Profiling of the Exosomal Cargo of Bovine Milk Reveals the Presence of Immune- and Growth-modulatory Non-coding RNAs (ncRNA)

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Summary and Implications

The objective of the study was to characterize non-coding RNAs (ncRNA) present in raw milk collected from one commercial dairy processing facility. Silo milk was selected because it should be representative of raw milk of "typical" lactating dairy cows. Our hypothesis was that raw bovine milk would contain immune- and developmental-related ncRNA that may support the health of the mammary gland of the cow and could aid in the maturation of the neonatal gut.

Four samples of raw silo milk were collected on separate days, and total RNA was purified and profiled by using next-generation sequencing (RNA-seq). Our findings indicate an over-representation of ncRNA that target genes related to both immune modulation and growth and development, supporting our hypothesis that ncRNA in raw milk may help support the health of both the cow and calf.

Introduction

Bovine milk contains exosomes that carry a variety of non-coding RNA (ncRNA). Exosomes play a role in intracellular communication and act in a paracrine or exocrine manner. Their unique properties protect the cargo against degradation. *In vitro* studies have demonstrated that gut epithelial cells can absorb exosomes and their cargo. It follows that exosomal ncRNA may play a role in mammary gland immune response of the cow and in the gut development of the pre-weaned calf through regulation of gene expression. We hypothesized that raw bovine milk would contain immune- and development-related ncRNA. Our objective was to purify exosomes from raw milk and profile ncRNA by using next-generation sequencing.

Four replicates of raw milk were collected from a commercial dairy processing facility balance tank, after exiting the raw milk silo, immediately prior to processing.

Exosomes were isolated, and total RNA was profiled by Illumina RNA-seq. MicroRNA (miRNA) was mapped to miRbase, normalized, and filtered. Panther gene ontology (GO) analysis on short-listed target genes identified associated biological processes.

Results and Discussion

Of the 90 short-listed target genes in bovine animals (*Bos taurus)*, 36 genes were related to developmental process with a mean fold-enrichment (FE) value of 2.05, meaning that those gene targets were over-represented when compared with the reference dataset (i.e., upregulated). Of those genes, 14 associated with defense response, 18 with immune response, and 24 with immune system processes were over-represented in the query set (Table 1; FE of 4.17, 5.43, and 3.94, respectively). Interestingly, the most highly represented miRNA was mir-125a, which is involved in host bacterial and viral immune response (Figure 1). Many genes had hits with GO biological processes related to cytokine response (inflammation), MAPK pathway (involved in cell cycle regulation and regulation of cellular proliferation), leukocyte activation (immune system activation), and developmental processes, which support our hypothesis. An interaction network of 18 genes associated with immune response were derived via Genemania, which predicted 18 additional closely regulated genes generating a gene-interaction network with clustering coefficient of 0.473 (Figure 2; an indicator of how closely the gene functions are clustered).

Because ncRNA are highly conserved, both mouse and human databases were probed and 5,015 target genes specific to humans (*Homo sapiens*) were identified; 1,344 of those genes were related to development and were over-represented with an FE of 1.18, consistent with support of mammary regeneration as well as gut epithelial development (Figure 3). Genes related to cellular pathways, such as TGF-β receptor signaling pathway which is involved in regulation of inflammation in the gut as well as immune and stem cell differentiation and regulation (99 genes), and the Wnt signaling pathway which is involved in intestinal epithelial regeneration (131 genes), also were catalogued. For 146 out of 414 genes in the query set from mice (*Mus musculus*), specific target genes associated with anatomical structure development were over-represented with an FE of 1.65, indicating the potential for these ncRNA to support mammary health and growth as well as pre-weaned calf gut maturation (Figure 4).

Of the 88 piwi-interacting RNA (piRNA) found, 37 had annotations in pirBASE, although their function remains

unclear. Furthermore, 305 instances of other RNA were found. By using the Ensembl database for identification, these transcripts were composed of 64 antisense RNA, 105 long-intergenic ncRNA, 11 protein-coding, and 50 processed transcripts, indicating a need for further exploration of possible roles of "other" RNA contained in bovine exosomes. Together, our results support our hypothesis that exosomes of bovine milk contain ncRNA associated with immune and developmental functions.

Table 1. Gene ontology (GO) IDS related to immune response and development over-represented in target gene-list specific to *Bos taurus.*

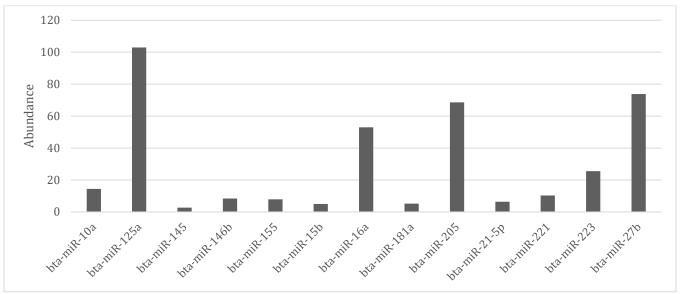| GO ID and associated biological process	Genes associated	Fold enrichment value	P-value
Regulation of cytokine production involved in immune response (GO:0002718)	6	26.51	0.0009
Regulation of production of molecular mediator of immune response (GO:0002700)	6	13.95	0.0372
Positive regulation of immune system process (GO:0002684)	19	7.53	<0.0001
Regulation of cytokine production (GO:0001817)	14	7.01	<0.0001
Cellular response to cytokine stimulus (GO:0071345)	13	6.6	0.0006
Regulation of defense response (GO:0031347)	10	6.1	0.0431
Leukocyte activation (GO:0045321)	10	6.05	0.0463
Regulation of immune system process (GO:0002682)	23	5.92	<0.0001
Immune response (GO:0006955)	18	5.43	<0.0001
Regulation of MAPK cascade (GO:0043408)	12	5.19	0.0251
Defense response (GO:0006952)	14	4.17	0.0450
Immune system process (GO:0002376)	24	3.94	<0.0001
Regulation of multicellular organismal development (GO:2000026)	23	3.89	<0.0002
Developmental process (GO:0032502)	36	2.05	0.0410

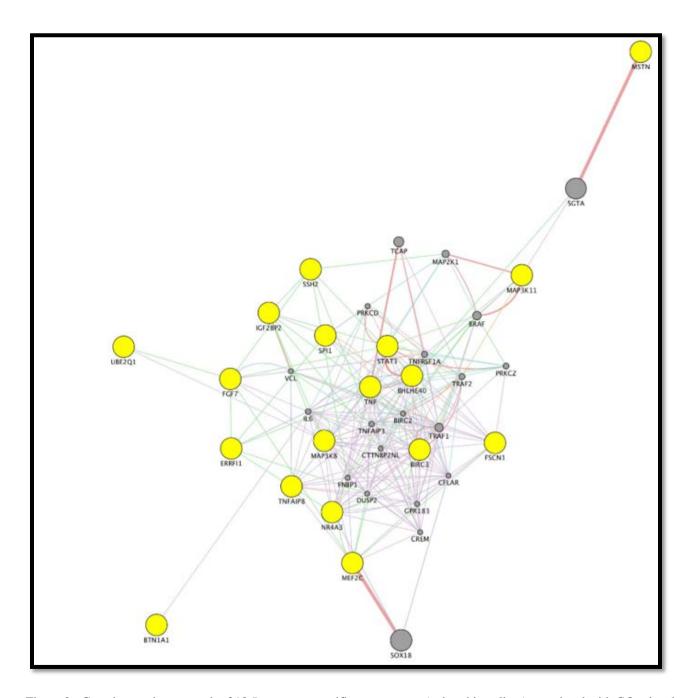Figure 1. Average abundance of shortlisted miRNAs found in raw milk (n = 4).

Figure 2: Gene interaction network of 18 *Bos taurus* specific target-genes (colored in yellow) associated with GO related to development; Genemania predicted 18 more closely related genes (colored in grey).
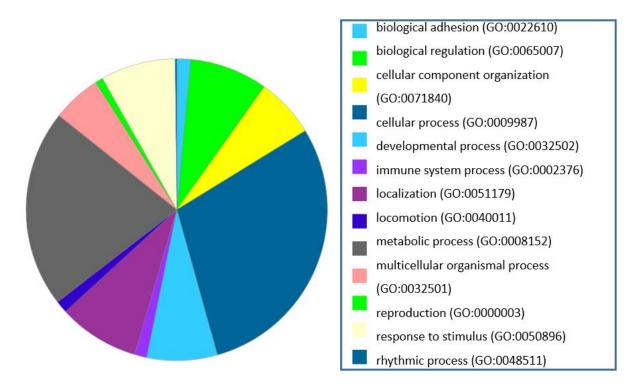
Figure 3: Pie chart depicting the percentage distribution of biological process-related GO IDs over-represented in *Homo sapiens*-specific target genes.
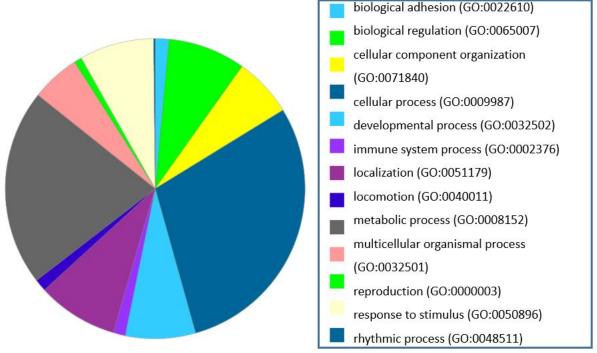

Figure 4: Pie chart depicting the percentage distribution of biological process-related GO IDs over-represented in *Mus musculus*-specific target genes.