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Review Article

microRNAs and the mammary gland: A new understanding of gene expression

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Abstract

MicroRNAs (miRNAs) have been identified in cells as well as in exosomes in biological fluids such as milk. In mammary gland, most of the miRNAs studied have functions related to immunity and show alterations in their pattern of expression during lactation. In mastitis, the inflammatory response caused by *Streptococcus uberis* alters the expression of miRNAs that may regulate the innate immune system. These small RNAs are stable at room temperature and are resistant to repeated freeze/thaw cycles, acidic conditions and degradation by RNAse, making them resistant to industrial procedures. These properties mean that miRNAs could have multiple applications in veterinary medicine and biotechnology. Indeed, lactoglobulin-free milk has been produced in transgenic cows expressing specific miRNAs. Although plant and animal miRNAs have undergone independent evolutionary adaptation recent studies have demonstrated a cross-kingdom passage in which rice miRNA was isolated from human serum. This finding raises questions about the possible effect that miRNAs present in foods consumed by humans could have on human gene regulation. Further studies are needed before applying miRNA biotechnology to the milk industry. New discoveries and a greater knowledge of gene expression will lead to a better understanding of the role of miRNAs in physiology, nutrition and evolution.

Keywords: lactation, mastitis, milk, miRNA.

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Introduction

The mammary gland is an interesting model for studying gene expression since this organ experiences different cycles of differentiation and regression during adult life. In eukaryotes, gene expression is a complex process that involves DNA methylation, chromatin modification, imprinting, and interfering RNA (Mattick and Makunin, 2006). Although Fire and Mello received the Nobel Prize for their discovery of double-strand interfering RNA in *Caenorhabditis elegans* (Fire *et al.*, 1998), the first miRNA (Lin 4) was in fact discovered years before by two groups and published simultaneously in 1993 (Lee *et al.*, 1993; Wightman *et al.*, 1993). In 2000, a second miRNA (Lin 7) was identified in the same nematode (Reinhart *et al.*, 2000) and soon after in many other species (Pasquinelli *et al.*, 2000). By 2001, it was clear that miRNAs represent a class of small conserved RNAs (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Three main categories of interfering RNAs have been recognized: microRNA (miRNA), short interfering RNA (siRNA) and piwi interactive RNA (piRNA). These three RNAs are of similar size (~23 nucleotides in length) but have sequence-specific inhibitory functions (Carthew and Sontheimer, 2009; Ambros, 2010). siRNAs are excised from long, fully complementary double-stranded RNAs (Tomari and Zamore, 2005; Ambros, 2010) and were initially considered to protect the host genome from foreign nucleic acids (such as viruses, transposons and transgenes) but that view changed with the discovery of abundantly expressed endogenous siRNAs in animal cells (Golden *et al.*, 2008). piRNAs are small RNAs that bind the piwi subfamily of argonaute proteins and protect the germline (Ambros and Chen, 2007) and embryonic stem cells (Morin *et al.*, 2008) from invasive transposable elements.

miRNAs regulate gene expression at a post-transcriptional level either by causing RNA degradation or by blocking translation through base-pairing with complementary sequences within mRNA. Since partial miRNA complementarities are enough to target an mRNA, each miRNA has the ability to regulate a large number of genes (Jackson *et al.*, 2003). Another feature is that a conserved miRNA can regulate different genetic pathways and developmental processes in various organisms (Ambros and Chen, 2007).

In recent years, many miRNAs have been identified in plants and animals by experimental and computational

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approaches. Adequate characterization of the actions of miRNAs in the mammary gland in different physiological states could provide new insights into the regulation of gene expression. The goals of this review are to outline and integrate the experimental and computational studies on miRNA in healthy and mastitic mammary gland and to address the stability of miRNA in milk and possible interspecies transmission.

Biogenesis of miRNA

miRNAs are DNA-derived RNA transcripts that are not translated into proteins. miRNA biosynthesis is a complex process that involves several steps. The canonical pathway for miRNA biogenesis requires two RNaseIII enzymes, Drosha and Dicer. In this process, miRNAs are initially transcribed by RNA polymerase II as long transcript hairpin-shaped units. As with mRNA, these molecules are spliced, capped and polyadenylated to produce primary miRNA (pri-miRNA). This precursor is processed by the enzyme Drosha to yield pre-miRNA that is then transported to the cytoplasm where it is cleaved by Dicer to the mature length. The functional strand of mature miRNA is loaded onto an argonaute (Ago) protein, the main constituent of the RNA-induced silencing complex (RISC), while the other strand is degraded (Lee *et al.*, 2003; Du and Zamore, 2005; Shabalina and Koonin, 2008) (Figure 1). Chong *et al.* (2010) used Dicer- and Drosha-knock-out mice to confirm the requirement of both enzymes for this canonical miRNA biosynthesis. However, not all miRNAs are formed by this pathway. It is now known that there are variations in many of the steps involved and that this can influence the biogenesis results (Okamura *et al.*, 2007; Winter *et al.*, 2009). Deep sequencing technology has revealed marked variability in miRNA biogenesis and has shown that many different sequences can share the same miRNA precursor.

Identification of miRNA in mammary gland and milk

miRNAs have been identified in cells and in fluids such as saliva, amniotic fluid, blood, urine and milk. In fluids, miRNAs are enclosed in exosomes (Zhou *et al.*, 2012).

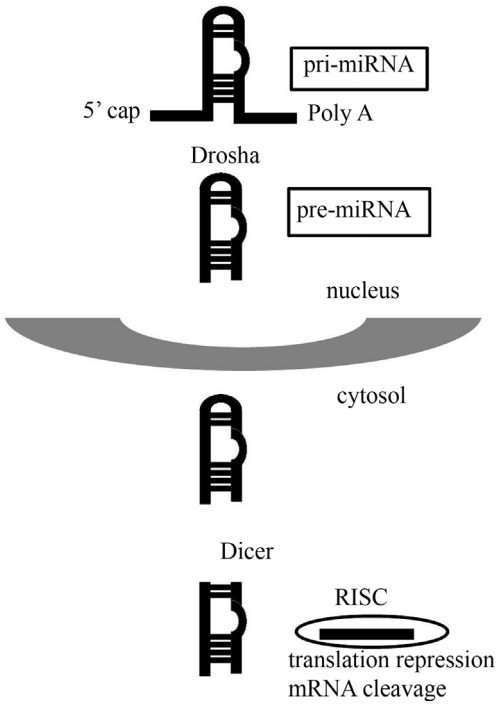

Figure 1 - A schematic representation of the canonical biogenesis of miRNA. Initially, a long hairpin-shaped (pri-miRNA) is transcribed by RNA polymerase II and then cleaved by Drosha (to yield pre-miRNA) prior to leaving the nucleus; the molecule is subsequently cleaved by a Dicer enzyme to yield double-stranded mature miRNA. Finally, miRNA is incorporated into the RNA-induced silencing complex (RISC), thereby allowing separation of the functional strand that interferes with mRNA by repressing translation or cleaving mRNA.

The number of miRNAs reported for mammary gland varies considerably (Table 1). Chen *at al.* (2010) used high-throughput sequencing technology to search for expressed small RNA in cow colostrum and milk. After classifying the molecules based on size, they found a similar number of reads in both fluids (1,594,965 in colostrum vs 1,418,136 in milk). A search in the miRBase (a database of published miRNA sequences and annotation) resulted in the identification of 230 and 213 known miRNAs in colostrum and mature milk, respectively. Among the differently expressed miRNAs, 108 were up-regulated and only eight were down-regulated in colostrum compared to mature milk. These results indicate a dynamic gene expression during

Table 1 - Total number of miRNAs reported in colostrum, milk and mammary gland in cow, goat and sheep.

	Number of miRNAs			Methods	Reference
Species	Colostrum	Milk	Mammary gland		
Cow	230	213		Solexa deep sequencing analysis	Chen *et al.* (2010)
Cow			921	Solexa deep sequencing analysis	Li *et al.* (2012d)
Goat	100	53		Microarray-real time PCR	Izumi *et al.* (2012)
Goat			328	Solexa deep sequencing analysis	Ji *et al.* (2012)
Goat		180	441	Solexa deep sequencing analysis	Li *et al.* (2012c)
Sheep			101	cDNA sequencing	Galio *et al.* (2012)

lactation. Izumi *et al.* (2012) identified a smaller number of miRNAs than Chen *et al.* (2010), with 100 being unique for colostrum and 53 for mature milk. It should be noted that whereas Chen *et al.* (2010) prepared RNA from a larger amount of milk and purified small RNA by PAGE, Izumi *et al.* (2012) started with a smaller amount of milk and used microarray analysis. Li *et al.* (2012d) reported 11,964,909 and 15,968,116 clean reads from mammary gland tissues in the lactating and non-lactating periods, respectively. After aligning the reads against the *Bos taurus* genome and miRBase, 885 pre-miRNAs were identified and encoded for 921 miRNAs; ~60% of these miRNAs were expressed during the lactation and non-lactation period; of these, 248 miRNAs were known, 57 were conserved and 239 were new identifications. In human milk, Zhou *et al.* (2012) identified exosomes containing pre-miRNA from four libraries. Four of the top ten miRNAs (30b, 182, 200a and 148a) were related to different aspects of the immune system, with the most abundant of them being miRNA 148a, which is also expressed in bovine milk (Chen *et al.*, 2010).

In a sheep mammary gland library constructed from early pregnancy tissue, Galio *et al.* (2012) identified 54 sequences already described in the miRBase; two of these miRNAs (27e and 36e) were identified for the first time in mammals and only miRNA 379 had previously been shown to occur in sheep. In goats, Ji *et al.* (2012) characterized miRNAs from a pool of five Laoshan breed animals. A total of 18,031,615 read sequences were obtained after discarding ~2.6% that did not meet the control criteria (*i.e.*, they were of low quality, had contaminants formed by adapter-adapter ligation, and contained reads without insert tags). Of these reads, 9,093,530 had a perfect match to the *Ovis aries* genome and 305,711 were new sequences. Furthermore, 290 conserved miRNAs and 38 novel miRNAs were identified, and this total of 338 miRNAs was very similar to that reported by Li *et al.* (2012c) for dairy goats (441 miRNAs).

The biological material (mammary gland tissue or milk), the amount of sample used, and the species and breed examined can influence the results. Moreover, differences in the quality control criteria used to classify the sequencing products (raw and clean reads) and discrepancies associated with library normalization may also contribute to variations in the number of miRNAs reported. Finally, some degree of variation may be introduced by the mode of sample preparation. Lee *et al.* (2010) observed no changes in the 3' end of a 22-nucleotide-long synthetic RNA introduced into RNA samples, but a fraction of the synthetic sequences were truncated at the 5' end. These authors concluded that the changes in the 5' end may have been caused by premature termination during the production of synthetic RNA. This finding suggests that at least part of the variation reported for end-region sequences may be linked to sample processing prior to sequence analysis. An additional source of diversity is that many miRNAs may vary from the published reference sequences. To address the latter phenomenon, Morin *et al.* (2008) proposed the terminology "isomers" to refer to sets of miRNAs that show similarity in their sequences. These authors suggested that isomer variability could be related to variation in the cleavage positions for the enzymes Dicer or Drosha within the pre-miRNA hairpin and showed results in which the variability among isomers influenced the differential expression of miRNA.

Functions of miRNA in mammary gland and milk

An important question in assessing miRNA function is whether the miRNAs present in milk are derived from blood or are specific for mammary gland. To address this issue, Chen *et al.* (2010) compared the miRNA profile of milk with that of serum from healthy cows and found that the total number of miRNAs in milk was about two-fold higher than in serum; they also identified 47 miRNAs unique to milk. Human breast milk also has a different pattern of miRNA expression compared to blood plasma (Kosaka *et al.*, 2010). These results clearly indicate that mammary alveolar cells express their own miRNAs.

Another question is whether the pattern of miRNA expression in mammary gland is constant throughout the lactation period. Of the 1,692,810 reads described by Li *et al.* (2012d), 34% were expressed only in the dry period compared to the peak period of milk production. Moreover, analysis of the expression patterns of 173 differentially expressed miRNAs showed that 165 were down-regulated during peak lactation compared to the dry period. Among the sequences reported by Li *et al.* (2012d), 56 showed significant differences in expression between lactating and non-lactating cows, as assessed using the IDEG6 package (Romualdi *et al.*, 2003); of these, nine were expressed only in lactating animals and six in non-lactating animals. However, 48 of these were confirmed by deep sequencing (Li *et al.*, 2012d), indicating that deep sequencing may be more sensitive and reliable than microarray analysis in identifying differentially expressed miRNAs. Together, these findings indicate that the pattern of miRNA expression varies according to the animals physiological state.

To examine the expression of specific miRNAs associated with cellular proliferation, metabolism and the innate immune response during lactation, Wang *et al.* (2012) assessed the expression of 13 miRNAs in cows during the dry period (30 d prepartum), fresh period (7 d postpartum) and early lactation (30 d postpartum). Twelve of the miRNAs identified (miRNAs 10a, 15b, 16, 21, 33b, 145, 146b, 155, 181a, 205, 221 and 223) were down-regulated in the dry period compared to during lactation. The exception was miRNA 31, which showed greater expression in early lactation compared to the dry period. Under normal conditions and using bioinformatic assays and biological experi-

ments, Xue *et al.* (2013) demonstrated that miRNA 31 up-regulated IL-2 (interleukin 2) expression by reducing the levels of the cytokines upstream kinase suppressor, KSR2 (kinase suppressor of ras 2). Interleukins are present in human milk (Bryan *et al.*, 2006) and have an important role in modulating the offsprings immunological system (Aspinall *et al.*, 2011). These findings indicate that miRNA 31 may have an indirect immunological role in the neonate.

Based on microarray analysis, Galio *et al.* (2012) identified three major patterns of miRNA expression in sheep. In pattern 1, expression was down-regulated during pregnancy, in pattern 2, miRNA expression was induced during pregnancy, and in pattern 3, miRNA expression was induced during lactation. The authors selected one miRNA from each pattern to confirm their expression by RT-qPCR in four animals per pattern. miRNA 21, which is expressed in alveolar epithelial cells, was up-regulated in non-pregnant sheep and at the beginning of pregnancy. This expression profile was attributed to a role for miRNA 21 in adipogenic differentiation. In this regard, Kim *et al.* (2009) showed that the activity of miRNA 21 in adipogenic tissue was mediated through TGF-β signaling. In contrast, miRNA 205 was expressed mainly in the basement membrane of normal mammary ducts and lobules during the first half of pregnancy and miRNA 200 was expressed in epithelial cells throughout pregnancy but was up-regulated at the end of pregnancy and lactation.

Bioinformatic analysis using the program RNA hybrid identified a miRNA 15a target sequence on the growth hormone receptor (Li *et al.*, 2012a). However, this miRNA was not identified in a sequence search by Chen *et al.* (2010). To confirm their finding, Li *et al.* (2012a) transfected this small RNA in mammary epithelial cells and observed a reduction in growth hormone receptor (GHR) transcription and in the expression of β casein. Growth hormone is the most relevant galactopoietic hormone in ruminants (Bauman and Vernon, 1993) and triggers casein expression (Sakamoto *et al.*, 2005). These results therefore indicated that miRNA 15a indirectly decreases milk production by blocking the expression of growth hormone receptor and thus identified a novel regulatory mechanism for GHR.

Some miRNAs have been suggested to have immunosuppressive roles. *In silico* analyses of two members of the miRNA 30 family (miRNAs 30a-5p and 30d-5p) predicted binding sites in several suppressors of cytokine signaling inhibitors of the JAK/STAT pathway that regulate IL-10 transcription (Gaziel-Sovran *et al.*, 2011). This miRNA has also been implicated in the formation of the adipose pad in mammary gland. Le Guillou *et al.* (2012) constructed transgenic mice over-expressing miRNA 30b and observed (based on histological analysis) that these animals had acinar structures with abnormally small lumens. Even when there were no differences in the concentration of major milk proteins, the number of lipid droplets was smaller and did not show the spherical shape seen in the wild type. Microarray analyses of animals that did not express miRNA 30b showed that 164 genes were up-regulated and 56 genes were down-regulated. All of the up-regulated genes were associated with tissue development, except for seven that were involved in the inflammatory response. It is remarkable that the blockade of just a single miRNA altered the expression levels of 222 genes.

Further studies are needed to understand the biological roles of most of the reported miRNAs. After identifying miRNAs in mammary gland by sequencing procedures or computational searches, it is important to validate the results in functional experiments and to study their expression pattern in physiological and pathological conditions (Huang *et al.*, 2010). The purpose of expression studies is to compare patterns between groups, *e.g.*, disease *vs.* healthy and lactation *vs.* dry. The subsequent variation observed between experimental groups should reflect differences in expression between the groups and not be attributable to other sources of variation such as sampling methods, stabilization procedures and extraction methods. A normalizer or internal control should be used. This control is generally an RNA that exhibits invariant expression across all samples, is expressed along with the target miRNA in the cells of interest, and demonstrates equivalent storage stability and efficiency of extraction and quantification as the target miRNA of interest (Vandesompele *et al.*, 2002; Peltier and Latham, 2008).

Selecting an optimal normalizer (an aspect that is frequently undervalued) may help to avoid inconsistent results. Gu *et al.* (2012) proposed a set of miRNAs for porcine milk studies. These authors compared the expression of six porcine milk miRNAs from different lactation periods and proposed three of them (miRNAs 17, 107 and 103) as internal controls because they were stabled throughout the periods studied (1 h and 3, 7, 14, 21 and 28 days postpartum). In cow, Izumi *et al.* (2012) normalized the samples using a synthetic cel-miRNA 39. Naeem *et al.* (2012) normalized the data using miRNA 320 and miRNA U6. The usefulness of the latter miRNA has been questioned because of its stability in serum (Chen *et al.*, 2008). For the next generation of sequencing technology, normalization will be challenging because different sequencing experiments may generate quite different total numbers of reads (Li *et al.*, 2012b). The total count normalization is inadequate for data generated by new generation sequencing technologies (Garmire and Subramaniam, 2012). To overcome this limitation various statistical models have been proposed for the normalization of data. Although there is still no general agreement about the most adequate internal controls and the best methods for normalizing data, it is nevertheless essential to establish criteria for selecting which controls should be used for each species and tissue and which method should be used to normalize the data in order to decrease the false discovery rate.

Functions of miRNA in mastitis

Mastitis, or inflammation of the mammary gland, is one of the most prevalent and costly diseases in dairy animals (for specific reviews see Halasa *et al.*, 2007; Akers and Nickerson, 2011; De Vliegher *et al.*, 2012). Intramammary infection occurs when bacteria cross the teat sphincter and reach the alveolar lumen after passing through the teat and gland cisterns. The first response of the immune system is a neutrophil influx via chemotaxis to establish the inflammatory process (Akers and Thompson, 1987). Understanding the molecular mechanisms involved in mastitis would be helpful in developing new strategies to prevent and treat this condition. *Streptococcus uberis* is one of the major etiological agents of mastitis. This Gram-positive bacterium can cause contagious or environmental mastitis (Rato *et al.*, 2008). Two recent studies examined the miRNA expression pattern in *S. uberis*-induced mastitis: one focused on *in vivo* infection and miRNA expression at 12 h post-infection while the other examined the expression pattern at different times in cultured mammary epithelial cells. Both studies provided interesting insights into the rapid and diverse response triggered after infection.

In the first of these studies, Naeem *et al.* (2012) examined the expression pattern of 14 miRNAs in mammary gland 12 h after a challenge with *S. uberis*. The resulting data plus the microarray gene expression patterns of 2,102 genes were used in bioinformatic analyses to identify miRNA targets and the biological pathways involved. The results showed down-regulation of miRNAs 15b, 16a, 31, 145 and 181a, and up-regulation of miRNA 223 in mastitis compared with healthy control animals (Figure 2, panel A). The target genes identified were mostly associated with immunological regulation, metabolic processes and cellular proliferation/differentiation. The change in miRNA 16a expression was associated with the up-regulation of some interleukins (IL-6, IL-8 and IL-10). The authors suggested that miRNA 16a might control the level of key inflammatory components in bovine mammary gland and could play a role in regulating the response to mastitis. Mastitis caused the down-regulation of miRNA 181a which has a role in the immune system (such as an increase in toll-like receptor and B cell receptor signaling). However, the decrease in miRNA 181a was the opposite of that observed in mice with an acute inflammatory response (Xie *et al.*, 2013). In the latter case, the levels of miRNA 181a increased within 2 h after the induction of inflammation and remained high for up to 6 h post-treatment; however, 12 h later the expression was lower than in control animals. The discrepancy between these two studies may be related to the sampling intervals since Naeem *et al.* (2012) obtained the mammary biopsies 20 h after the bacterial challenge. However, differences among species should also be considered. The only miRNA that was up-regulated in udder mastitis was miRNA 223, which can inhibit several cellular signaling pathways mediated via the down-regulation of IGF1R (insulin growth factor 1 receptor). Negative modulation of the immune response may be required to avoid damage to the host tissue.

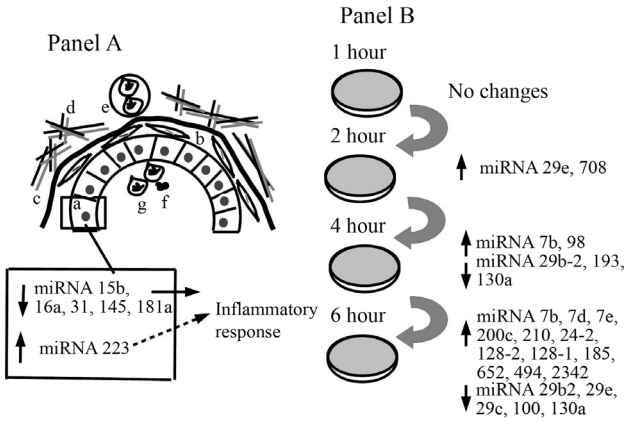

Figure 2 - Diagram of the mammary epithelial cell response to infection by *Streptococcus uberis in vivo* and *in vitro*. (A) Mammary alveolus showing (a) epithelial cells, (b) myoepithelial cells, (c) basement membrane, (d) extracellular matrix and (e) capillary. In the alveolar lumen: (f) bacterial infiltration and (g) neutrophil infiltration. The accompanying box shows that 12 h after inoculation with *S. uberis* there was a decrease in the expression of miRNAs 15b, 16a, 31, 145 and 181a and an increase in miRNA 223 to modulate the inflammatory response (Naeem *et al.*, 2012). (B) Mammary epithelial cells inoculated with *S. uberis*. No changes were observed at 1 h but there was an increase in miRNAs 29e and 708 at 2 h, an increase in miRNAs 7b and 98 and a decrease in miRNAs 29b-2, 193 and 130a at 4 h and, finally, an increase in 12 miRNAs (7b, 7d, 7e, 200c, 210, 24-2, 128-2, 128-1, 185, 652, 494 and 2342) concomitantly with a decrease in miRNA 29b2, 29e, 29c, 100 and 130a at 6 h (Lawless *et al.*, 2013).

In the second study, Lawless *et al.* (2013) identified 15 miRNAs that showed altered expression in cultured bovine mammary epithelial cells challenged with *S. uberis.* No changes were observed in the first hour after inoculation but at 2 h, miRNA 29e and miRNA 708 were up-regulated. This was followed by the up-regulation of miRNA 7b and miRNA 98 at 4 h post-challenge. At 6 h, 12 miRNAs were up-regulated (miRNAs 7b, 7d, 7e, 24-2, 128-1, 128-2, 185, 200c, 210, 494, 652 and 2342). Down-regulation was first observed only at 4 h and involved miRNAs 29b-2, 193a and 130a. Two miRNAs (29b-2 and 130a) were down-regulated at 4 h and 6 h (Figure 2, Panel B). Although a large number of 5' isomers were identified, they were expressed at a low rate. The prediction of target genes showed that only the miRNAs down-regulated at 4 h and 6 h post-inoculation were significantly enriched in genes with a role in innate immunity. Table 2 summarizes the different miRNAs reported in mammary gland and milk and their level of expression in cow and sheep.

The mammary alveolus cell is a three-dimensional structure in which the extracellular matrix plays an active role in epithelial function such as casein expression (Katz and Streuli, 2007). Therefore, the time intervals studied and the biological model used (*in vivo* vs. cell culture) could account for the divergent miRNA expression reported in these studies. Nevertheless, the results clearly suggest that

S. uberis can coordinate different processes by regulating target miRNAs. Another aspect to emphasize is the variability in miRNA expression and their rapid and dynamic temporal expression patterns that can modulate the ability of mammary epithelial cells to mobilize the innate immune system.

Table 2 - Summary of miRNA expression in mammary gland, mammary epithelial cell lines and milk in cow and sheep.

miRNA	Tissue or biological fluid	Species	Expression level	Reference
miRNA 7b	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 7d	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 7e	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 10a	MGT	Cow	Elevated in lactation	Wang *et al.* (2012)
miRNA 15a	MECL	Cow	Elevated in transfected cells	Li *et al.* (2012a)
miRNA 15b	MGT	Cow	Elevated in lactation and reduced in mastitis	Wang *et al.* (2012); Naem *et al.* (2012)
miRNA 16	MGT	Cow	Elevated in lactation	Wang *et al.* (2012)
miRNA 16a	MGT	Cow	Reduced in mastitis	Naem *et al.* (2012)
miRNA 21	MGT	Cow/sheep	Elevated in cow during lactation and in sheep during early pregnancy	Galio *et al.* (2012); Wang *et al.* (2012)
miRNA 24-2	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 29-b2	Milk	Cow	Reduced in mastitis	Lawless *et al.* (2013)
miRNA 29c	Milk	Cow	Reduced in mastitis	Lawless *et al.* (2013)
miRNA 29e	Milk	Cow	Elevated and then reduced in mastitis	Lawless *et al.* (2013)
miRNA 31	MGT/milk	Cow	Reduced in lactation and mastitis	Wang *et al.* (2012); Naem *et al.* (2012); Lawless *et al.* (2013)
miRNA 33b	MGT	Cow	Elevated in lactation	Wang *et al.* (2012)
miRNA 98	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 100	Milk	Cow	Reduced in lactation	Lawless *et al.* (2013)
miRNA 128-1	Milk	Cow	Elevated in lactation	Lawless *et al.* (2013)
miRNA 128-2	Milk	Cow	Elevated in lactation	Lawless *et al.* (2013)
miRNA 130a	MGT	Cow	Reduced in mastitis	Lawless *et al.* (2013)
miRNA145	MGT	Cow	Elevated in lactation and reduced in mastitis	Wang *et al.* (2012); Naem *et al.* (2012)
miRNA 146b	MGT	Cow	Elevated in lactation	Wang *et al.* (2012)
miRNA 148a	Milk	Cow	Elevated in lactation	Chen *et al.* (2010)
miRNA 155	MGT	Cow	Elevated in lactation	Wang *et al.* (2012)
miRNA 181a	MGT	Cow	Elevated in lactation and reduced in mastitis	Wang *et al.* (2012); Naem *et al.* (2012)
miRNA 185	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 193a	MGT	Cow	Reduced in mastitis	Lawless *et al.* (2013)
miRNA 200	MGT	Sheep	Elevated in lactation	Galio *et al.* (2012)
miRNA 200c	MGT/milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 205	MGT	Cow/sheep	Elevated in cow lactation and mastitis and second half of sheep pregnancy	Galio *et al.* (2012); Wang *et al.* (2012)
miRNA 210	MGT/milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 221	MGT	Cow	Elevated in lactation	Wang *et al.* (2012)
miRNA 223	MGT	Cow	Elevated in lactation and mastitis	Wang *et al.* (2012); Naem *et al.* (2012)
miRNA 494	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 652	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 708	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)
miRNA 2342	Milk	Cow	Elevated in mastitis	Lawless *et al.* (2013)

ECL: mammary epithelial cell line, MGT: mammary gland tissue.

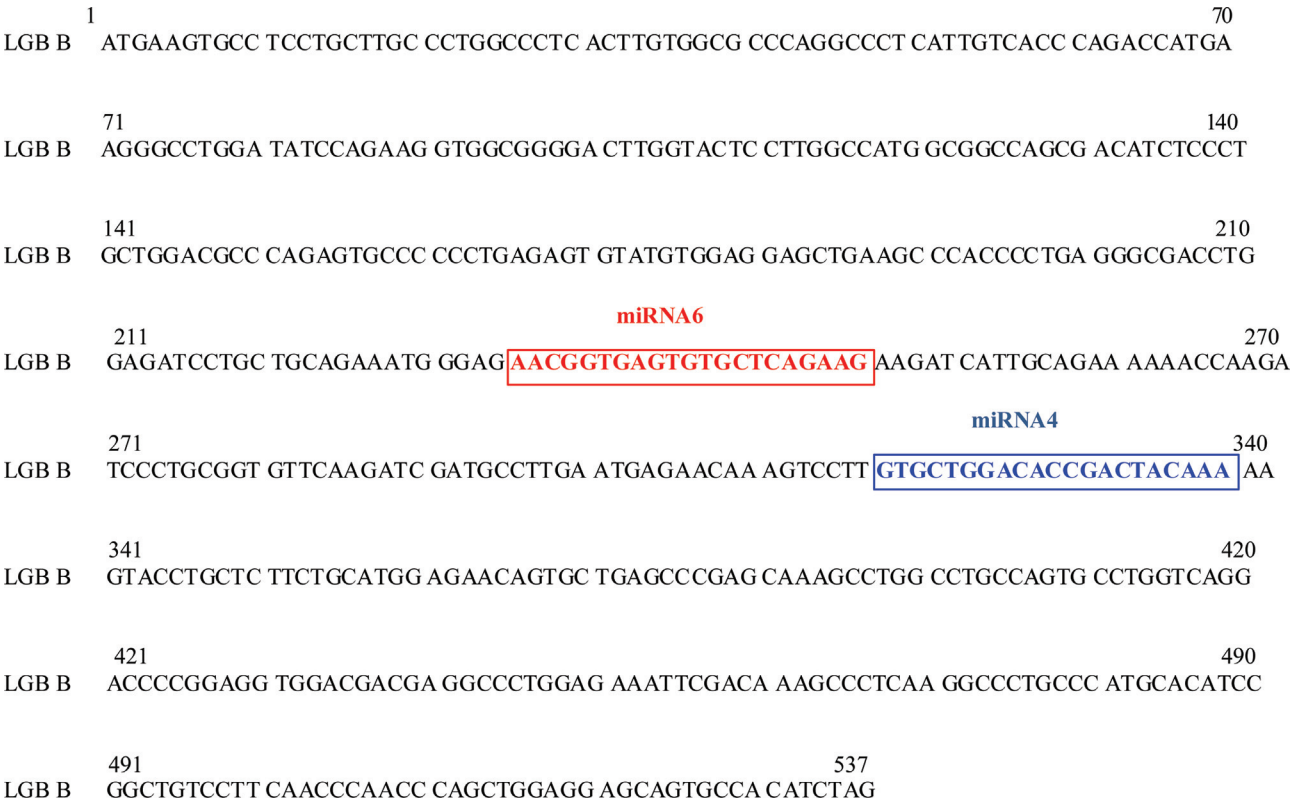

Figure 3 - *Bos taurus* lactoglobulin B (LGB) mRNA sequence (GenBank accession number BC108213.1) showing the positions of miRNA6 and miRNA4 that targeted LGB, as designed by Jabed *et al.* (2012). The numbers refer to the nucleotide positions.

miRNA and biotechnology

Lactoglobulin (LGB) is the major whey protein in ruminants whereas human milk contains no LGB (Anderson *et al.*, 1982). This difference in milk composition accounts for some of the milk allergy problems in infants. LGB intolerance in infants has been known since 1965 (Davidson *et al.*, 1965). One strategy to reduce the allergenic potential of milk is to produce LGB-free milk. In pigs, Merkl *et al.* (2011) reported the effectiveness of short hairpin RNAs and artificial miRNAs in blocking LGB. Jabed *et al.* (2012) designed a successful strategy to block LGB synthesis by constructing a transgenic cow that expressed a tandem miRNAs construct (miRNA 6 and miRNA 4) against bovine LGB (see Figure 3). By controlling the artificial miRNAs through a lactation-specific-promoter it is possible to express miRNAs only during the lactation period when LGB is being produced. The analysis of milk samples by SDS-PAGE and HPLC showed that milk from miRNA 4 and 6 calves contained no LGB, but there was a strong, compensatory effect on the levels of other milk proteins. Consequently, artificial miRNAs could be an alternative for abolishing the production of specific proteins (Jabed *et al.*, 2012).

Stability of miRNA in milk

One of the interesting features of miRNAs is their stability. miRNAs are resistant to acidic conditions, digestion by RNAse, incubation at room temperature and various freeze/thaw cycles (Hata *et al.*, 2010; Kosaka *et al.*, 2010; Gu *et al.*, 2012; Izumi *et al.;* 2012; Zhou *et al.*, 2012). In milk, this resistance to degradation is explained by the fact that miRNAs are contained in exosomes or microvesicles. Treatment with Triton-X, a detergent that disrupts lipid membranes, results in the degradation of miRNAs by RNAse (Zhang *et al.*, 2010). The resistance to acidic conditions ensures passage through the stomach and absorption into the bloodstream, and this in turn allows the exchange of genetic information between mother and offspring.

An important recent finding is the isolation of miRNAs from industrially processed foods for infants (Izumi *et al.*, 2012). This raises new questions about the possible role that cow miRNAs could have in the end-consumer, especially children. Could cross-species miRNAs have an epigenetic role? In this regard, the study by Zhang *et al.* (2012) have provided interesting results regarding cross-kingdom transmission and regulation since these authors isolated a plant miRNA (miRNA168a) from the blood of mice fed with rice. They also demonstrated that this miRNA could bind to low density lipoprotein receptor adapter 1 mRNA in liver and that this binding led to a de-

crease in low density proteins in mouse plasma. This plant-derived miRNA was also identified in people consuming a rice-based diet. Even though plant and animal miRNAs have undergone independent evolutionary adaptations and are unrelated (Ambros, 2011), these findings show that plant miRNA can still exert a cross-kingdom effect.

The identification of cross-kingdom miRNA transmission raises significant questions. To what extent can miRNAs present in the diet regulate mammalian genes? How many miRNAs are incorporated through a normal diet? What are the effects of these miRNAs in health and disease? Milk provides a variety of bioactive components such as lactoferrin, defensin and immunoglobulin. In this regard, miRNAs represent yet another group of molecules transported by milk that could influence the immunological system in neonates.

Conclusion

The identification of small non-coding RNAs has provided new insights into cell regulation and intercellular communication. Studies of the mammary gland in lactation and mastitis show that the temporal expression of miRNAs regulates the innate immune system. The discovery of cross-species and cross-kingdom miRNA regulation provides a basis for new research into the regulatory mechanisms involved and the impact of epigenetic regulation. As stated by Zhang *et al.* (2012): "like vitamins, minerals and other essential nutrients derived from food sources, plant (or milk) miRNAs may serve as a novel functional component of food and make a critical contribution to maintaining and shaping animal body structure and function". We are in an exciting time of new discoveries and a new understanding of gene regulation and epigenetic effects. Future research will improve our understanding of the role of miRNAs in health and disease and their importance as food resources.

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