In addition, miRNA absorption is related to the permeability of the intestinal epithelium. Alterations or damage to the gut are associated with higher absorption of dietary miRNAs (Yang et al., 2015b). Because the calf's gut is more permeable in the first 24 h of life (Lora et al., 2018), higher absorption of miRNAs in this short period can be presumed. This hypothesis requires thorough investigation.

Biological Effect of miRNAs Delivered to the Neonate

Intestines. MicroRNAs seem to be important in the differentiation and functional development of the intestinal epithelium. McKenna et al. (2010) used knockout mice deficient in the gene encoding Dicer 1, an enzyme crucial for activity of the miRNA (Figure 2). The mutant miRNA-deficient mice were smaller than control littermates in early life, probably because of the less-efficient digestion of fat molecules present in milk (the feces of mutant mice contained large fat droplets when they were fed a fat-rich diet; McKenna et al., 2010). The intestinal epithelium of mutant mice was completely disorganized, with an increase in lymphocyte infiltration, a severe increase in apoptosis of epithelial cells, and decreased function of the intestinal epithelial barrier (McKenna et al., 2010). This suggests that miRNAs are crucial for the normal morphology and function of the intestines. Moreover, it is likely that miRNAs present in colostrum and milk are important for intestinal development. Chen et al. (2014) suggested that miRNAs present in porcine milk exosomes play a

role in the development of the digestive tract. In an in vivo experiment in mice, daily administration of porcine milk exosomes significantly promoted villus height and crypt depth of the duodenum and jejunum (Chen et al., 2016). This effect is probably induced by RNA molecules, which stimulate expression of several transcription and proliferation factors (CDX2, proliferating cell nuclear antigen, and IGF-1R) in the jejunum and reduce expression of protein p53 (Chen et al., 2016), an important regulator of cell death (Kruiswijk et al., 2015). Exosomes present in rat milk are also associated with a beneficial effect on intestinal development. It is suggested that they stimulate cell viability, proliferation, and stem cell activity of the intestinal epithelium (Hock et al., 2017).

In the study of Liang et al. (2014), temporal and regional differences in miRNA expression profiles of the GIT in dairy calves were investigated from birth to 42 d of age (samples taken at 0, 7, 21, or 42 d of age). The small intestines showed the largest temporal change in miRNA expression during the first week of life, which can be linked to the rapid development of the intestines in this period (Liang et al., 2014). One of the factors related to the temporal alterations of miRNA expression patterns is dietary change (Liang et al., 2014). Colostrum and, to a lesser extent, mature milk contain relevant amounts of development-related miRNAs (Izumi et al., 2012). Moreover, the 10 highest-expressed miRNAs in the calf's intestines are represented in colostrum and milk, with 6 of them (miR-192, miR-194, miR-143, miR-378, miR-10a, and miR-181a) being upregulated in colostrum (Chen et al., 2010; Liang et al., 2014). These findings could signify the contribution of colostrum and milk feeding in the temporal changes of miRNA expression, as well as in the development of the intestines. However, it remains unknown what concentration of miRNAs in milk or colostrum is needed to exert a biological effect in the recipient, in this case the calf. When adult humans consumed 1 L of milk containing 148 ± 42 and 680 ± 151 pmol/L of miR-29b and miR-200c, respectively, their plasma concentrations of miR-29b increased from 232 ± 51 to 624 ± 83 fmol/L and that of miR-200c increased from 458 ± 37 to 924 ± 121 fmol/L (Baier et al., 2014). These maximal plasma concentrations were measured 3.6 $\pm$ 0.6 h after consumption (Baier et al., 2014). Gene expression in human PBMCs of the *RUNX2* gene was elevated by $31 \pm 13\%$ after milk consumption (Baier et al., 2014). MicroRNA-29b is a positive regulator of the *RUNX2* gene (Li et al., 2009), which could explain these findings. Thus, bovine milk contains certain miRNAs that exert a biological effect in the recipient. However, similar studies are lacking in dairy calves. Further research is needed to confirm the hypothesis that colostrum and

milk contain miRNAs that are biologically available in the calf. In addition to the dietary influence, the density of bacteria (mainly *Bifidobacterium* and *Lactobacillus* spp.) present in the GIT is correlated with the expression of miRNAs (Liang et al., 2014). This again highlights the complexity of the contribution of miRNAs in the organism's physiology and metabolism.

Immunology. MicroRNAs can affect many cell types and regulate numerous target genes from close range or at a distance (Chen et al., 2012). Hata et al. (2010) pointed out the presence of immune-related miRNAs in bovine milk-derived microvesicles. Moreover, immune-related miRNAs seemed to be upregulated in colostrum compared with mature milk in cows (Izumi et al., 2012; Sun et al., 2013) and pigs (Gu et al., 2012). This phenomenon was first seen in human breast milk, where these miRNAs are upregulated during the first 6 mo

of lactation (Kosaka et al., 2010). In Li et al. (2012a), 4 of the top 20 most abundant miRNAs (miR-29a, miR-230a, miR-2101, and miR-2146b) in the mammary gland of dairy goats are immune-related miRNAs. The expression levels of these miRNAs were similar between the mammary gland and the milk (Li et al., 2012a). We can hypothesize that the higher levels of immune-related miRNAs are correlated with the needs of the neonate, especially in the early stages of life when their immune system still needs to mature.

MicroRNAs are believed to participate in the development and regulation of the immune system (Xiao and Rajewsky, 2009; O'Connell et al., 2010; Chen et al., 2014; Alsaweed et al., 2015, 2016). This effect is thought to act through alterations in gene expression of several immune cells, such as T and B cells, monocytes, macrophages, neutrophils, and dendritic cells (Kosaka

Figure 2. Genes coding for miRNAs are transcribed by RNA polymerase II (RNA pol II) or RNA pol III into a primary miRNA (pri-miRNA) transcript (Lee et al., 2004; Borchert et al., 2006). The pri-miRNAs are long and are cleaved in the nucleus by Drosha, an RNase III ribonuclease, into an approximately 60- to 70-nt precursor hairpin, better known as the pre-miRNA (Lee et al., 2002, 2003). The pre-miRNA is actively transported by exportin-5 to the cytoplasm, where it is processed into a 20-bp miRNA/miRNA* duplex by another RNase III ribonuclease, called Dicer (Lee et al., 2002; Yi et al., 2003; Krol et al., 2010), where the asterisk denotes the strand of the duplex that will be degraded as the other one will be loaded into the RNA-induced silencing complex (RISC). Eventually, 1 of the 2 miRNA strands is loaded into the RISC, the other strand will be degraded (Lodish et al., 2008). In mammals, RISC is composed by the mature miRNA and the Argonaute 2 protein (AGO2) (Liu et al., 2004). This complex will bind to a corresponding mRNA, which will lead to degradation or repression of the translation of the mRNA (Lodish et al., 2008). The main purpose is to reduce the production of targeted proteins. This is why miRNAs are called regulators of gene expression at a post-transcriptional level (Lodish et al., 2008). This figure was created using Servier Medical Art templates (<https://smart.servier.com>), licensed under a Creative Commons Attribution 3.0 Unported License. Adapted by permission from Springer Nature: Nature Reviews Immunology, Micromanagement of the immune system by microRNAs. H. F. Lodish, B. Zhou, G. Liu, and C. Z. Chen. © 2008.

et al., 2010; Alsaweed et al., 2015; Arntz et al., 2015). It is suggested that milk miRNAs play an important role in the maturation of the neonate's immune system (Izumi et al., 2014). For example, miR-181 and miR-155 are both regulators of B- and T-cell differentiation and are abundant in human breast milk (Kosaka et al., 2010). Arntz et al. (2015) showed that bovine-milk derived microvesicles can have an anti-inflammatory function and may suppress the adaptive immune response. Moreover, colostrum-derived vesicles play a regulating role in cytokine production (Sun et al., 2013). When cultured macrophages were challenged with LPS after exposure to colostrum vesicles, IL-1 and IL-6 production increased IL-10 production decreased; however, this effect was only seen when the macrophages were challenged with a low concentration of LPS (Sun et al., 2013). In addition, if colostrum-derived vesicles would exert a positive effect on phagocytosis in incubated macrophages, this effect would be higher than with milk-derived vesicles (Sun et al., 2013).

The most abundant miRNAs in porcine milk exosomes are thought to be involved in the IgA immune network and may target several immune-related genes (Chen et al., 2014). The *let-7* miRNA family members, for example, are assumed to affect IgA production in the digestive tract of the piglet (Chen et al., 2014). When isolated murine splenocytes were incubated with BMEV, a reduction in the LPS-induced tumor necrosis factor α (TNF- α) and monocyte chemoattractant protein 1 (MCP-1) production could be seen (Arntz et al., 2015). Furthermore, BMEV seemed to exert a therapeutic effect when they were orally administered to mice suffering from autoimmune arthritis (Arntz et al., 2015). Additionally, Liang et al. (2014) suggest that miRNAs are involved in development of the mucosal immune system in the small intestine.

All these findings suggest that maternal signaling through miRNAs may be important in the development of the newborn and that they contribute to a variety of biological processes. Still, literature on this matter is relatively sparse, especially in bovine research. Previous research focused mainly on the importance of miRNA expression in milk for human consumption. Studies on the effect of miRNA delivery through colostrum and milk ingestion in neonatal calves are still rare. Moreover, it should be acknowledged that current studies on miRNA expression in bovine colostrum deviated from the true definition of colostrum and sampled transition milk. Colostrum is the first milk a cow produces after giving birth, and the transition to mature milk takes approximately 5 d. Future research on the miRNA expression in colostrum of first milking would be more

interesting as this is the neonate's very first meal. In addition, more research should be done on the possible absorption of miRNAs in the neonate's gut; if these miRNAs are absorbed, then possible biological effects should be investigated.

CONCLUSIONS

Milk and especially colostrum are complex fluids that contain essential nutrients for the newborn. Colostrum and milk are the neonate's first source of nutrition and are therefore crucial for development and growth. There is evidence that miRNAs are abundant in both colostrum and milk. They are stored in exosomes, which makes it possible to survive in the GIT. In the small intestines, miRNAs can be absorbed and transferred to the bloodstream. Because endogenous synthesis cannot compensate for dietary deficiencies of certain miRNAs, the supply of miRNAs through feed intake could be more important than initially thought. There are indications that these colostrum- and milk-delivered miRNAs exert an effect on the recipient. They can act locally in the intestines or be transported via the bloodstream to affect biological processes in other parts of the body, such as the immune system. The abundance of miRNAs in colostrum and milk and the alteration of their expression during these lactational stages indicate that this pattern follows the neonate's needs. For example, immune-related miRNAs are expressed more in colostrum than in mature milk, which might be related to the immature immune system of the newborn calf. However, true links between these findings are still missing. If the presence of miRNAs in colostrum were important for the development of the newborn calf, it would be interesting to study whether the expression could be altered due to management factors, such as nutrition. So far, dietary effects on the miRNA expression patterns in bovine milk have not received very much attention. In general, studies on the effect of ration composition on expression of miRNAs are scarce. The few studies that are published on this matter report an effect of dietary fat content in humans and mice. However, further research is needed to determine ways to optimize miRNA expression through maternal nutrition. Overall, we conclude that miRNAs present in colostrum and milk represent a mechanism of postnatal signaling from the mother to the neonate. They are important regulators in the development of the newborn that can influence different biological processes. Future research should focus on the underlying mechanisms of expression in colostrum and milk and the effects on the (neonatal) recipient.

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