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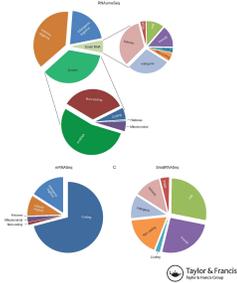
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# No effect of an elevated miR-30b level in mouse milk on its level in pup tissues

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**Keywords:** diet, horizontal genetic transfer, microRNA, milk, transgenic mouse

Recent reports have shown that ingested microRNAs may be transferred to blood, accumulate in tissues and exert canonical regulation on endogenous transcripts. In spite of several attempts to replicate these findings, they have not been confirmed and several questions remain. By using a transgenic mouse model presenting a high level of miR-30b in milk, the horizontal delivery of this microRNA *via* oral ingestion was studied in pups. Our findings demonstrated that, although very high levels of miR-30b were found in milk and in stomach contents of the pups, we did not detect an increase in miR-30b in tissues of pups fed by transgenic females compared to pups fed by wild-type females.

## Introduction

Interest in microRNAs has grown rapidly during the past decade, and some groups have recently reported investigations into the effects of diet-derived microRNAs. In 2012, Zhang et al.<sup>1</sup> described the uptake of plant microRNAs by the serum, liver and some other organs of mice following the consumption of rice, suggesting that exogenous plant microRNAs present in foods might regulate the expression of target genes in mammals. Plant microRNAs that cross the gut in sufficient quantities to regulate specific animal genes could be considered as a new type of nutrient. Baier et al.<sup>2</sup> showed recently that microRNAs contained in bovine milk could affect gene expression in adult humans. But other, contradictory, studies have also been published (for a review see refs.<sup>3,4</sup>). Dickinson et al.<sup>5</sup> replicated the original experiments performed by Zhang et al.<sup>1</sup> but were not able to reproduce their findings. An examination of publicly available small RNA sequencing datasets on various tissues from mammals, chickens and insects could not confirm the presence of high levels of plant-derived microRNAs in these species.<sup>6</sup> Several specific feeding studies found only small quantities of plant microRNAs in human, bee, mouse and non-human primate tissues.<sup>7,8</sup> However, Snow et al.<sup>7</sup> were able to show that when MIR156a was expressed at higher levels in a food when compared with MIR159a and MIR169a, it was more easily detected in recipient organisms, albeit at extremely low levels. They therefore considered that an improvement in delivery might theoretically be possible through increased ingestion, but making such dietary alterations would be challenging under normal circumstances.<sup>7</sup> Indeed, it is considered that the diet contains insufficient quantities of microRNAs for biological activity to occur in numerous tissue compartments.

Some hypotheses to explain the differences between findings of Zhang et al.<sup>1</sup> and those obtained more recently<sup>5,7,8</sup> have been put forward. One is that the plant microRNA-mappable reads observed in some studies could be explained by sequence errors or cross-contamination.<sup>5</sup> Moreover, Chen et al.<sup>9</sup> proposed that the sequencing procedure might be biased against plant microRNAs rather than animal microRNAs because of the 2'-O-methyl modification at the 3' terminal nucleotide of plant microRNAs. To explain some of these discrepancies, Snow et al.<sup>7</sup> suggested that particular circumstances might facilitate transfer and perhaps even target microRNAs to specific tissue niches after oral ingestion, as is the case for microRNAs from breast milk.<sup>2,10,11</sup>

Obtaining a clearer understanding of the biological activity of diet-derived microRNAs in multiple tissue compartments remains an important issue in several respects. For example, human therapeutics based on nucleic acid targeting are based on sequence-specific interactions between effectors and target molecules to achieve beneficial effects through specific modification of the expression of targeted genes. Oral delivery might constitute an excellent treatment strategy that could offer convenient and patient-friendly features; however, progress in this approach has been hampered by substantial challenges associated with biological barriers that limit the oral activity of nucleic acid therapeutics.<sup>12</sup>

In this context, we used a pertinent transgenic mouse model over-expressing the microRNA miR-30b in the mammary gland<sup>13</sup> in order to study the horizontal delivery of microRNAs *via* the oral ingestion of miR-30b-enriched milk by the pups. Sensitive, mature microRNA-specific RT-qPCR assays were used to quantify miR-30b variations in the blood, small intestine, liver, lung and kidney of pups fed by transgenic or non-transgenic females. Our findings were able to demonstrate that,

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although very high levels of miR-30b were found in milk and in stomach contents of the pups, we did not detect an increase in miR-30b in tissues of pups fed by transgenic females compared to pups fed by wild-type females.

## Results

The miR-30b transgenic mice used during this study specifically over-expressed the mouse precursor of miR-30b in mammary epithelial cells which produce and secrete milk components during lactation.<sup>13</sup> miR-30b over-expression is significantly increased in the mammary gland during lactation and is estimated at 15–20-fold the mean level.<sup>13</sup>

miR-30b levels in milk were analyzed using RT-qPCR (Fig. 1A and S1). Endogenous miR-30b was present at a very low level, but detectable using RT-qPCR in milk samples obtained from wild-type mice. In milk samples from transgenic females, the miR-30b level was higher than in those from wild-type mice, the difference being estimated at 134-fold of the mean. Although marked variations were observed between animals, all transgenic mice presented higher miR-30b levels than all wild-type mice.

Because miR-30b is expressed ubiquitously and strongly in all mouse tissues, it is easy to detect using the RT-qPCR technique.<sup>13</sup> Thus tissues (liver, small intestine (jejunum), lung and kidney) and blood from 10-day old pups fed by wild-type or transgenic mice were harvested for miR-30b quantification. No significant differences were observed between the tissues and blood from pups fed by the 2 different mother models (Figs. 2 and S1). Individual variations were observed with respect to other microRNA such as miR-145 (Fig. S2), so they were not correlated to the quality of the sample treatments (RNA extraction). It is possible that during ingestion, the microRNAs present in milk might be degraded. In order to evaluate the impact of digestion

on miR-30b, its level was measured in the stomach contents. miR-30b was still detectable, and the levels were higher in samples from pups fed by transgenic mice than those from pups fed by wild-type mice (Fig. 1B and S1), the difference being estimated at 31-fold of the mean. Although this ratio was lower than in milk, a marked difference still persisted in the stomach contents. In the same way as for milk samples, and although important variations were observed, all the stomach contents collected from pups fed by transgenic mice contained more miR-30b than those fed by wild-type mice.

## Discussion

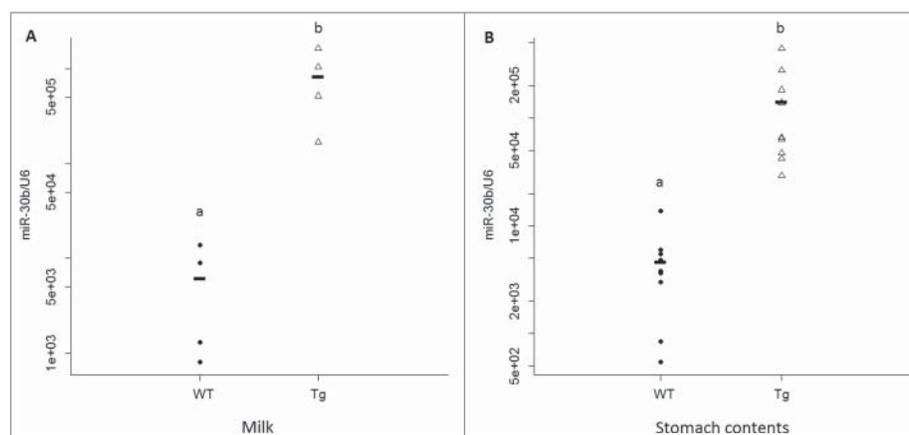
Through the use of an original transgenic mouse model which generates an artificially high level of miR-30b in milk, we have been able to show that miR-30b is not increased in tissues of pups fed by transgenic females compared to pups fed by wild-type females.

Our data confirm recent results<sup>5,7,8</sup> obtained regarding the transfer of diet-derived microRNAs into the circulation or tissues which disagreed with the first study on this subject published in 2012 by Zhang et al.<sup>1</sup> and the recent data published by Baier et al.<sup>2</sup>

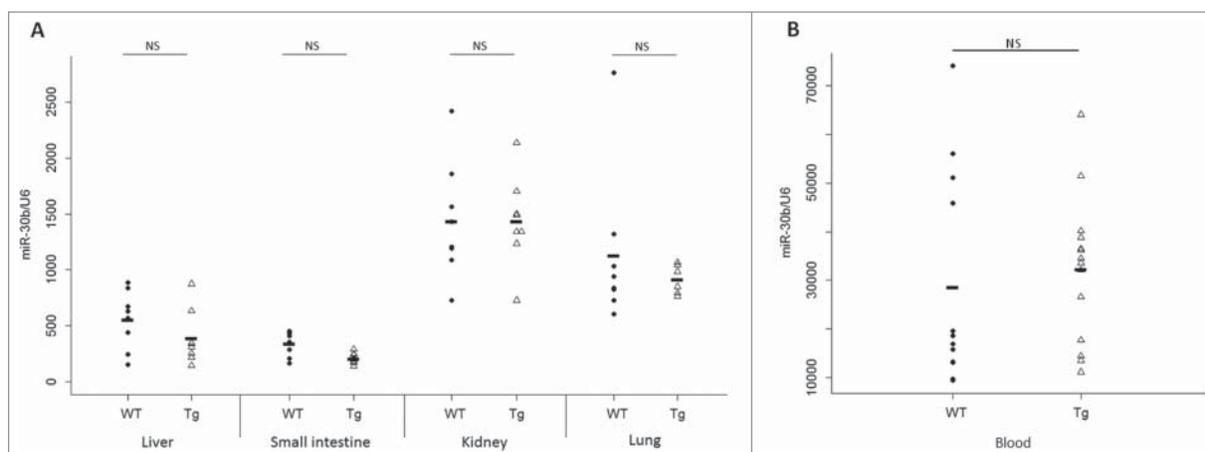
Moreover, our model has helped to answer some of the questions raised by previous studies.<sup>5,7,8</sup> Recent findings<sup>7,14</sup> included several calculations which suggested that humans could not ingest sufficient plant material to reach regulatory levels of plant microRNAs. Here, the miR-30b level present in the diet received by the pups from birth and for the subsequent 10 d was higher than that seen in previous studies.<sup>1,5,7,8</sup> In fact, the body of day-10 old pups (4g) contains  $4 \times 10^8$  cells<sup>15</sup> and each miRNA on average must reach at least 100 copies per cell to induce canonical target gene repression.<sup>16</sup> At least  $4 \times 10^{10}$  copies of miR-30b must be

ingested for widespread biological activity. Based on our findings, milk from transgenic mice contains  $2.3 \times 10^8$  copies of miR-30b per  $\mu\text{L}$ . A pup suckles 800  $\mu\text{L}$  per day (20% of its weight) which corresponds here to the ingestion of  $1.8 \times 10^{11}$  copies of miR-30b. Our mouse model would permit a sufficient absorption of exogenous miR-30b to affect gene expression. Our study confirmed the finding that diet-derived microRNAs, even in large quantities, are not systematically detected in blood or tissues.

Previous studies<sup>1,2,5,7,8</sup> had focused on the detection of plant microRNA. To avoid problems due to the difference between animal and plant microRNAs, we chose to study animal microRNAs only. For example, until now, it was not known whether native animal protein packaging such as Argonaute proteins,



**Figure 1.** miR-30b levels in milk and stomach contents. Relative levels of miR-30b, as assessed using RT-qPCR on RNA purified (A) from milk from wild-type (WT, N=4) and transgenic (Tg, N=4) mice collected at mid-lactation (day-12) and (B) from stomach contents harvested from 10-day old pups fed by wild-type animals (WT, N=9 pups from 3 different females) and transgenic animals (Tg, N=9 pups from 3 different females). miR-30b levels were quantified relative to U6. a, b: significant differences (ANOVA test with one factor and a threshold of 0.05). ●: wild-type, △: transgenic, —: mean.



**Figure 2.** miR-30b levels in pup tissues. Relative levels of microRNA, assessed using RT-qPCR on RNA purified from tissues from pups fed by wild-type (WT) and transgenic (Tg) mice harvested at mid-lactation (day-10): in the liver, small intestine, kidney (WT: N=8 pups from 3 females and Tg: N=8 pups from 3 females), lung (WT: N=8 pups from 3 females and Tg: N=6 pups from 2 females) and blood (WT: N=12 pups from 4 females and Tg: N=14 pups from 4 females). microRNA levels were quantified relative to U6. NS: no significant difference (ANOVA test with one factor and a threshold of 0.05). ●: wild-type, △: transgenic, —: mean.

were able to retain plant microRNAs. In addition, although previous studies considered that diet-derived microRNAs from plants are essential, the contribution of animal microRNAs could be equally important. In fact, microRNAs and their targets are strongly conserved in mammals.

Our study of miR-30b transgenic mice avoided the problem of undetectable levels of microRNA in the samples harvested. Endogenous miR-30b, which is ubiquitous and highly expressed<sup>13</sup> allowed to work at a scale of detection that was highly compatible with the RT-qPCR technique. Moreover, change higher than 2-fold was searched, in fact, modest changes (2-fold) to cellular microRNA concentrations or to individual microRNAs will only have minor effects on change in target levels (only a few percent).<sup>16,17</sup>

In our study, the presence of elevated levels of miR-30b in milk does not appear to affect its levels in pup tissues after ingestion. It is nevertheless possible that such a diet-derived microRNA effect occurs under certain circumstances, as suggested by Baier et al.<sup>2</sup> The differences between their data and our findings might be explained by the biological model used, because they studied transfer i) in adults, ii) between microRNAs from species other than human, and iii) microRNAs other than miR-30b.

Because we observed that miR-30b levels were still high in the stomach contents, we could not exclude the possibility that miR-30b has a function in the gastrointestinal tract. Recent studies have shown that immune-related microRNAs are present and enriched in milk<sup>18-21</sup> suggesting that microRNAs in breast milk may affect development of the immune system, although at present there is no evidence to support these hypotheses.

To protect extracellular microRNAs from degradation, they are incorporated in extracellular vehicles.<sup>22,23</sup> Future studies will address the presence of miR-30b in these different milk extracellular vehicles which thought to serve for genetic exchange between cells.

The data from our study have improved our knowledge of the relationship between diet-derived microRNAs and their presence in tissues of recipient animals.

## Materials and Methods

### Animals and tissue collection

miR-30b transgenic mice (line Tg12) were as previously described.<sup>13</sup> The day of parturition was counted as day-0 of lactation. The number of pups per litter was reduced to 8 at birth and wild-type pups were used. All experiments involving animals were performed in strict accordance with the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 86/609EC) and the recommendations of the French Commission de Génie Génétique (Permit number 12931 (01.06.2003)) which approved this study. Every effort was made to minimize animal suffering. For the collection of milk samples, the pups were removed (4h beforehand) and oxytocin (150  $\mu$ l per female at a dose of 10 IU/ml) was administered via intraperitoneal injection. All samples were collected at mid-lactation (day-10 for tissues and blood, day-12 for milk), then frozen as small aliquots and stored at  $-80^{\circ}\text{C}$  until use.

### RNA isolation

Total RNA was isolated from mouse tissue biopsies using the RNA NOW kit (Ozyme, # BX101) with overnight precipitation, so as to guarantee a maximum yield of microRNAs. For blood samples (20–200  $\mu$ l), 200  $\mu$ l of PBS were added prior to RNA extraction. The quantity and quality of RNA were assessed using an Agilent BioAnalyzer and quantified by Nanodrop ND1000.

## RT-qPCR

The reverse transcription (RT) and quantitative PCR (qPCR) steps were performed according to the manufacturer's protocol. miR-30b were quantified using RT-qPCR with TaqMan MicroRNA Expression Assays (Applied Biosystem, #4427975 Assay ID 000602). Briefly, 10 ng of total RNA for tissue and milk samples, and 100 ng for blood and stomach content samples, were reverse transcribed under the following conditions: 16°C for 30 min., 42°C for 30 min., 85°C for 5 min. PCR reactions were as follows: 95°C for 10 min. followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. using the Mastercycler ep Realplex (Eppendorf). After normalization with the small RNA RNU6B (Applied Biosystems, #4427975 Assay ID 001973), used to calculate the relative abundance of microRNA, the data were analyzed using the Delta-Delta Ct method ( $2^{-\Delta\Delta C_t}$ ).<sup>24</sup>

## Statistical analysis

Differences between samples were compared using one-way analysis of variance (ANOVA). Mean and Standard Errors

(SEM) were calculated for each group. A p-value of 0.05 was considered to be statistically significant.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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