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Non-Coding RNA Roles in Ruminant Mammary Gland Development and Lactation

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Abstract

The ruminant mammary gland (MG) is an important organ charged with the production of milk for young and human nourishment. Many factors influence MG productivity, including nutrition, genetics, breed, epigenetics (including non-coding RNA [ncRNA]), disease pathogens and other environmental factors. In recent years, increasing research is beginning to determine the role of non-coding RNA in MG functions. Non-coding RNAs (small interfering RNA [siRNA], microRNA [miRNA], PIWI-interacting RNA [piRNA], small nucleolar RNA [snoRNA] and long non-coding RNA [lncRNA]) are a class of untranslated RNA molecules that function to regulate gene expression, associated biochemical pathways and cellular functions and are involved in many biological processes. This chapter presents a review of the current state of knowledge on the role of ncRNAs (particularly miRNAs and lncRNAs) in the MG and lactation processes, lactation signalling pathways, lipid metabolism, MG health of ruminants as well as miRNA roles in milk recipients. Finally, the potential application of new genome editing technology for ncRNA studies in MG development, the lactation process and milk components is presented.

Keywords: non-coding RNA, microRNA, long non-coding RNA, mammary gland, lactation, genome editing, signalling pathways

1. Introduction

As one of the remarkable products of evolution, lactation is a very dynamic and complex process. The process of lactation involves the development of the mammary gland (MG) and the synthesis and secretion of milk. The lactation process is affected by many factors, including genetics, epigenetics, non-genetics and environmental factors. The knowledge of lactation regulation is not only important for improvement of milk production and quality

but also provides a model for basic cellular processes (proliferation, differentiation, survival and death) [1], which may have important implications for productivity (milk yield) and disease status (e.g. breast cancer, mastitis, etc.). The endocrine regulation and physiological processes as well as the signalling pathways involved in these processes are fairly understood [1, 2]. Facilitated by the release of the whole genome sequences of cattle, sheep and goat [3–6] as well as availability of single nucleotide polymorphism (SNP) genotyping chips [7–11], the genetic mechanisms of ruminant lactation have been extensively explored (**Figure 1**). As a consequence, many quantitative trait loci (QTL) and genetic markers for lactation-related traits (for instance, milk yield, milk components, lactation persistency, etc.) have been detected and catalogued in the animal QTL database (<http://www.animalgenome.org/cgi-bin/QTLdb/index>).

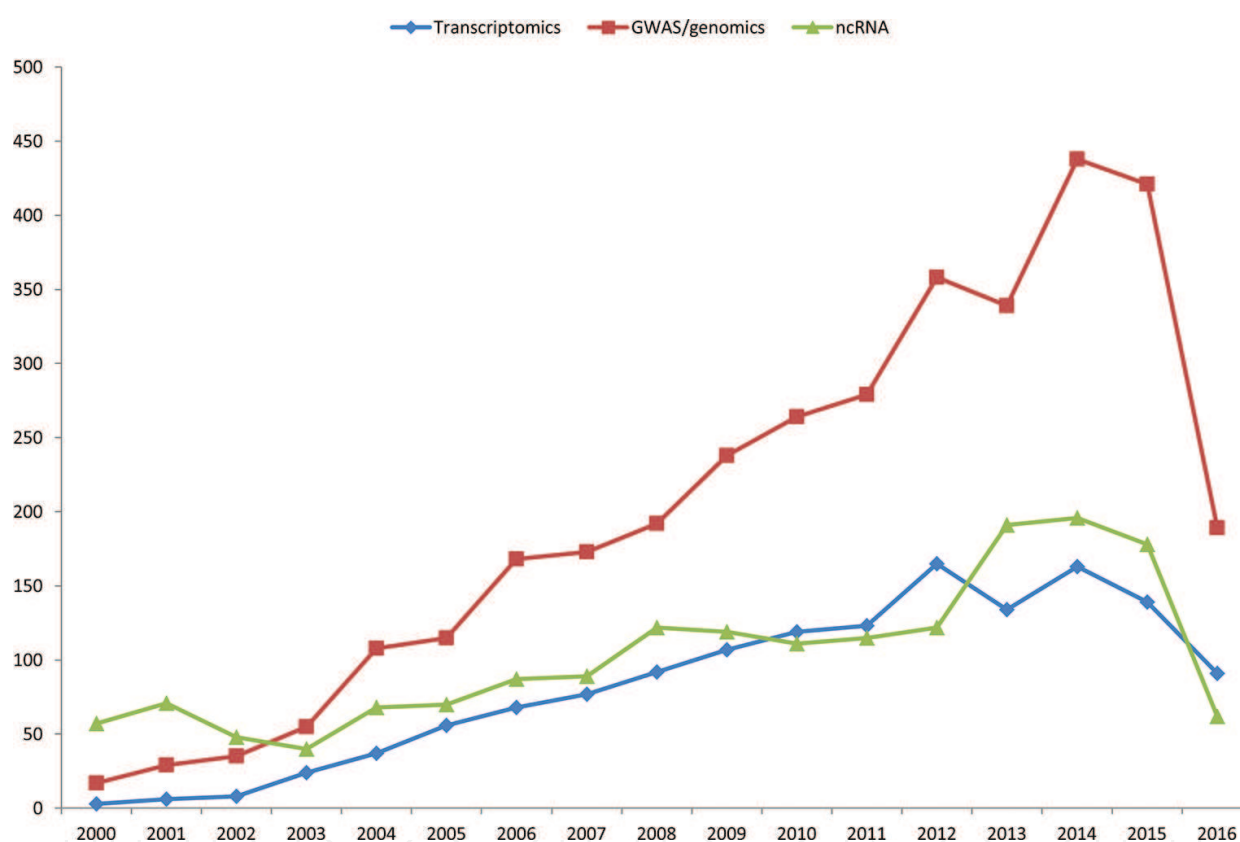


Figure 1. Growing research by year in the field of cattle genomics and transcriptomics (including non-coding RNA) from January 2000 to August 2016.

Transcriptomics research by both microarray and RNA sequencing methods has allowed for a better understanding of the genes and regulatory networks of complex traits in animals [12], such as the biosynthesis of major milk components (reviewed in Refs. [13, 14]). Emerging studies now suggest that non-coding RNAs (ncRNAs) are key regulators of mammary gland development and lactation processes [15–17]. The results from the ENCODE (ENCyclopedia of DNA elements) project [18, 19] indicate that only a small portion of the genome, about

1.5%, codes for proteins while most of the genome is transcribed into non-coding regulatory elements or ncRNA. This indicates that ncRNAs play significant regulatory roles in complex animal traits. A similar project to functionally annotate regulatory elements in animal genomes (FAANG project, www.faang.org) started in 2014 [20] and will generate data that will foster understanding of how the genome is read and translated into complex animal traits of economic importance. Indeed, the recent explosion of data on the regulatory functions of ncRNAs proves their importance in the regulation of multiple/major biological processes impacting development, differentiation and metabolism. This chapter explores recent developments on the expression, regulation and functions of ncRNAs, in particular microRNA (miRNA) and long non-coding RNA (lncRNA), in ruminant (cattle, sheep and goat) mammary gland development and the lactation process, as well as illustrate our own studies on the roles of ncRNAs in these processes.

2. Non-coding RNAs: biosynthesis and classification

Non-coding RNAs are transcribed RNA molecules that are not translated into proteins. They play a remarkable variety of biological functions by engaging target transcripts through sequence-specific interactions. They regulate many biological processes, including gene expression (transcription, RNA processing and translation), protect genomes from foreign nucleic acids and can guide DNA synthesis or genome rearrangement [21]. In general, ncRNAs are classified according to size or function. According to size, ncRNAs are classified as (1) small or short ncRNA: <200 nucleotides in their mature forms (e.g. miRNA, PIWI-interacting RNA [piRNA], small nuclear RNA [snRNA], small nucleolar RNA [snoRNA] and endogenous small interfering RNA [siRNA]) and (2) long ncRNA: >200 nucleotides long (e.g. lncRNA). According to function, ncRNAs are classified as (1) housekeeping or translation-related ncRNAs: they are constitutively expressed and crucial for normal cellular function and viability and include tRNA, rRNA and snoRNA and (2) regulatory ncRNAs and include miRNA, lncRNA, siRNA and piRNA [22, 23]. The biogenesis of these various types of ncRNAs has been discussed extensively [23–26]. This chapter focuses particularly on the involvement of miRNA and lncRNA in ruminant mammary gland development and lactation.

2.1. MicroRNAs

MiRNAs are an abundant class of short ncRNAs of about 22 nucleotides long. They regulate a variety of cellular processes through post-transcriptional repression of gene expression. MiRNAs consequently control the activities of about 60% of all protein-coding genes and participate in the regulation of almost every cellular process investigated in mammals [25]. Mature miRNAs are generated from a series of biochemical events beginning in the nucleus and culminating in the cytoplasm [24, 27, 28]. Briefly, these events occur in several main steps as follows: (1) nuclear processing of primary miRNA transcripts (pri-miRNAs) into precursor miRNAs (pre-miRNAs) by the DiGeorge Syndrome Critical Region Gene 8 (DGCR8)/Drosha complex, (2) cytoplasmic processing of pre-miRNAs into imperfectly paired miRNA duplexes by dicer,

and (3) preferential incorporation of one strand (the ‘guide’ miRNA strand) onto the RNA-induced silencing complex (RISC) [25]. Most miRNA genes located in introns of protein-coding genes share the promoter of the host gene [29]. MiRNAs often have multiple transcription start sites and regulate gene expression through inhibition of translation initiation or elongation, co-translational protein degradation and premature termination of translation [25, 30].

Since the discovery of the first miRNA, lin-4, in 1993 [31] and aided by deep sequencing technologies and developments in bioinformatics processing of deep sequence data, thousands of miRNAs have been detected in humans, mouse, farm animal species and plants and deposited in the miRNA data base (**Table 1**). Due to the crucial regulatory roles of miRNAs in many biological processes across species, they are being considered as candidate biomarkers of various human diseases, such as autoimmune [32], metabolic [33] and cardiovascular diseases [34], and various types of cancers [35–37].

Species	MiRNA		lncRNA	
	Precursor	Mature	Transcripts	Genes
Cattle	808	793	22,386	23,696
Sheep	106	153	–	
Goat	267	436	–	
Pig	382	411	–	
Chicken	740	994	13,085	9681
Human	1881	2588	141,353	90,062
Mouse	1193	1915	117,405	79,940

*Data source: MiRBase release 21 (<http://www.mirbase.org/>[38], and NONCODE database (www.noncode.org, Noncode 2016 [39]).

Table 1. Number of detected miRNAs and lncRNAs in farm animal species, mouse and human*.

2.2. Long non-coding RNAs

Long non-coding RNAs are a diverse collection of non-coding RNAs with emerging regulatory roles in many biological processes in every branch of life [26, 40–42]. LncRNA transcripts are >200 nucleotides long and constitute the largest portion of the mammalian non-coding RNA transcriptome [40]. LncRNA closely resembles mRNA than other classes of ncRNA in terms of their biogenesis pathways and form. Most lncRNAs are transcribed by the activities of RNA polymerase II, have a 5’ terminal methylguanosine cap and are often spliced and polyadenylated [41]. Some non-polyadenylated lncRNAs arise through alternative pathways probably expressed from RNA polymerase III promoters [43, 44] or arise during splicing and small nucleolar RNA production [45]. Furthermore, some lncRNAs are regulated in different ways at different stages of their biogenesis, maturation and decay [26]. Thousands of genes encoding lncRNAs have been identified in mammalian genomes (including livestock species),

birds and plants studied so far and deposited in the NONCODE database (www.noncode.org [39], **Table 1**).

3. MicroRNA in mammary gland development and lactation biology

3.1. Occurrence of microRNA in ruminant mammary gland and in milk

The regulatory roles of miRNAs in livestock species have emerged and are growing quickly [46, 47]. The most recent release of miRBase (release 21, <http://www.mirbase.org/>, [38]) contains 793 mature miRNAs for cattle, 436 for goat and 153 for sheep [38] (**Table 1**). However, with the increase in the application of RNA sequencing in expression profiling of miRNAs in different livestock species, the number of novel livestock miRNAs is expected to increase.

3.1.1. Cattle

The profiles of miRNAs in bovine MG tissue or milk have been investigated using different approaches, such as microarray [48, 49], genome sequencing [4] and RNA sequencing [50–57]. A total of 496 miRNA genes were identified following sequencing of the cattle genome of which 135 were novel [4]. The expression profiles of miRNAs in MG tissues and cells facilitate discovery of novel miRNAs and also identification of candidate miRNAs for different cell types, lactation stages, periods, disease response and so on. Before the release of the bovine genome sequence, Gu et al. [49] pioneered miRNA discovery in the bovine MG by cloning and sequencing small RNAs from MG tissue followed by identification of 59 distinct bovine miRNAs. Using next-generation sequencing techniques, Chen et al. [58] identified 230 and 213 known miRNAs in cow colostrum and mature milk, respectively. The authors also observed that 108 and 8 miRNAs were upregulated and downregulated, respectively, in colostrum compared to mature milk [58]. Using microarray, Izumi et al. [59] identified 100 and 53 known miRNAs in colostrum and mature milk, respectively. Using Solexa sequencing method, Li et al. [60] reported 884 unique miRNAs sequences in the bovine MG (283 known, 505 novel and 96 conserved miRNAs). Le Guillou et al. [61] identify 167 novel miRNAs in the bovine MG, many of which were also detected in mouse MG. Analysing three milk fractions (fat, whey and cells) and mammary gland tissues, we reported 210, 200 and 249 known and 33, 31 and 36 novel miRNAs in milk fat, whey and cells, respectively, and 321 known and 176 novel miRNAs in mammary gland tissues [62]. Deep sequencing the milk fat across the lactation curve, we also identified a total of 475 known and 238 novel miRNAs [63].

3.1.2. Goat

A total of 487 miRNAs were identified when the goat genome was sequenced and the largest miRNA clusters were found on chromosome 21 [6]. Using the Illumina-Solexa high-throughput sequencing technology to analyse goat MG tissues during early lactation, Ji et al. [64]

reported 131 novel and 300 conserved miRNAs. Using the same method (Illumina-Solexa sequencing), Li et al. [65] reported 346 conserved and 95 novel miRNAs in goat MG tissues from dry off and peak lactation does.

3.1.3. Sheep

Most miRNAs identified in sheep come from tissues other than the MG. For example, Caiment et al. [66] identified 747 miRNAs from the skeletal muscle through deep sequencing, whereas McBride et al. [67] reported 212 miRNAs from sheep ovarian follicles and corpus lutea at various reproductive stages. In the MG, Galio et al. [68] showed the presence of three known miRNAs including miR-21, miR-205 and miR-200 family in pregnant and lactating sheep.

3.2. MicroRNA function in ruminant mammary gland and milk synthesis

3.2.1. Expression patterns of microRNAs in lactation stages

3.2.1.1. Temporal and spatial expression of microRNAs

Indication of involvement of miRNAs in MG functions was gained through observation of differences in type and expression levels of miRNAs between lactation stages, under different nutritional regimes and presence of disease pathogens. Li et al. [50] identified 56 miRNAs that were significantly differentially expressed between lactation and non-lactation periods. Similarly, Wang et al. [48] detected 12 downregulated miRNAs (miR-10a, miR-15b, miR-16, miR-21, miR-33b, miR-145, miR-146b, miR-155, miR-181a, miR-205, miR-221 and miR-223) in the dry period (30 days prepartum) compared to early lactation period (7 days postpartum) and one upregulated miRNA (miR-31) in early lactation compared to the dry period. Previously, we examined miRNA expression pattern during a lactation cycle to explore its regulatory mechanisms during lactation using milk fat as input tissue for sampling [63]. In a previous investigation, we have shown that milk fat miRNA transcriptome closely resembles the miRNome of MG tissue [62]. We collected samples at the lactogenesis (LAC) (day 1 and 7), galactopoiesis (GAL) (day 30, 70, 130, 170 and 230) and involution (INV) (day 290 and when milk production dropped to 5 kg/day) stages from nine cows for deep sequencing [63]. We observed that 15 miRNAs (miR-30a-5p, miR-30d, miR-21-5p, miR-26a, miR-148a, let-7a-5p, let-7b, let-7f, let-7g, miR-99a-5p, miR-191, miR-200a, miR-200c, miR-186, miR-92a) were highly expressed across lactation stages [63]. MiR-148a and miR-26a were the most abundantly expressed accounting for more than 10% of the read counts in each stage of lactation. We also performed a differential expression (DE) analysis and detected miR-29b/miR-363 and miR-874/miR-6254 as important mediators of transition signals from LAC to GAL and from GAL to INV stages, respectively [63]. Furthermore, DE analysis indicated various patterns of miRNA expression across the lactation curve. For instance, some miRNAs were highly expressed during early lactation (lactogenesis) followed by decreased expression at later stages, whereas others were slightly expressed during early lactation but showed increased expression during mid-lactation and decreased expression during late lactation and vice versa [63] (**Figure 2**).

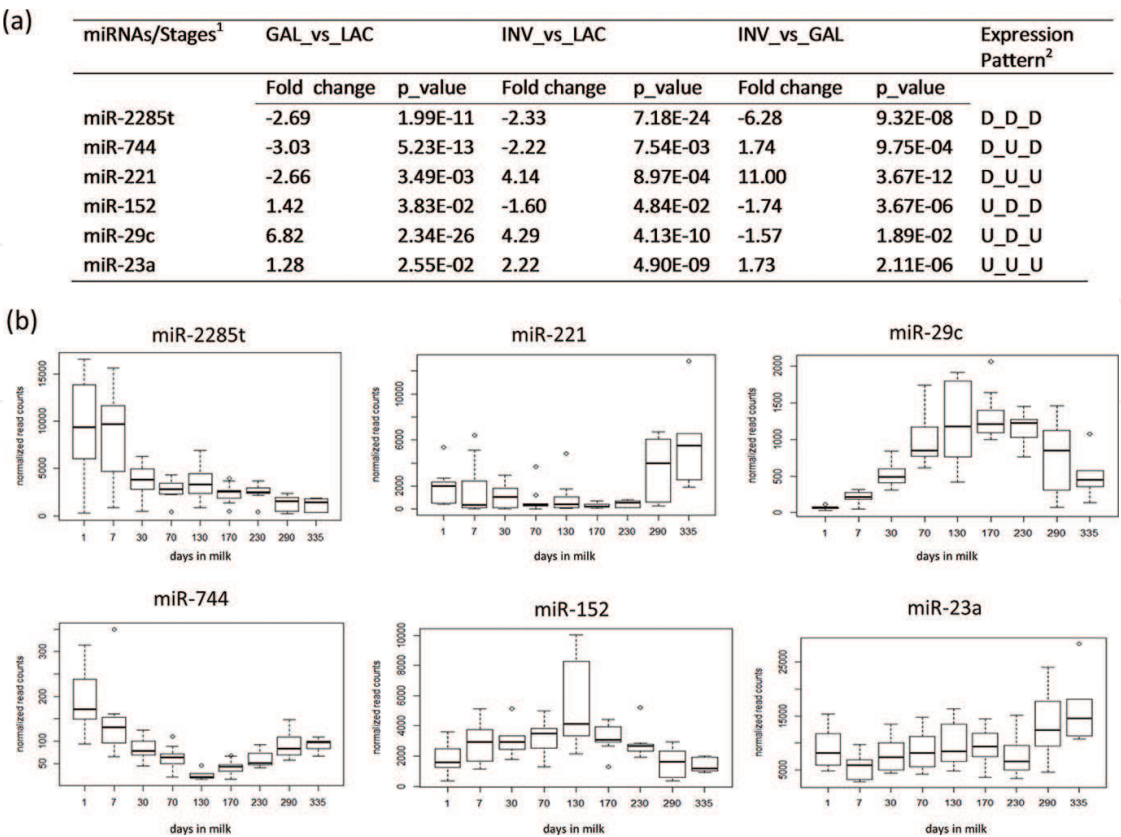


Figure 2. Differential miRNA expression patterns during a bovine lactation curve. (a) Fold change values of six miRNAs whose expression patterns changed significantly during each lactation switch and (b) box plots of their normalized read count values by lactation day. ¹LAC: lactogenesis; GAL: galactopoiesis; INV: involution; ²D: downregulated and U: upregulated.

The temporal expression pattern of miRNAs has been reported in other ruminant species. For example, Galio et al. [68] reported a change in the expression pattern of miR-21, miR-205 and miR-200 family in MG tissues from pregnant and lactating sheep. From the early, middle and late stages of pregnancy and during lactation, the expression of miR-21 and miR-25 decreased, whereas miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) showed increased expression [68]. Similarly, investigating the expression pattern of miRNAs during early and peak lactation and dry period, Li et al. [65] identified 15 differentially expressed miRNAs when comparing peak lactation and dry period including three significantly highly expressed miRNAs (miR-2887, miR-451 and miR-2478) during peak lactation and 12 significantly highly expressed miRNAs (miR-199b, miR-128, miR-25, miR-145, miR-98, miR-222, miR-181b, miR-199a-3p, miR-93, miR-221, let-7b and let-7c) during the dry period.

3.2.1.2. MicroRNAs synergistically regulate lactation control mechanisms

A wealth of evidence indicates that several miRNAs can work together to regulate target genes in the same or different biological pathways [69, 70]. We have successfully characterized a group of highly interacting miRNAs (modules) using a weighted co-expression network analysis [71] and correlated important miRNA modules to milk yield and milk components [72].

We identified three consensus (BLUE [62 miRNAs], TURQUOISE [133 miRNAs] and BROWN [59 miRNAs]) modules and the GREY module reserved for unclassified genes, throughout lactation stages (**Figure 3**). Based on module trait relationship, we were able to determine important modules (with absolute correlation >0.6) for milk components at each lactation stage. The BROWN and BLUE modules were highly related to protein and somatic cell count, respectively, in early lactation, the BLUE module to somatic cells in middle lactation and the BLUE module to urea and lactose in late lactation stage. We also found the most important component or hub miRNAs, which potentially coordinated miRNA synergetic mechanisms in their respective modules. MiR-149-5b and miR-874 were hub miRNAs in the BLUE module for milk somatic cells at early and middle lactation, respectively, whereas miR-330 was the hub miRNA in the BLUE module for milk urea and lactose at late lactation (**Figure 3**). Three miRNAs (mir-149-5b, miR-874 and miR-30) in the BLUE module play important roles in cell cycle [73–77], so it could be expected that these miRNAs regulate secretion of somatic cells in milk from MG.

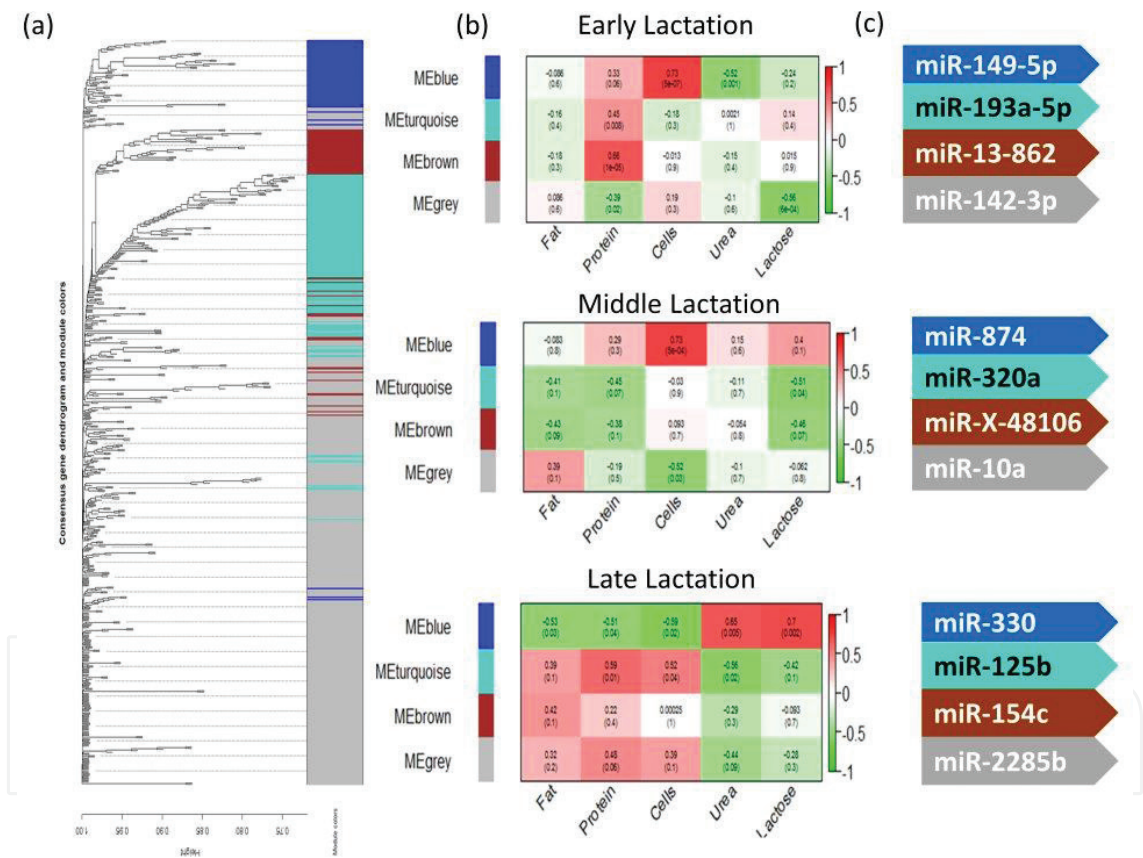


Figure 3. Important consensus modules and their hub miRNAs for milk component traits in different lactation periods. (a) Dynamic cut tree (dendrogram) based on topological overlap distance in gene expression profile; (b) module trait relationship in early, middle and late lactation and (c) hub miRNAs in the modules. GREY colour is for genes that do not belong to a specific module.

3.2.2. Networks and pathways regulated by microRNAs during a lactation cycle

Through their target genes, miRNAs have been shown to control signal transduction in different species [78]. MiRNA roles in important pathways such as transforming growth factor beta

(TGF- β), prolactin and protein kinase signalling in MG development and lactation have been reviewed by several authors [79–83]. MiRNA regulation of three important signalling pathways (NOTCH, PTEN and HIPPO) in MG and breast cancer cells was recently reviewed [15]. Important miRNAs regulating these pathways include mir-34, mir-29, mir-146, mir-199 and mir-200 families for NOTCH signalling pathway, miR-21 and miR-155 for PTEN signalling pathway and miR-934 for HIPPO pathway. In Canadian Holstein cows, we performed the enrichment of differentially expressed miRNA target genes to signalling pathways and noted that relevant signalling pathways for transition between lactation stages are involved in apoptosis (PTEN and SAPK/JNK), intracellular signalling (protein kinase A, TGF- β and ERK5), cell cycle regulation (STAT3), cytokines (prolactin), hormone and growth factors (growth hormone and glucocorticoid receptor). *PTEN* is an important target gene for miR-29b in the regulation of mammary gland development [84]. PTEN signalling is crucial for the activities of prolactin autocrine [85]. The initiation of lactation is known to require induction of autocrine prolactin, and the level of this autocrine is known to be endogenously regulated by the signal of PTEN-PI3K-AKT pathway [85]. **Figure 4** is an illustration of some miRNAs that target genes in relevant signalling pathways during lactation [63]. Pathways, such as PTEN and growth hormone signalling, have been identified as important for regulatory mechanisms during lactation [85, 86].

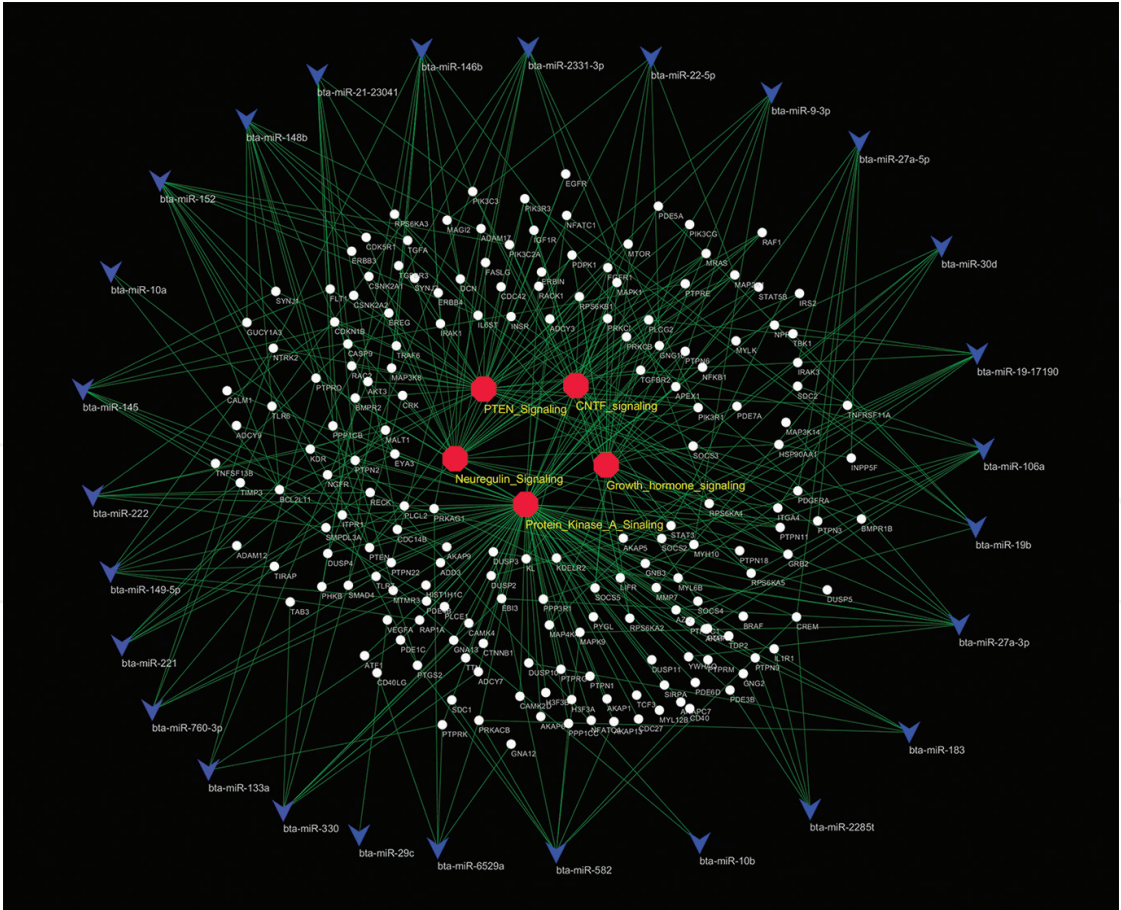


Figure 4. Illustration of miRNA-gene-pathway networks obtained from dynamic differentially expressed miRNAs during a bovine lactation curve. The outer layer shows miRNAs (blue arrow heads), which targets at least two genes (white dots) in significantly enriched pathways (red dots).

3.2.3. Functional validation of microRNA target genes

Since *in vivo* experiments for functional validation of MG miRNAs are not feasible, such studies have mostly relied on the use of knock-out/mimics and MG-specific cell types. Using bovine mammary epithelial cells (BMEC), miR-15a was shown to regulate growth hormone receptor, viability of BMEC and the expression of casein genes [86]. MiR-486 regulation of lactation by targeting the PTEN gene in cow MGs has been demonstrated [87]. Bian et al. [88] recently reported that epigenetic regulation of miR-29s affects the lactation activity of BMEC. MiR-181a was shown to regulate the biosynthesis of bovine milk fat through targeting acyl-CoA synthetase long-chain family member 1 (*ACSL1*) [89]. MiR-103 was reported to control milk fat accumulation in goat MG during lactation [90]. Moreover, miR-27a was shown to suppress triglyceride accumulation as well as altered gene expression associated with fat metabolism in dairy goat mammary epithelial cells (GMEC) [91]. In another study, miR-135a was reported to target and regulate prolactin receptor (*PRLR*) gene in GMEC [92]. Inhibition of the expression of miR-145 in GMEC was shown to increase methylation levels of fatty acid synthase (*FASN*), stearoyl-CoA desaturase 1 (*SCD1*), peroxisome proliferator-activated receptor gamma (*PPARG*) and sterol regulatory element binding transcription factor 1 (*SREBF1*) [93]. MiR-24 control of triacylglycerol synthesis in goat mammary epithelial cells by targeting *FASN* gene has been demonstrated [94]. The ability of miR-145 to regulate lipogenesis in GMEC through targeting insulin-induced gene 1 (*INSIG1*) and epigenetic regulation of lipid-related genes has been demonstrated [93]. MiR-143 was shown to inhibit proliferation as well as induce apoptosis of GMEC [95]. MiR130b regulation of PPAR γ coactivator-1 α suppressed fat metabolism in GMEC [96]. In non-ruminant species, many miRNAs, including let-7 family members, mir-17/92, miR-30b, miR-93, miR-99a and miR-b, miR-101a, miR-126-3p, miR-138, miR-146b, miR-200 family members, mir-203, miR-205, miR-206, miR-210, miR-212/132, miR-221 and miR-424/50, have been reported to play roles in mammary gland development and disease [15]. Some miRNAs with functionally validated targets are summarized in **Table 2**.

3.3. Nutritional modulation of microRNA expression and function

The miRNA expression profile in response to dietary treatments has been studied in adipose tissues of lambs and cattle and bovine mammary gland tissues [56, 100–102]. A change in diet that interferes with energy balance has been shown to change miRNA expression pattern in cow liver [103]. Wang et al. [104] fed cows with high- and low-quality forage diets (corn stover and rice straw) and showed that miR-125b, miR-141, miR-181a, miR-221 and miR-15b changed their expression patterns across different tissues including MG. We have examined the expression pattern of miRNAs following MG adaptation to dietary supplementation with 5% linseed oil or 5% safflower oil using miRNA sequencing and identified seven differentially regulated miRNAs, including six upregulated (miR-199c, miR-199a-3p, miR-98, miR-378, miR-148b and miR-21-5p) and one downregulated (miR-200a) by both linseed and safflower oil. The target genes of these seven miRNAs have functions related to gene expression and general cellular metabolism and are enriched in four pathways of lipid metabolism (3-phosphoinositide biosynthesis, 3-phosphoinositide degradation, D-myo-inositol-5-phosphate metabolism and the superpathway of inositol phosphate compounds) [51]. The largest number of target genes

(39) were associated with two functions (synthesis of lipid and concentration of lipid) related with lipogenesis. In goat, Mobuchon et al. [105] detected 30 miRNAs with expression patterns potentially modulated by food deprivation (14 and 16 were upregulated and downregulated, respectively). Among them, miR-204-5p and miR-223-3p were most remarkably affected by food deprivation and potentially played roles in the nutritional regulation of gene expression in the MG.

MiRNAs	Target genes	Main consequence	Cell	References
miR-181	<i>ACSL1</i>	Decrease lipid synthesis	BMEC	[89]
miR-29 family	<i>DNMT3A DNMT3B</i>	Decrease global DNA methylation	BMEC	[88]
miR-152	<i>DNMT1</i>	Decrease global DNA methylation and increase expression of Akt and PPAR γ	BMEC	[97]
miR-486	<i>PTEN</i>	Alter expression of downstream genes of PTEN (AKT, mTOR pathways)	BMEC	[87]
miR-181b	<i>IRS2</i>	Wnt signalling pathway in GMEC	GMEC	[98]
miR-27a	<i>PPARγ</i>	Decrease triglyceride accumulation	GMEC	[91]
miR-26a and b	<i>INSIG1</i>	Decrease triacylglycerol synthesis	GMEC	[99]
miR-24	<i>FASN, SREBF1, ACACA</i>	Decrease triacylglycerol synthesis	GMEC	[94]
miR-15a	<i>GHR</i>	Inhibit viability of mammary epithelial cells	BMEC	[86]
miR-130b	<i>PPARGC1A</i>	Repress PPARGC1A expression	GMEC	[96]
miR-143	<i>BAX and BCL-2</i>	Inhibit proliferation and induce apoptosis	GMEC	[95]
miR145	<i>INSIG1</i>	Increase fat droplet formation, triacylglycerol accumulation and proportion of unsaturated fatty acids	GMEC	[93]

Table 2. MicroRNAs with functionally validated target genes using ruminant mammary gland cells.

3.4. MicroRNA functions in mammary gland health

MiRNAs have been shown to play roles in bovine infection and immunity in a wide range of tissues [54, 106–113]. For mammary gland, Naeem et al. [114] studied the expression of 14 miRNAs (miR-10a, miR-15b, miR-16a, miR-17, miR-21, miR-31, miR-145, miR-146a, miR-146b, miR-155, miR-181a, miR-205, miR-221 and miR-223) in MG tissue challenged with *Streptococcus uberis* and identified three downregulated miRNAs (miR-181a, miR-16 and miR-31) and one upregulated miRNA (miR-223) in infected versus healthy tissue. Lawless et al. [107] showed that 21 miRNAs were differentially expressed upon *Streptococcus uberis* infection of bovine primary epithelial cells. Using BMEC, Jin et al. [108] reported a differential expression of nine miRNAs (miR-184, miR-24-3p, miR-148, miR-486, let-7a-5p, miR-2339, miR-499, miR-23a and miR-99b) upon challenge with heat inactivated *Escherichia coli* and *Staphylococcus aureus* bacteria. Hou et al. [115] identified three upregulated miRNAs (miR-296, miR-2430 and miR-671) and one downregulated miRNA (miR-2318) in mastitis affected compared with healthy mammary gland tissues. Li et al. [111] sequenced RNA isolated

from *S. aureus*-induced mastitis and control cows and identified 77 miRNAs with significant expression differences between the two groups. Li et al. [116] showed that miR-23 might be an important immune miRNA through its target mastitis candidate gene, high mobility group box 1 (*HMGB1*).

3.5. MicroRNA function in milk recipients

Recent evidence suggesting that milk-derived miRNAs may have potential regulatory roles in modulating the immune system or metabolic processes of milk recipients still remain controversial [117–124]. Currently, there are two hypotheses about miRNA function in infants/offspring: the first proposes that milk miRNAs exert physiological regulatory functions after transferring to offspring, and the second assumes that miRNAs do not have any function but merely provide nutrition. According to Zhang et al. [117], the rice-derived miRNA, miR-168a, can bind to the mRNA of human/mouse low-density lipoprotein receptor adapter protein 1 (*LDLRAP1*) and inhibit its expression in the liver, and consequently decrease LDL removal from mouse plasma. Baier et al. [118] reported that miR-29b-3p and miR-200c-3p could be absorbed by humans in biologically meaningful amounts, which could affect related gene expression in peripheral blood mononuclear cells while Izumi et al. [125] confirmed that whey exosomes containing miRNAs and mRNA could be absorbed by human macrophages. These results opened a new aspect of the nutritional control of metabolism [119]. However, other studies have not succeeded to validate the hypothesis that milk miRNAs exert physiological regulatory functions after transferring to offspring [126–129]. For instance, Auerbach et al. [129] observed that drinking bovine milk increased circulating levels of miRNAs (miR-29b-3p and miR-200c-3p) but found no evidence that they significantly altered miRNA signals after milk ingestion. These authors concluded that milk miRNAs likely serve as a source of nutrition but not as post-transcriptional regulators in recipients.

4. Long non-coding RNA in mammary gland development and lactation biology

4.1. Prolife and expression of long non-coding RNAs

A limited number of studies have examined the occurrence and potential functions of lncRNAs in ruminant livestock species [130–132]. A pioneer study screened reconstructed transcript assemblies of bovine-specific expressed sequence tags and identified 449 putative lncRNAs located in 405 intergenic regions [130]. Following this initial study, Weikard et al. [131] used RNA sequencing technique and identified 4848 potential lncRNAs, which were predominantly intergenic (4365) in bovine skin. In another study, Billerey et al. [132] characterized 584 lncRNAs in bovine muscle in addition to significant correlated expression between 2083 pairs of lncRNA/protein encoding genes. Koufariotis et al. [133] characterized the lncRNA repertoire across 18 bovine tissues including the mammary gland and reported 9778 transcripts. Ibeagha-Awemu et al. [134] studied the lncRNA profile of the

bovine mammary gland by RNA sequencing and identified 4227 lncRNAs (338 known and 3889 novel). In goats, Zhan et al. [135] sequenced libraries from developing longissimus dorsi fetal (45, 60 and 105 days of gestation) and postnatal (3 days after birth) muscles and identified 3981 lncRNA transcripts corresponding to 2739 lncRNA genes. Ren et al. [136] identified 1336 specific lncRNAs in fetal skin of Youzhou dark goat (dark skin) and Yudong white goat (white skin). Similarly, Chao et al. [137] in a study with aim to identify and classify new transcripts in Dorper and small-tail Han sheep muscle transcriptomes predicted with high confidence 1520 transcripts to be lncRNAs.

4.2. Function of long non-coding RNAs

While the regulatory roles of lncRNAs have been associated with several human disease conditions including tumourigenesis, cardiac development, aging and immune system development [138–143], little information exist on livestock species. Our previous study on bovine mammary gland identified 26 lncRNAs that were significantly differentially regulated in response to a diet rich in α -linolenic acid thus suggesting potential regulatory roles of lncRNAs in fatty acid synthesis and lipid metabolism [134]. In a study with goat fetal muscle tissues at different stages of development, Zhan et al. [135] identified 577 significantly differentially expressed lncRNA transcripts thus suggesting roles in muscle development.

5. Genome editing technology and non-coding RNA

Genome engineering has been considered as the next genomic revolution [144], and it is expected to significantly improve livestock production by precision genome editing [145–147] favouring markers associated with improved productivity, reproduction and health status. The history of genome editing in livestock has been extensively reviewed [145, 148–150]. The advent of engineered endonucleases (EENs), including zinc finger nucleases (ZFNs) [151], transcription activator-like effector nucleases (TALENs) [152] and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) [153]), allows to cut a specific position in DNA sequence and then use endogenous cellular pathways to direct DNA repair to introduce specified alterations to the DNA sequence. Genome-editing approaches have been successfully used in different livestock species, such as pig [154, 155], goat [156], cattle [157] and sheep [158]. In dairy cows, these technologies have been used to manipulate the genome so that they produce specific milk types, such as milk that causes less allergic problems (e.g. milk with less β -lactoglobulin protein) [159, 160]. These genome-editing tools also helped to improve mammary gland health by generating mastitis-resistant cattle [161, 162]. From an animal breeding perspective, a simulation study showed that genomic prediction combined with genome editing could be of benefit [163]. A total of 10,000 additive loci were simulated and shown to contribute to the variation in selected traits and benefits could be achieved with only 20 of those loci being edited in each selected sire [163]. Similar to other genome sequences, miRNA gene sequences within mammalian genomes can be easily edited with high efficacy and precision [144]. Targeted miRNA editing will enable revelation of the

complex regulatory circuits governed by miRNAs and realization, in the long term, of their full diagnostic and therapeutic potentials. For instance, Chen et al. [164] successfully used TALEN to disrupt the function of miR-21 in cancerous cells. A transgenic calf engineered to express miRNA-4 and miR-6 showed an absence of β -lactoglobulin and a concurrent increase in casein proteins in milk [165].

6. Conclusion and remarks

Up to now, it is well known that the mammalian genome encodes thousands of ncRNAs and these ncRNAs play important roles in many processes related to MG development, health and disease as well as roles in milk secretion and lactation processes. Regarding animal breeding, several ncRNAs target specific processes and their target genes could be important biomarkers for specific traits of interest. Therefore, the application of ncRNA to improve mammary gland health and milk production as well as enhance milk quality is very promising. However, the first step is a better understanding of ncRNA function in MG development and lactation. In fact, the MG is a complex tissue and lactation is a complicated process, but what we know about the regulatory networks underling MG function and the lactation process is very limited. For instance, through RNA sequencing, many novel ncRNAs have been detected in the MG but knowledge of their actual functions remains elusive. Therefore, integrated 'omics' approaches (genomics, transcriptomics, epigenomics and proteomics) should be used to identify and explore the potential roles of ncRNAs in mammary gland development and lactation biology. Moreover, a miRNA can target hundreds of genes thus making it difficult, costly and labour-intensive to functionally validate each miRNA gene target. Thus, integrative approaches such as combination of miRNA and mRNA expression in the same sample will refine computational predictions and increase our understanding of miRNA function and its application.

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