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LETTER TO THE EDITOR

Detection of dietary plant-based small RNAs in animals

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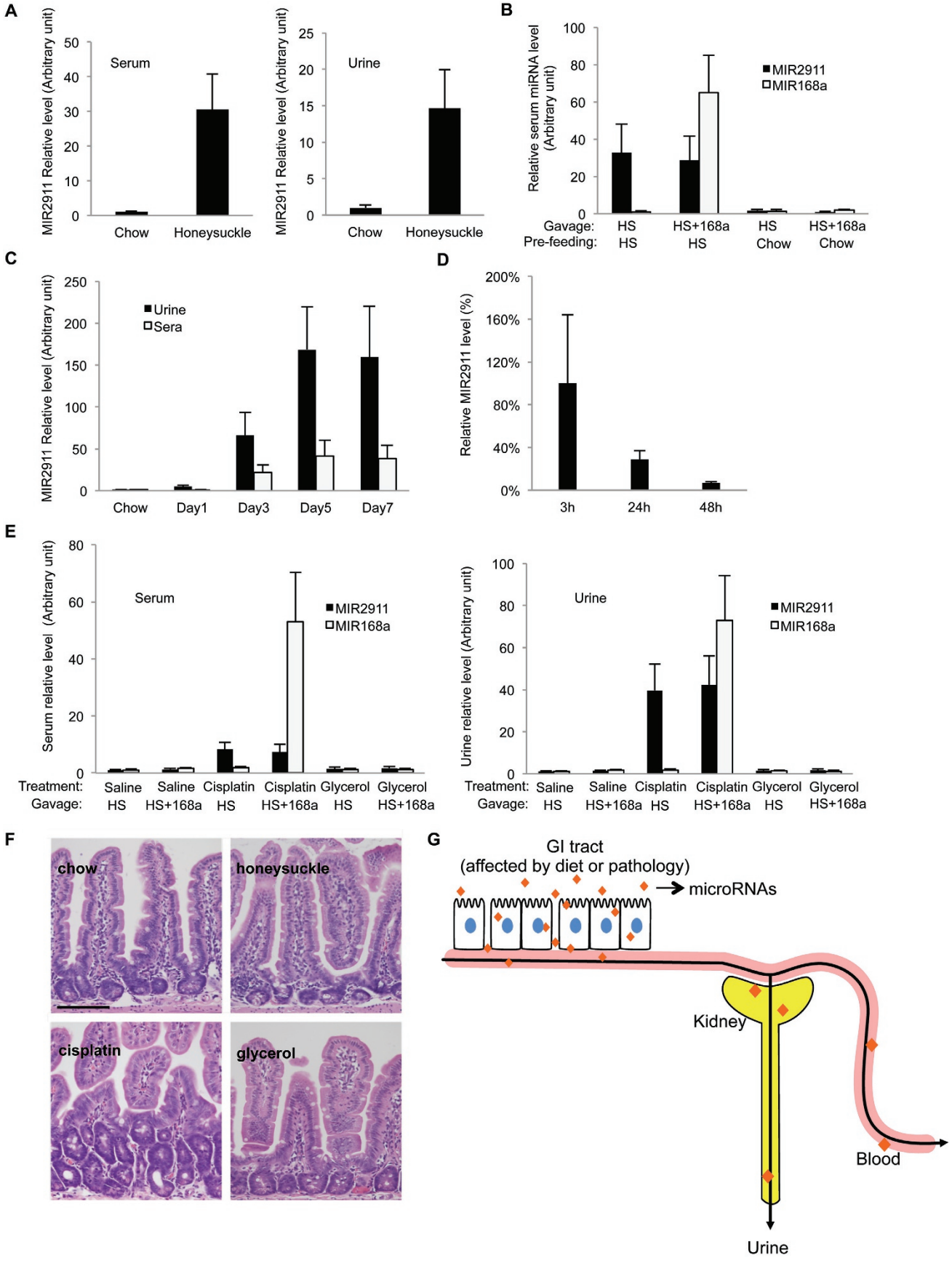
Dear Editor,

Disease and nutritional status are important factors controlling consumer nutrient requirements [1]. An estimated 4 billion people worldwide live primarily on plant-based diets. Approximately 10%-15% of these people suffer from chronic kidney disease or gastrointestinal (GI) health issues [2]. We hypothesize that diet preferences and health issues may converge to influence uptake of plant-based genetic material in a subpopulation of consumers. Here we have identified separate dietary and pharmacological regimes that facilitate the detection of diet-derived microRNAs (miRNAs) in consuming animals. Using a chow diet containing honeysuckle (*Lonicera japonica*), we could detect plant-based small RNAs in the sera and urine of mice (Figure 1A). Initially, we measured levels of a 26S ribosomal RNA-derived miRNA, previously referred to as MIR2911 [3]. This RNA was found in the sera and urine of all animals 3.5 days after starting this plant-based diet (Figure 1A). Using the same feeding regime, we gavage-fed these animals with a concentrated dose of honeysuckle (honeysuckle decoction) supplemented with a synthetic plant miRNA MIR168a, and we detected both MIR2911 and MIR168a in the sera of mice by quantitative reverse transcription-PCR (qRT-PCR) (Figure 1B). MIR168a is represented in some animal public high-throughput miRNA deep sequencing databases and has been investigated intensively [4-6]. We used digital droplet PCR to show that the amplified products are likely to be specific and not due, for example, to nonspecific amplification of endogenous RNAs (Supplementary information, Figure S1A). No dietary RNAs could be detected above background level in mice gavage-fed with the same honeysuckle but pre-fed with chow devoid of the honeysuckle (Figure 1B). A time course analysis of all animals pre-fed with the honeysuckle-containing chow revealed that the dietary RNAs were detectable in urine and sera after several days (Figure 1C). Furthermore, MIR2911 was no longer detectable 48 h after the honeysuckle was removed from the diet (Figure 1D), enforcing the idea that these small RNAs were of dietary origin. When we gavage-fed honeysuckle-pre-fed animals with MIR168a-supplemented honeysuckle decoction, we detected dose-dependent serum accumulation of MIR168a in mice with heightened response (showing elevated serum levels of MIR2911) to the honeysuckle diet (Supplementary information, Figure S1B and S1C). To assess physiological changes in the responding cohort, we performed clinical blood chemistry analysis. This subpopulation appeared to have some kidney failure and minor liver damage (Supplementary information, Figure S1D). However, no histopathological differences were detected in liver and kidney tissues between these responding mice and mice fed with the control diet (data not shown). Using synthetic miRNAs as standards, we estimated the concentrations of MIR2911 in blood and urine to be approximately 60 and 260 fM, respectively (Supplementary information, Figure S1E), similar to those of circulating endogenous mammalian miRNAs such as let-7d.

To further investigate the role of kidney damage in miRNA retention in the consuming animals, we treated mice with cisplatin, a platinum derivative and important chemotherapeutic agent [7]. A known effect of cisplatin administration is acute renal failure (ARF). Three days after cisplatin administration, kidney damage was confirmed by elevated blood urea nitrogen (BUN) levels resembling those of the most responsive honeysuckle-fed animals (data not shown). When we gavage-fed the cisplatin-treated mice with either honeysuckle decoction or honeysuckle decoction supplemented with MIR168a, these mice showed measurable levels of dietary miRNAs in their sera and urine, whereas untreated mice gavage-fed with the same cocktail did not display measurable levels of exogenous miRNAs (Figure 1E). Using a glycerol-induced model for ARF [7], we obtained similar BUN levels, but were unable to detect exogenous dietary miRNAs in the sera of gavage-fed mice (Figure 1E and Supplementary information, Figure S1F). This suggests that kidney damage alone did not lead to enhanced miRNA retention in the mouse circulation. Microscopic investigation of the intestinal villi stained with hematoxylin and eosin (H&E) showed that cisplatin treatment, but not glycerol treatment or honeysuckle feeding, disrupted the organization of small intestine epithelial cells (Figure 1F). This suggests that, first, cisplatin promotes the ele-


A
MIR2911 Relative level (Arbitrary unit)
Serum
Chow
Honeysuckle
Urine
B
Relative serum miRNA level (Arbitrary unit)
MIR2911
MIR168a
Gavage:
Pre-feeding:
HS
HS+168a
Chow
C
Urine
Sera
Day1
Day3
Day5
Day7
D
Relative MIR2911 level (%)
3h
24h
48h
E
Serum relative level (Arbitrary unit)
Urine relative level (Arbitrary unit)
Treatment:
Saline
Cisplatin
Glycerol
F
chow
honeysuckle
cisplatin
glycerol
G
GI tract
(affected by diet or pathology)
microRNAs
Kidney
Blood
Urine

vation of blood dietary miRNA levels by modulating gut function and architecture, and second, long-term honeysuckle feeding potentiates absorption of dietary miRNA through a mechanism that likely differs from cisplatin treatment.

In summary, our work suggests that consumers of particular diets and/or with alterations in intestinal permeability have an improved capacity to absorb dietary small RNAs (Figure 1G). We hypothesize that the cisplatin experiments exemplify phenotypes that may occur during various disease states that cause "leaky gut"-type symptoms. Cisplatin does not exclusively damage cancer cells and has diverse side effects, including changes in the organization of the GI tract [9]. Our results suggest that altered or damaged guts resulting from illness and/or therapeutic treatments could enhance dietary RNA uptake.

It is noteworthy that we never obtained an enhanced detection of rice-encoded miRNAs in sera from large numbers of mice or in our limited human feeding trials (Supplementary information, Figure S1G and S1H). This agrees with numerous reports from studies done in the United States [4, 10, 11]. It remains a formal possibility that the variance noted among research groups could be a product of dietary differences, environmental exposures and/or genetic or physiological dissimilarities among the consumers [3, 5].

The dietary regimes and dosages of exogenous miRNAs tested here were not designed to recapitulate standard dietary intake levels [1]. Previous studies estimate that a standard bowl of rice may contain 1.3 pmol of MIR168a per serving [4], and here we are using 300 times that amount. Rather, we have sought to establish initial delivery parameters. Plant miRNAs are known to have 2-O-methyl modifications on the 3′-terminal ribose, which are absent from animal miRNAs [12]. In our feeding experiments, we have used synthetic MIR168a with 2-O-methylation, and have determined that the plant-specific methylation of MIR168a does not appear to inhibit detection in sera (Figure 1B). However, future work will need to be done to discern which forms of dietary miRNAs are most efficiently absorbed.

As our work highlights, identical dietary intake does not necessarily result in the same concentrations of a specific nutrient in the blood or tissue because of the variability in the absorption, distribution, metabolism and elimination [1]. This variability further cautions that measuring serum miRNA levels in consumers should not be the sole means of evaluating absorption [4, 10, 11]. To date, the field has generally failed to follow the basic tenets of the balance technique for studying dietary miRNAs. That is, many groups have only measured serum levels of dietary miRNAs, and few groups have examined miRNA turnover or the potential deposition of dietary miRNAs in other body tissues. Further work will need to be done to optimize detection of dietary enhancement of plant-based small RNAs in organs such as liver, lung and heart from the honeysuckle-fed mice. However, we demonstrated that dietary small RNAs can survive circulation and are excreted in urine. Future work is needed to understand the exact mechanisms by which kidneys clear dietary miRNAs from circulation [13]. Establishing conditions whereby diet-derived nucleic acids are absorbed can be useful in transforming our understanding of the relationships between disease and diet, while enhancing the therapeutic potential of plant-based foods.

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Figure 1 Honeysuckle diet or cisplatin treatment promotes detection of MIR2911 and MIR168a in body fluids of mice. **(A)** MIR2911 detection in the sera and urine of honeysuckle-fed vs chow-fed mice. Mice were pre-fed with honeysuckle diet (Honeysuckle) or chow alone (Chow) for 3.5 days before tissue collection. **(B)** MIR2911 and MIR168a detection in mice fed with honeysuckle diet. Mice were pre-fed with honeysuckle (HS) or chow (Chow) for 3.5 days prior to gavage feeding with either honeysuckle decoction alone (HS) or honeysuckle decoction supplemented with 400 pmol of synthetic MIR168a (HS+168a). Blood was collected 3 h after gavage feeding. **(C)** Time course analysis of MIR2911 levels in mice pre-fed with honeysuckle for 0, 1, 3, 5, or 7 days. **(D)** Clearance dynamics of MIR2911 from circulation after honeysuckle feeding. Mice were pre-fed with a honeysuckle diet for 3.5 days and were then gavage-fed with honeysuckle decoction. These mice were then switched to chow diets and serum MIR2911 levels were analyzed 3, 24, or 48 h after removal of honeysuckle from the diet. n = 5 for each time point. **(E)** Detection of dietary MIR2911 and MIR168a in sera and urine of cisplatin-treated mice. Mice were treated with saline, cisplatin (15 mg/kg, intraperitoneal, 3 days), or 50% glycerol (8 ml/kg, intramuscular, 1 day). All mice were fed with standard chow diets during the study and gavage-fed with either honeysuckle decoction (HS), or honeysuckle decoction supplemented with 400 pmol of synthetic MIR168a (HS+168a). Blood was collected 3 h after gavage feeding. **(F)** Histology of intestinal villi from mice fed with chow, honeysuckle, or treated with cisplatin or glycerol. Panels, clockwise from top left corner: H&E-stained epithelial layer of small intestines isolated from mice pre-fed with chow or 5 days post honeysuckle feeding, 3.5 days post injection (intraperitoneal) with cisplatin, and 1 day post injection (intramuscular) with glycerol. Scale bar, 100 μm. **(G)** A model of dietary-induced miRNA absorption and excretion in consumers. The model of the gut-kidney axis posits that specific diets or gut pathologies cause dietary RNA to pass into the blood, whereupon the kidney acts to filter the RNA. n = 5 **(A-E)**. Error bars in all bar graphs represent standard error.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)