bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

miR-21-5p and miR-30a-5p are identical in human and bovine, have similar isomiR distribution, and cannot be used to identify xenomiR uptake from cow milk

Bastian Fromm[1], Juan Pablo Tosar[2,3], Lin Yu[4], Marc K. Halushka[4], Kenneth W. Witwer[5,6,*]

[1]Department of Tumor Biology, Institute for Cancer Research, Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway

[2]Functional Genomics Unit, Institut Pasteur de Montevideo, Montevideo, Uruguay;

[3]Nuclear Research Center, Faculty of Science, Universidad de la República, Montevideo, Uruguay

[4]Department of Pathology, [5]Department of Molecular and Comparative Pathobiology, and [6]Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore , MD , USA

*Corresponding Author:

Kenneth W. Witwer

733 N. Broadway, MRB 829

Baltimore, MD 21205

USA

Phone: 1-410-955-9770

Fax: 1-410-955-9823

Email: kwitwer1@jhmi.edu

Authors' last names:

Fromm, Tosar, Yu, Halushka, Witwer

Word Count: 763

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Figures: 1

Running title:

No evidence of dietary xenomiR uptake from milk

Source of financial support:

No specific funding sources contributed to this study.

Conflict of interest statement:

The authors declare that they have no conflicts of interest.

Keywords:

microRNA; isomiR; xenomiR; milk; database

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Abstract

microRNAs (miRNAs) are often highly conserved across species, but species-specific sequences are known. In addition, miRNA "isomiRs" arise from the same precursor molecule but differ in post-processing length and modification, usually at the 3' end. A recently published feeding study reported the intriguing result that two bovine milk-specific miRNAs were taken up into human circulation after ingestion of bovine milk. Unfortunately, this interpretation is based on annotation errors in a public microRNA database. Reanalysis using databses including the MirGeneDB database reveals that the miRNAs in question, miR-21-5p and miR-30a-5p, arise from 100% identical 5' precursor sequences in human and bovine, and the putative bovine-specific isomiRs appear to be depleted, not enriched, in bovine milk. Thus, enrichment of these isomiRs in human blood is inconsistent with uptake of xenomiRs and likely betrays endogenous miRNA regulation in response to diet or technical artifact.

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

It was recently reported (1) that two bovine microRNAs (miRNAs) were detected in human blood plasma after ingestion of 1 liter of 1%-fat milk. Unfortunately, the premise of a bovine-human difference in these miRNAs is based on annotation errors in a public microRNA database (2).

Mature miRNAs are formed through consecutive cleavage events of a primary miRNA transcript by the ribonucleases Drosha and Dicer (3,4). Each mature miRNA has consensus 5' and 3' cleavage sites. However, instead of a single sequence defining a miRNA, a collection of "isomiRs" exist, with different 3' end lengths and modifications. Even the most abundant specific sequence of a given miRNA is, on average, only 45% of total reads (5). Additionally, for 204 miRNAs in the public miRBase repository used by Wang et al (2), the designated "canonical" sequence is different from the most abundant sequence.

Wang et al (1) make the unexpected claim that miR-21-5p and miR-30a-5p have unique sequences in bovine and human. Specifically, the bovine version of each is said to include two additional 3' nucleotides (nt) not found on the human miRNA, presumably based on miRBase (**Figure 1A**). However, at the DNA level, the human and bovine sequences are identical through the longer purported bovine miRNA. Thus, the 3' isomiR families of human and bovine cannot be distinguished by nt differences, as also supported by the actual read counts in miRBase (not shown).

Wang et al (1) would have been better served to view these miRNAs at MirGeneDB (6,7), an updated database. miR-21-5p and miR-30a-5p have the same mature miRNA sequence for both species (the longer, 24-nt sequence), which is shared across all vertebrates (not shown). MirGeneDB decay plots of miR-21-5p (Figure 1B) and miR-30a-5p (not shown) show the same 3' cut site in bovine and human and suggest a high proportion of the 24-nt miR-21-5p with terminal 'U/T' in human.

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Despite this clarification, it would still be possible that different isomiRs are favored in different species, akin to what we noted in cells (5). If there were extreme skewing towards a 22-nt miR-21-5p or miR-30a-5p in humans or a 24-nt version in cow milk, the data in this report would be intriguing. However, that is not the case. From small RNA-seq data processed in miRge (8), we obtained the miR-21-5p isomiR spectrum from 122 human samples (6 studies) and 114 bovine samples (4 studies), of which 48 were from milk. Reported data are limited to samples with >1,000 reads of the specific miRNA (data available upon request). As seen in Figure 1C, cow milk appears to be depleted, not enriched, in the 24-nt, pan-vertebrate consensus isomiR of miR-21. This apparent 3' decay may occur in milk-secreting cells, in the biofluid, during industrial processing of milk, or a combination of the above. Transfer of milk isomiRs into human blood, if detectable, would tend to dilute, not increase, levels of 24-nt isomiRs in human circulation after milk intake. These data are counter to the proposal of Wang et al (1) that milk ingestion specifically increased the 24-nt isomiRs (their proposed bovine-exclusive miRNAs). Data for miR-30a are similar, with the highest values for the full-length isomiR being in human, not cow milk (not shown).

It is not clear why 24-nt isomiRs would appear to increase slightly after a milk meal. We suspect either technical factors or perhaps real biology of human miRNAs changing their levels during the day (9) or in response to a food bolus (10). Concerning technical issues, rhPCR (11) was designed to distinguish single nucleotide polymorphisms (SNPs), but in the application by Wang et al, no true SNPs exist. Perhaps this method could be applied to molecules with internal nt differences, such as the passenger strand (miR-21-3p) that was not studied (Figure 1A), but necessary assay development and controls would need to be done (11). We also note that if the longer ("bovine") versions of these miRNAs were truly unique to bovine or cow milk, they should not have been present in humans at time 0; yet they were detected.

In conclusion, Wang et al (1) performed a study of dietary miRNA uptake based on annotation mistakes in one public database (2) and failed to take into account the distribution of reads of two isomiR families.

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Public data show 100% sequence identity for the two miRNAs, and cow milk is depleted, not enriched, in putative milk-specific isomiRs. This manuscript does not further inform us of whether or not xenomiRs enter mammalian circulation, but rather adds to the questionable science advancing that narrative (12).

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Acknowledgements

All authors analyzed data. MKH and KWW wrote the paper and had primary responsibility for final content. All authors read and approved the final manuscript.

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Figure Legend

Figure 1. miR-21 in cow and human. A) bta- and hsa-miR-21-5p are 100% identical in bovine and human, (miRBase misannotation highlighted purple). Note the nt difference in the -3p arm, not studied by Wang et al. B) MirGeneDB isomiR coverage shows that human isomiRs are at least at the same length and level as in bovine. C) miR-21-5p isomiR distribution data from 122 human samples, 66 cow samples and 48 cow milk samples based on the canonical isomiR ending in A, AC or ACU. Other isomiRs are not shown.

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Reference list

1. Wang L, Sadri M, Giraud D, Zempleni J. RNase H2-Dependent Polymerase Chain Reaction and Elimination of Confounders in Sample Collection, Storage, and Analysis Strengthen Evidence That microRNAs in Bovine Milk Are Bioavailable in Humans. J Nutr [Internet]. 2018 Jan 1 [cited 2018 Feb 1];148(1):153–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29378054
2. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res [Internet]. 2005/12/31. 2006;34(Database issue):D140-4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16381832
3. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature [Internet]. 2003 Sep 25 [cited 2018 Feb 4];425(6956):415–9. Available from: http://www.nature.com/articles/nature01957
4. Hutvágner G, McLachlan J, Pasquinelli AE, Bálint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science [Internet]. 2001 Aug 3 [cited 2018 Feb 4];293(5531):834–8. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1062961
5. McCall MN, Kim M-S, Adil M, Patil AH, Lu Y, Mitchell CJ, et al. Toward the human cellular microRNAome. Genome Res [Internet]. 2017 Oct [cited 2018 Feb 4];27(10):1769–81. Available from: http://genome.cshlp.org/lookup/doi/10.1101/gr.222067.117
6. Fromm B, Billipp T, Peck LE, Johansen M, Tarver JE, King BL, et al. A Uniform System for the Annotation of Vertebrate microRNA Genes and the Evolution of the Human microRNAome. Annu Rev Genet [Internet]. 2015 Nov 23 [cited 2018 Feb 4];49(1):213–42. Available from: http://www.annualreviews.org/doi/10.1146/annurev-genet-120213-092023
7. Fromm B, Domanska D, Hackenberg M, Mathelier A, Hoye E, Johansen M, et al. MirGeneDB2.0: the curated microRNA Gene Database. bioRxiv [Internet]. 2018 Feb 5 [cited 2018 Feb 5];258749.

bioRxiv preprint doi: https://doi.org/10.1101/275834; this version posted March 5, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Available from: https://www.biorxiv.org/content/early/2018/02/05/258749

8. Baras AS, Mitchell CJ, Myers JR, Gupta S, Weng L-C, Ashton JM, et al. miRge - A Multiplexed Method of Processing Small RNA-Seq Data to Determine MicroRNA Entropy. PLoS One [Internet]. 2015 Jan [cited 2016 Feb 16];10(11):e0143066. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4646525&tool=pmcentrez&rendertype=abstract

9. Bartman CM, Oyama Y, Brodsky K, Khailova L, Walker L, Koeppen M, et al. Intense light-elicited upregulation of miR-21 facilitates glycolysis and cardioprotection through Per2-dependent mechanisms. Wang M, editor. PLoS One [Internet]. 2017 Apr 27 [cited 2018 Feb 4];12(4):e0176243. Available from: http://dx.plos.org/10.1371/journal.pone.0176243

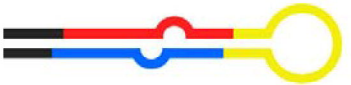
10. Witwer KW. Diet-responsive mammalian miRNAs are likely endogenous. Vol. 144, The Journal of nutrition. United States; 2014. p. 1880–1.

11. Dobosy JR, Rose SD, Beltz KR, Rupp SM, Powers KM, Behlke MA, et al. RNase H-dependent PCR (rhPCR): improved specificity and single nucleotide polymorphism detection using blocked cleavable primers. BMC Biotechnol [Internet]. 2011 Aug 10 [cited 2018 Feb 4];11(1):80. Available from: http://bmcbiotechnol.biomedcentral.com/articles/10.1186/1472-6750-11-80

12. Kang W, Bang-Bertelsen CH, Holm A, Houben A, Müller AH, Thymann T, et al. Survey of 800+ datasets from human tissue and body fluid reveals XenomiRs are likely artifacts. RNA [Internet]. 2017 Jan 6 [cited 2017 Jan 12];rna.059725.116. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28062594

A

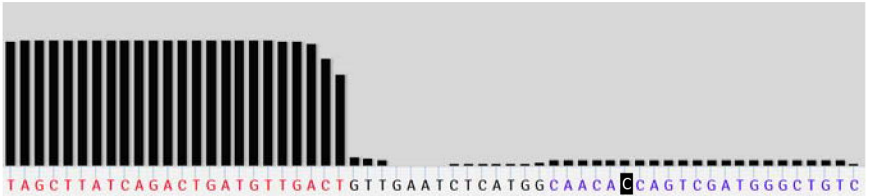
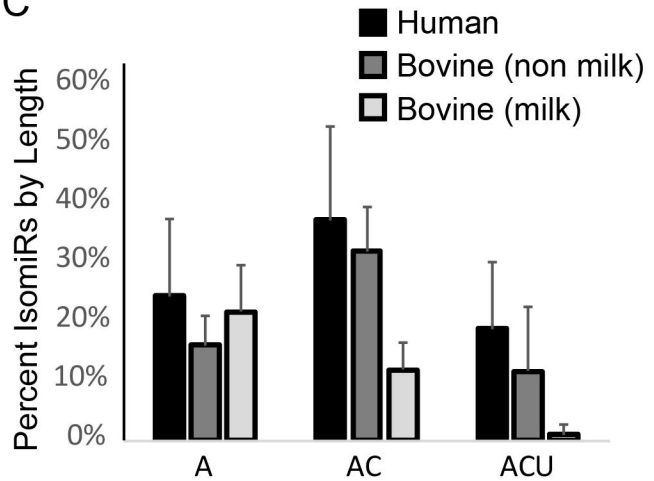
```
                 ************************ ************** ***** ****************
bta-Mir-21 UAGCUUAUCAGACUGAUGUUGACU-GUUGAAUCUCAUGG-CAACAGCAGUCGAUGGGCUGUC
hsa-Mir-21 UAGCUUAUCAGACUGAUGUUGACU-GUUGAAUCUCAUGG-CAACACCAGUCGAUGGGCUGUC
```



B

bta-miR-21

hsa-miR-21



C