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The flavonoid apigenin improves glucose tolerance through inhibition of microRNA maturation in miRNA103 transgenic mice

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Polyphenols are representative bioactive substances with diverse biological effects. Here, we show that apigenin, a flavonoid, has suppressive effects on microRNA (miRNA) function. The effects were mediated by impaired maturation of a subset of miRNAs, probably through inhibition of the phosphorylation of TRBP, a component of miRNA-generating complexes via impaired mitogen-activated protein kinase (MAPK) Erk activation. While glucose intolerance was observed in miRNA103 (miR103)-overexpressing transgenic mice, administration of apigenin improved this pathogenic status likely through suppression of matured miR103 expression levels. These results suggest that apigenin may have favorable effects on the pathogenic status induced by overexpression of miRNA103, whose maturation is mediated by phosphorylated TRBP.

Polyphenols, common components of many popular drinks and foods, and caffeine, an alkaloid in various seeds and leaves, are representative bioactive substances with diverse biological effects^{1,2}. However, while some effects have been examined in detail³, the molecular mechanisms underlining these biological effects are mostly undetermined.

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs expressed in most organisms ranging from plants to vertebrates⁴. Primary miRNAs, which possess stem-loop structures, are processed into mature miRNAs by Drosha, Dicer, RNA polymerase III, and other related molecules. These mature miRNAs then bind the RNA-induced silencing complex (RISC), and the resulting co-complex directly binds the 3'-untranslated regions (3'-UTRs) of target mRNAs to act as suppressors of translation and gene expression. Thus, dependent upon the identity of the target mRNAs, miRNAs are responsible for the control of various biological functions, including cell proliferation, apoptosis, differentiation, metabolism, oncogenesis, and oncogenic suppression⁵⁻⁹. For example, it was reported recently that expression of miRNA103 and 107 (miR103 and 107) was upregulated in obese mice, and that the gain of miR103 function in either liver or fat was sufficient to induce impaired glucose homeostasis¹⁰.

Because the effects of bioactive substances are diverse and the functions of miRNAs result in diverse biological consequences, we hypothesized that some effects of bioactive substances may depend on modulation of miRNA function. In this study, we examined whether polyphenols and caffeine affect miRNA function and determined the molecular mechanisms underlying these effects. In addition, we applied the results obtained here to clinically relevant models to facilitate their use in practical applications.

Results

Apigenin suppresses miRNA function. To determine the effects of polyphenols and caffeine on miRNA function, we determined the luciferase activities of several types of reporters constructed containing



miRNA-binding sites (the function of which is suppressed by corresponding miRNAs) upon treatment with caffeine or polyphenols. The polyphenols used here were apigenin, procyanidin A2 and procyanidin B2 from flavonoids, and chlorogenic acid from phenolic acid. A cell line derived from the liver, Huh7, was used because substances in food theoretically flow into the liver first through the portal vein immediately after intestinal absorption. Among the bioactive substances examined, only apigenin significantly inhibited the effects of miRNAs such as miR122, miR185

and miR103 (Figure 1a), which are highly expressed in the liver¹¹. The effects were similarly observed irrespective of endogenous miRNAs or exogenous overexpression of corresponding miRNAs (Figure 1a and b) in a dose-dependent manner (Figure 1c). Another liver cell line, Hep3B, showed similar results, suggesting that the effects were not cell line-specific (Supplementary Figure 1a, b and c). The effects were detected with 5 μ M apigenin; this concentration is physiologically attainable^{12–14}. These results suggest that apigenin has suppressive effects on miRNA function.

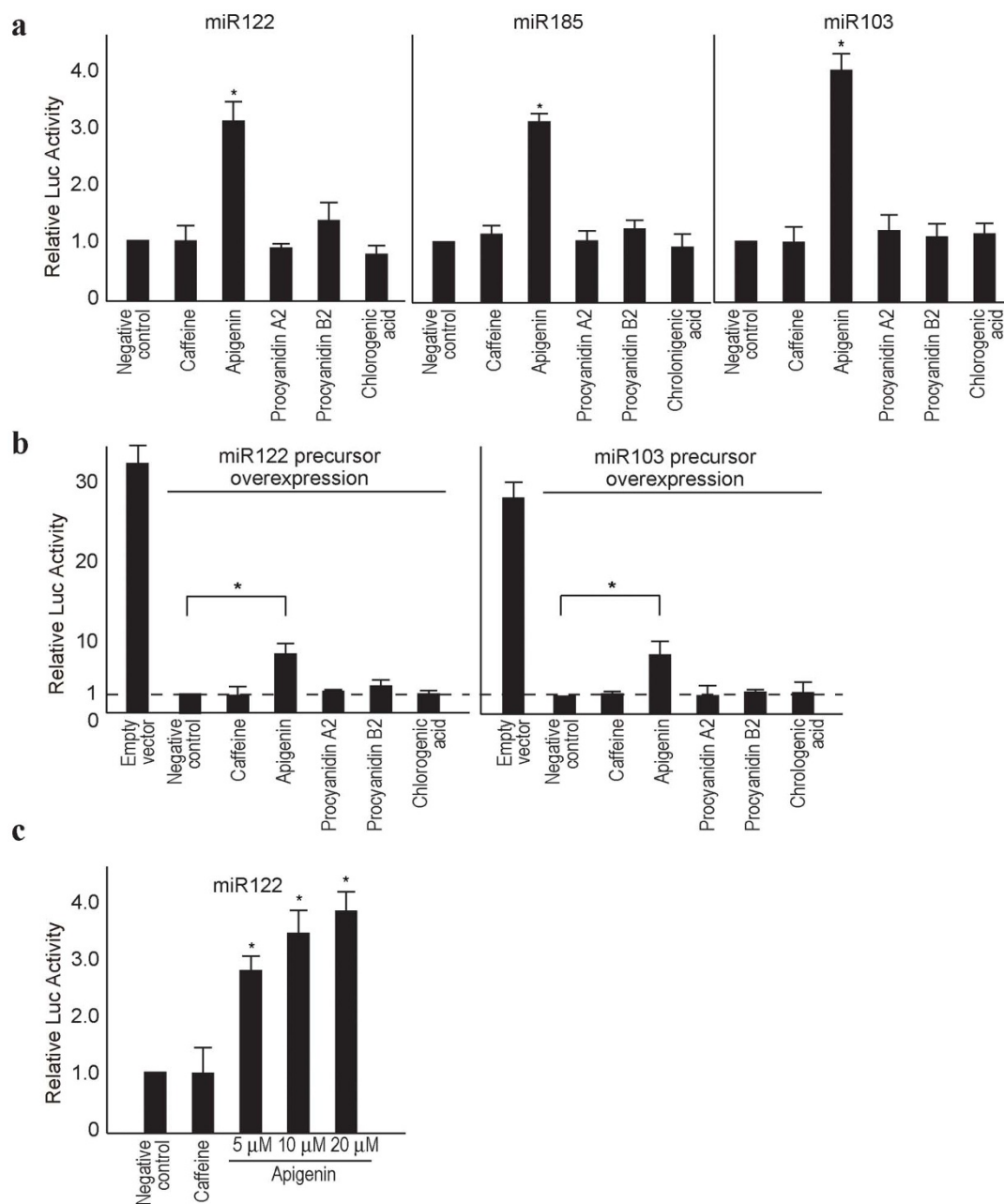


Figure 1 | Apigenin inhibits miRNA function. (a), Apigenin inhibits endogenous miRNA function. Huh7 cells were transfected with reporters to determine the functions of the indicated miRNAs. Twenty-four hours after treatment with the indicated substances, reporter assays were performed. Data represent the means \pm standard deviation (s.d.) from three independent experiments. *, $p < 0.05$ (t -test). (b), Apigenin inhibits the function of exogenously overexpressed miRNAs. Huh7 cells were transfected with reporters and corresponding miRNA precursor-expressing plasmids or an empty vector. Twenty-four hours after treatment with the indicated substances, reporter assays were performed. Data represent the means \pm s.d. from three independent experiments. *, $p < 0.05$ (t -test). (c), Dose-dependent effects of apigenin on miRNA function. Huh7 cells were transfected with reporter plasmids to determine miR122 function. Cells were treated with indicated doses of apigenin for 24 h and luciferase assays were performed. Caffeine was included as a negative control. Data represent the means \pm s.d. from three independent experiments. *, $p < 0.05$ (t -test) compared with the negative control.



Apigenin inhibits miRNA maturation from miRNA precursors.

To elucidate the molecular mechanisms underlying the inhibitory effects of apigenin on miRNA function, we first determined the expression levels of miRNA pathway-related molecules including Drosha, DGCR8, KSRP, Argonaute 2 (Ago2), and Dicer in the presence of apigenin. While the expression levels of Drosha, Ago2 and Dicer proteins appeared to decrease slightly after a high dose of apigenin, no significant changes were observed in the expression levels of these proteins (Figure 2a and Supplementary Figure 2a). Next, we examined the expression and maturation of miRNAs by quantitative real-time polymerase chain reaction (qRT-PCR) and Northern blotting (Figure 2b and Supplementary Figure 2b). Expression levels of mature endogenous miR122, miR103, and miR185 decreased and accumulation of precursor miRNAs was also observed after apigenin treatment (Figure 2b), suggesting that maturation from miRNA precursors was decreased. In addition, a comprehensive miRNA microarray analysis confirmed that apigenin altered the expression levels of a major subset of miRNAs (Supplementary Figure 2c; the raw data were deposited in the GEO database; GSE46526). However, some miRNAs, such as let-7, were not affected by apigenin treatment, which was confirmed by qRT-PCR (Figure 2b). These results suggest that apigenin has an inhibitory effect on the maturation of a subset of miRNAs.

Apigenin inhibits phosphorylation of TRBP. The microRNA-generating complex is composed of Dicer and phospho-TRBP isoforms¹⁵, and TRBP phosphorylation enhances the maturation of a subset of miRNAs through stabilization of the microRNA-generating complexes¹⁵. Phosphorylation of TRBP is mediated by mitogen-activated protein kinase (MAPK) Erk¹⁵. Because apigenin is known to inhibit Erk activity^{16–19}, we hypothesized that the inhibitory effects of apigenin on miRNA maturation may be mediated by decreased phosphorylation of TRBP through inhibition of Erk. Consistent with previous reports, although caffeine had no effect on the Erk phosphorylation status, apigenin clearly inhibited Erk phosphorylation 24 h post-treatment without changes in total Erk levels (Figure 3a). Concordantly, SRE-driven reporter activities were diminished by apigenin treatment (Figure 3b), suggesting that apigenin indeed inhibited an Erk-mediated intracellular signaling pathway, consistent with previous reports^{16–19}. While TRBP was

phosphorylated under normal serum culture conditions, and its phosphorylation status did not change with caffeine treatment, its phosphorylation was inhibited by apigenin (Figure 3c). This effect was confirmed by electrophoresis in a phos-tag gel, which showed a clear slow-migrating band, indicating that TRBP was phosphorylated in control and caffeine-treated conditions, but its phosphorylation was inhibited upon treatment with apigenin (Figure 3d). To confirm that Erk activity was inhibited by apigenin following TRBP phosphorylation, we examined the effects of apigenin using Huh7 cells stably expressing constitutively active Mek1 (CA-MEK) on TRBP phosphorylation. As shown in Figure 3e, the degree of TRBP phosphorylation was increased only by CA-MEK expression, and the augmented phosphorylation was not diminished by apigenin treatment (Figure 3e), suggesting that the effects of apigenin could not be observed under the induced Erk activation. That is, the effects of apigenin were most probably mediated by inhibition of Erk activation. In addition, we established Huh7 cells stably expressing dominant negative Erk (DN-Erk). As predicted, the levels of mature miRNA103, 122, and 185, were decreased in DN-Erk expressing cells, but were slightly increased in CA-MEK expressing cells, irrespective of apigenin treatment (Figure 3f). The expression levels of mature let-7, which were examined as a representative miRNA that was not affected by apigenin treatment in the miRNA microarray (Figure 3f), were not changed by enforced expression of DN-Erk or CA-MEK, suggesting that this miRNA maturation is not significantly regulated by MAPK activity or TRBP phosphorylation, consistent with a previous report¹⁵. These results suggest that apigenin inhibits Erk phosphorylation, and subsequent decreased MAPK activity leads to a decrease in TRBP phosphorylation, which may result in decreased maturation of a subset of miRNAs.

Apigenin improves glucose tolerance through inhibition of miRNA function.

To apply the above results in a clinical setting, we focused on recent findings demonstrating that a gain of miR103/107 expression induces impaired glucose homeostasis *in vivo*¹⁰. To utilize this, we generated transgenic mice expressing a miR103 precursor under control of the CMV promoter (Supplementary Figure 3a). Overexpression of miR103 in these mice was confirmed by Northern blotting against mature miR103 in liver tissues (Figure 4a and Supplementary Figure 3b). No significant over-saturation of RISC

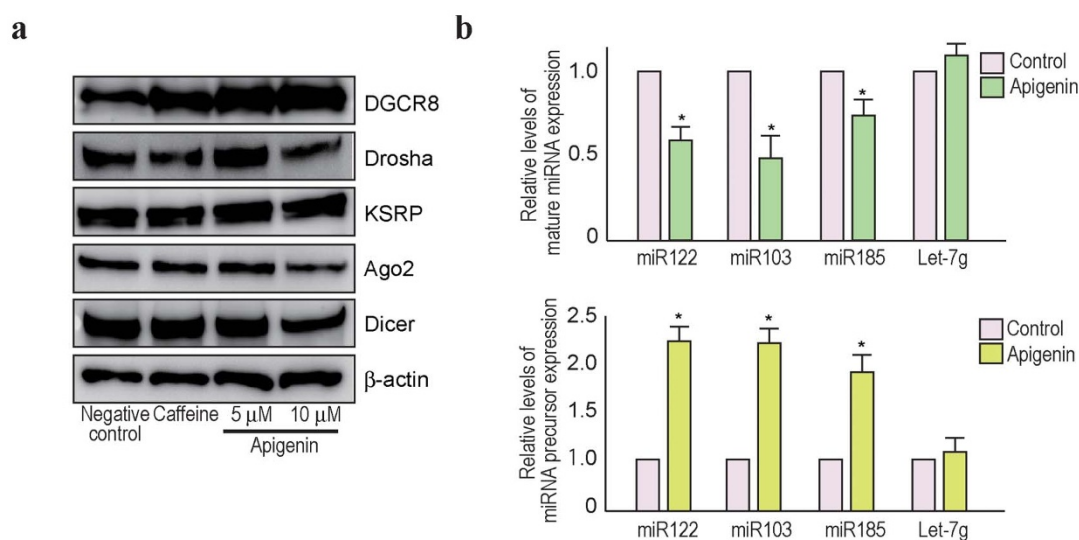


Figure 2 | Apigenin impairs miRNA maturation. (a), Cells were treated with the appropriate substances for 24 h and the indicated proteins were blotted. Representative results from three independent experiments using Huh7 cells are shown. Full-length blot images are available in Supplementary Figure 5a. (b), The expression levels of mature miRNAs and miRNA precursors were determined by qRT-PCR using Huh7 cells with or without apigenin treatment for 24 h. Data represent the means \pm s.d. from three independent experiments. *, $p < 0.05$ (t -test) compared with the control (DMSO only) treatment.

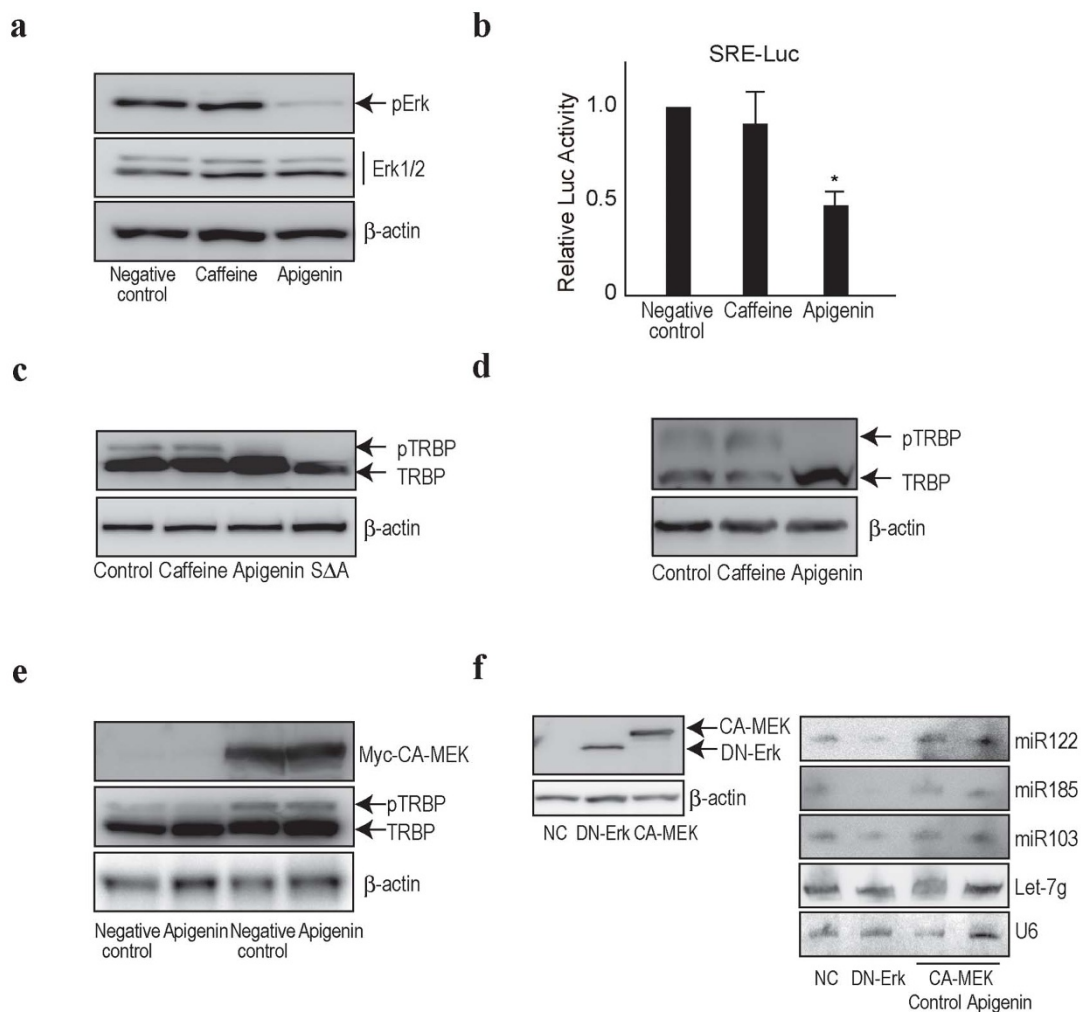


Figure 3 | Apigenin inhibits TRBP phosphorylation. (a), Cells were treated with caffeine or apigenin for 24 h. Cell lysates were blotted with anti-phosphorylated Erk and anti-total Erk1/2. Representative results from three independent experiments using Huh7 cells are shown. Similar results were obtained using Hep3B cells. (b), A luciferase assay was performed to determine SRE-driven transcription under apigenin treatment. Caffeine was included as a comparison. Data represent the means \pm s.d. from three independent experiments using Huh7 cells. *, $p < 0.05$ (t -test) compared to the negative control. (c), Huh7 cells were transfected with wild-type TRBP-expressing plasmids followed by treatment with the indicated substances for 24 h. Serine-to-alanine mutant TRBP (S Δ A) indicates non-phosphorylated TRBP. Representative results from three independent experiments using Huh7 cells are shown. (d), Substance-treated Huh7 cell lysates were separated using a Mn $^{2+}$ -Phos-tag gel to discriminate the phosphorylated form of TRBP. Representative results from three independent experiments using Huh7 cells are shown. (e), TRBP-expressing Huh7 cells were stably transfected with myc-tagged CA-MEK-expressing plasmids followed by apigenin treatment for 24 h. Phosphorylation status of TRBP was determined by Western blotting. Representative results from three independent experiments are shown. (f), Huh7 cells were stably transfected with myc-tagged CA-MEK-expressing plasmids or myc-tagged DN-Erk-expressing plasmids. The expression of the transfected constructs was confirmed by Western blotting using anti-myc antibodies (left panels). Expression levels of mature miRNAs in those cells with or without apigenin treatment were determined by Northern blotting (right panels). Representative results of at least three independent experiments are shown. Full-length blot images in a, b, c, d, e, and f are available in Supplementary Figure 5b, c, d, e, and f.

complexes due to overexpressing miR103 in these mice was confirmed by a lack of significant changes in the expression levels of other mature miRNAs, such as miR122 and miR185 (Figure 4a). As expected from a previous report¹⁰, these miR103 transgenic mice showed an increase in both random and fasting blood-glucose levels and insulin levels (Supplementary Figure 3c and d). The mean size of adipocytes in visceral fat was larger in normal chow fed miR103 transgenic mice than in control mice, and their size became larger nearly in parallel in both control and miR103 transgenic mice under a high-fat diet (Supplementary Figure 3e).

To determine the effect of apigenin in these models, 40 mg/kg apigenin was intraperitoneally injected daily for 14 days in miR103 transgenic mice. The level of mature miR103 was decreased, and precursors accumulated in apigenin-treated mice, as determined

by Northern blotting and qRT-PCR (Figure 4b and Supplementary Figure 4a and b). Similar to the *in vitro* results, levels of mature miR122 and miR185, but not let-7, in the liver tissues were also decreased by apigenin treatment (Supplementary Figure 4a and b). Phosphorylated TRBP in the liver tissues was decreased in apigenin-treated mice, as determined by a retarded band in the phos-tag gel (Figure 4c), consistent with the *in vitro* results (Figure 3d). Erk phosphorylation was consistently decreased following apigenin treatment (Supplementary Figure 4c). In addition, we confirmed the upregulated expression level of caveolin-1, a major regulator of the insulin receptor, which is a direct target gene of miR103¹⁰ in these tissues (Supplementary Figure 4c). As expected from these results, apigenin-treated miR103 transgenic mice showed decreased random and fasting blood glucose-levels (Figure 4d). While miR103 transgenic mice

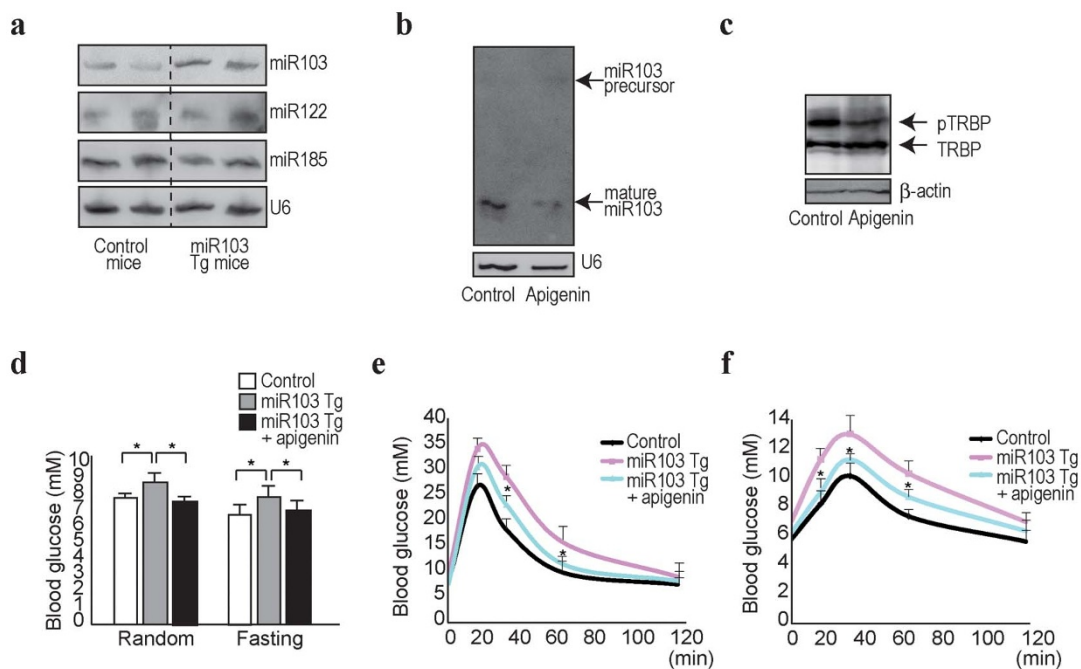


Figure 4 | Apigenin improves glucose tolerance in miR103 transgenic mice. (a), Expression levels of mature miR103, miR122, and miR185 in liver tissues of miR103 transgenic mice (miR103 Tg) were determined by Northern blotting. (b), Expression levels of mature miR103 and its precursor in liver tissues of miR10-transgenic mice treated with apigenin were determined by Northern blotting. Control (DMSO) or apigenin (40 mg/kg) was injected intraperitoneally daily for 14 days. Representative results from three independent mouse sets are shown. (c), Liver tissue homogenates from miR103 transgenic mice were separated using a phos-tag gel to determine the phosphorylation status of TRBP. Representative results from three independent mouse sets are shown. Full-length blot image is available in Supplementary Figure 5g. (d), Blood glucose levels were determined at random times or after 12 h fasting in control and miR103 transgenic (miR103 Tg) mice ($n = 8$ in each group). Data represent the means \pm s.d. *, $p < 0.05$ (t -test). (e), (f), Glucose and pyruvate tolerance tests in control, miR103 transgenic (miR103 Tg), and miR103 transgenic with apigenin treatment (miR103 Tg + apigenin) mice ($n = 6$ in each group). Data represent the means \pm s.d. *, $p < 0.05$ (t -test).

showed impaired glucose tolerance after an intraperitoneal glucose injection, apigenin treatment significantly suppressed these phenomena (Figure 4e). Similarly, while miR103 transgenic mice showed increased glucose production during an intraperitoneal pyruvate-tolerance test, apigenin treatment also suppressed these effects (Figure 4f). In addition, an increased number of small adipocytes and a decreased number of large adipocytes were observed in apigenin-treated miR103 transgenic mice (Supplementary Figure 3e and f). These results suggest that apigenin may have beneficial effects on pathological conditions in miR103 transgenic mice.

Discussion

In this study, we demonstrated that apigenin (4',5,7-trihydroxyflavone) has inhibitory effects on the maturation processes of a subset of miRNAs and subsequent miRNA function. These effects may be mediated through inhibition of TRBP phosphorylation, possibly through inhibition of Erk activation. These results suggest that apigenin may be utilized to improve miRNA-mediated pathogenic states, such as glucose tolerance, induced by the over-expression of miRNA103.

Bioactive substances, such as caffeine and polyphenols, have been reported to have pleiotropic physiological effects^{3,20}. However, those phenomena are descriptive in most cases and the underlying mechanisms are largely unclear. Apigenin, which is present in many fruits and vegetables, also has diverse biological effects, including improvement of the cancer cell response to chemotherapy²¹, tumorigenesis^{13,22}, modulating immune cell function²³, and anti-platelet activity²⁴. In this study, we showed that apigenin inhibits TRBP phosphorylation and its related miRNA maturation through inhibition of MAPK Erk activation. This modulation of miRNA function

by apigenin may account, at least in part, for its various reported biological effects.

Phosphorylation of TRBP is mediated by Erk¹⁵. We showed clear inhibition of Erk phosphorylation by apigenin. Although previous studies have reported the inhibition of Erk activation by apigenin^{16–19}, the underlying mechanisms were unknown. Because Erk has many biological functions in intracellular signaling, modulation of TRBP phosphorylation and miRNA expression induced by Erk inhibition through apigenin is likely a part of the phenotype. To clarify the biological function of apigenin, identification of molecules on which apigenin directly acts must be the next step.

Another important finding in this study was the impaired glucose tolerance observed in miR103 transgenic mice. Previous studies showed that recombinant adenoviruses expressing the miR103/107 family (only one nucleotide difference in miR103 and miR107 at position 21) and a gain of miR103/107 function by transient infection in mice was sufficient to induce impaired glucose homeostasis, and these miRNAs play a central role in insulin sensitivity¹⁰. In this study, we confirmed that constitutive expression of miR103 in mice resulted in impaired glucose tolerance and increased size of adipocytes. These mice may represent a new *in vivo* model of metabolic disorders and facilitate development of new drugs targeting impaired glucose tolerance. In fact, we found that apigenin reversed impaired glucose tolerance in miR103-transgenic mice. Because apigenin is one of the flavonoids and is present in high content in celery and parsley, intake of apigenin from foods or dietary supplements may have some favorable effects on glucose intolerance induced by overexpression of miRNA levels, even if it does not completely overcome impaired glucose tolerance.



Phosphorylated TRBP is not related to the maturation of all miRNAs, but rather a subset of miRNAs¹⁵. With this respect, apigenin may have favorable effects on the pathogenic status induced by overexpression or overfunction of the miRNAs to which phosphorylated TRBP is related. Maturation of miR122, a liver-specific miRNA, is at least partly regulated by apigenin, as shown in our study, its crucial function in cholesterol synthesis^{11,25–28}, and hepatitis c viral replication^{29,30}. Therefore, apigenin may also have beneficial effects on these conditions. Other effects of apigenin on the pathological state may be necessary to reconsider from the point of view of overexpression or overfunction of specific miRNAs.

Simultaneously, one should be cautious about the modulation of miRNA function by apigenin. Because some miRNAs may have favorable effects on human health, apigenin might be harmful if it inhibits the maturation and function of such miRNAs. For example, inhibitory effects on tumor-suppressive miRNAs should be avoided. Caution regarding these issues is necessary and, in parallel, the biological functions of miRNAs in general should be further examined.

In summary, we showed that apigenin displays inhibitory effects on the phosphorylation of TRBP and its subsequent miRNA maturation and function through regulation of Erk activity. Decreasing miRNA function may be used for treatment of conditions induced by over-functioning of miRNAs. Moreover, clarifying the as-yet-undiscovered functions of bioactive substances is important. Similar strategies to those used here may also be applied to other bioactive substances whose effects have been reported but the mechanisms are as yet undetermined.

Methods

Cell culture. The human hepatocellular carcinoma cell lines, Huh7 and Hep3B, were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Reagents. Caffeine, apigenin and chlorogenic acid were purchased from Wako Chemicals (Osaka, Japan). Procyanidin A2 and B2 were purchased from Indofine Chemical (Hillsborough, NJ) and ChromaDex (Irvine, CA). Caffeine, chlorogenic acid and procyanidin B2 were dissolved in water. Apigenin and procyanidin A2 were dissolved in dimethyl sulfoxide (DMSO). Caffeine, chlorogenic acid and procyanidin A2 and B2 were added at concentrations of 20 μ M, 10 μ M, 50 μ g/mL, and 50 μ g/mL, respectively, as reported previously^{31–34}. Apigenin was used at 10 μ M unless otherwise specified for *in vitro* studies, and 40 mg/kg was used for intraperitoneal injection daily for *in vivo* studies. An equal volume of DMSO only was used as a negative control.

Mouse experiments. Experimental protocols were approved by the Ethics Committee for Animal Experimentation at the Graduate School of Medicine, the University of Tokyo and the Institute for Adult Disease, Asahi Life Foundation, Japan and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Department of Medicine, the University of Tokyo, and the Institute for Adult Disease, Asahi Life Foundation.

Plasmids, transfection and dual luciferase assays. Plasmids expressing miR122 and miR185 precursors and the corresponding firefly luciferase-based reporters have been described previously^{8,35}. Plasmids expressing miRNA-103 precursors and the corresponding luciferase reporter were newly constructed according to protocols reported previously⁸. To determine MAPK pathway activity, SRE-driven luciferase was transfected, and dual luciferase assays were performed as described previously⁹, with the exception that pGL4.74, a control plasmid containing *Renilla reniformis* (sea pansy) luciferase under control of the herpes simplex virus thymidine kinase promoter (Promega), was used as an internal control. Chemicals were added at 24 h and the reporter assays were done 48 h post-transfection. Constitutively active MEK1(DD) and dominant negative Erk(K/N) constructs with zeocin resistance genes were kindly provided by Prof. Takekawa (The Institute of Medical Sciences, the University of Tokyo)³⁶. After transfection, the cells were selected with 6 μ g/mL zeocin to establish cells stably expressing those constructs.

Western blot analysis. Protein lysates were prepared from cells or mouse liver tissues for immunoblotting analyses. Western blotting was performed as described previously⁸. Primary antibodies were purchased from Sigma (DGCR8, #SAB4200088; Dicer, #SAB4200087; TRBP2, #SAB4200111; β -actin, #A5441), Bethyl (KSRP, A302-021), Wako (Ago2, 015-22031), and Cell Signaling (Drosha, #D28B1; Phospho-Erk, #9101; Total Erk, #4695; myc-tag, #2276; Caveolin, #3267).

Northern blotting of miRNAs. Northern blotting of miRNAs was performed as described previously⁹. Briefly, total RNA was extracted using TRIzol Reagent

(Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ten micrograms of RNA were resolved in denaturing 15% polyacrylamide gels containing 7 M urea in 1 \times TBE and then transferred to a Hybond N+ membrane (GE Healthcare) in 0.25 \times TBE. Membranes were UV-crosslinked and prehybridized in hybridization buffer. Hybridization was performed overnight at 42°C in ULTRAhyb-Oligo Buffer (Ambion) containing a biotinylated probe specific for miR122 (CAA ACA CCA TTG TCA CAC TCC A), miR103 (TCA TAG CCC TGT ACA ATG CTG CT), miR185 (TCA GGA ACT GCC TTT CTC TCC A), and let-7g (AAC TGT ACA AAC TAC TAC CTC A), which had been heated to 95°C for 2 min. Membranes were washed at 42°C in 2 \times SSC containing 0.1% SDS, and the bound probe was visualized using a BrightStar BioDetect Kit (Ambion). Blots were stripped by boiling in a solution containing 0.1% SDS and 5 mM EDTA for 10 min prior to rehybridization with a U6 probe (CAC GAA TTT GCG TGT CAT CCT T).

Quantitative RT-PCR analysis of miRNA expression. To determine miR122, miR103, miR185, and let-7g expression levels, cDNA was first synthesized from RNA, and quantitative PCR was then performed using Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR Kit (Clontech). The expression levels of miRNA precursor were determined according to the previous report³⁷ using the reported primers. Relative expression values were calculated by the CT-based calibrated standard curve method. These calculated values were then normalized to the expression of U6 snRNA. The reverse primer was provided in the kit.

Determining TRBP phosphorylation status. Plasmids expressing wild-type TRBP and kinase-dead TRBP (TRBP SAA) were kindly provided by Professor Liu¹⁵. Twenty-four hours after transfection into Huh7 cells with corresponding plasmids, substances were treated for 24 h, and cell lysates were collected for subsequent Western blotting. To better discriminate the phosphorylated form of TRBP from the unphosphorylated form, a Mn2+ -Phos-tag SDS-PAGE gel (Wako) was used according to the manufacturer's instructions.

Generation of miR103-expressing transgenic mice. To construct transgenic mice, plasmids expressing miRNA-103 precursors were modified as follows: to add the SV40 poly(A) tail signal downstream of the miR103 precursor sequences, the pCDH-miR103 precursor-expressing plasmid was digested at the *NotI* restriction site, and PCR-amplified poly(A) tail signal sequences were digested with *Clai* from the original plasmid as a template was inserted by the Infusion cloning system (Clontech, Mountain View, CA). A DNA fragment of 1,125 bp, containing the CMV promoter region, the 470-bp genomic region for the miR103 precursor, and a SV40 poly(A) tail signal, was resected from the constructed plasmid by digestion with *Clai*. Stable C57BL/6 embryonic stem (ES) cell lines were generated by electroporation of the linearized transgene, and the resulting cells were injected into blastocysts by the UNITECH Company (Chiba, Japan). Genotyping was performed by PCR using DNA isolated from tail snips. Four different mouse lines were maintained and the male littermates were used in the experiments.

Glucose test. Blood glucose was tested using a Glucose Pilot system (Iwai Chemical, Japan). Glucose tolerance and pyruvate tolerance tests were performed by intraperitoneal injection of glucose (2 g/kg) or pyruvate (2 g/kg) after fasting overnight. Blood glucose levels were measured before injection (0 min) and at 15, 30, 60, and 120 min after injection.

Adipocyte size. Visceral fat tissues stained with hematoxylin and eosin were analyzed using the Image-J software. One hundred adipocytes were measured per animal to determine adipocyte size. The high-fat diet was purchased from CLEA-Japan (Tokyo, Japan).

miRNA microarray analyses. miRNA microarray analysis was performed using miRNA oligo chips (Toray Industries, Tokyo, Japan). Normalization was performed using the intensities from U6, instead of the standard global normalization. The data and the protocols were deposited in a public database (Please refer the following link during the review process; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=frwpomkicoic&acc=GSE46526>).

Statistical analysis. Statistically significant differences between groups were determined using Student's *t*-test when variances were equal. When variances were unequal, Welch's *t*-test was used. *P*-values less than 0.05 were considered to indicate statistical significance.

1. Surh, Y. J. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* **3**, 768–780 (2003).
2. D'Incalci, M., Steward, W. P. & Gescher, A. J. Use of cancer chemopreventive phytochemicals as antineoplastic agents. *Lancet Oncol* **6**, 899–904 (2005).
3. Baur, J. A. *et al.* Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444**, 337–342 (2006).
4. Carrington, J. & Ambros, V. Role of microRNAs in plant and animal development. *Science* **301**, 336–338 (2003).
5. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
6. Ambros, V. The functions of animal microRNAs. *Nature* **431**, 350–355 (2004).



7. Lu, J. *et al.* MicroRNA expression profiles classify human cancers. *Nature* **435**, 834–838 (2005).
8. Kojima, K. *et al.* MicroRNA122 is a key regulator of α -fetoprotein expression and influences the aggressiveness of hepatocellular carcinoma. *Nat Commun* **2**, 338 (2011).
9. Takata, A. *et al.* MicroRNA-140 acts as a liver tumor suppressor by controlling NF- κ B activity by directly targeting DNA methyltransferase 1 (Dnmt1) expression. *Hepatology* **57**, 162–170 (2013).
10. Trajkovski, M. *et al.* MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* **474**, 649–653 (2011).
11. Krützfeldt, J. *et al.* Silencing of microRNAs in vivo with ‘antagomirs’. *Nature* **438**, 685–689 (2005).
12. Budhraj, A. *et al.* Apigenin induces apoptosis in human leukemia cells and exhibits anti-leukemic activity in vivo. *Mol Cancer Ther* **11**, 132–142 (2012).
13. Shukla, S. *et al.* Blockade of beta-catenin signaling by plant flavonoid apigenin suppresses prostate carcinogenesis in TRAMP mice. *Cancer Res* **67**, 6925–6935 (2007).
14. Wang, W. *et al.* Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Mol Carcinog* **28**, 102–110 (2000).
15. Paroo, Z., Ye, X., Chen, S. & Liu, Q. Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. *Cell* **139**, 112–122 (2009).
16. Yi Lau, G. T. & Leung, L. K. The dietary flavonoid apigenin blocks phorbol 12-myristate 13-acetate-induced COX-2 transcriptional activity in breast cell lines. *Food Chem Toxicol* **48**, 3022–3027 (2010).
17. Tatsuta, A. *et al.* Suppression by apigenin of peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane in Wistar rats. *Clin Exp Metastasis* **18**, 657–662 (2000).
18. Yin, F., Giuliano, A. E. & Van Herle, A. J. Signal pathways involved in apigenin inhibition of growth and induction of apoptosis of human anaplastic thyroid cancer cells (ARO). *Anticancer Res* **19**, 4297–4303 (1999).
19. Kuo, M. L. & Yang, N. C. Reversion of v-H-ras-transformed NIH 3T3 cells by apigenin through inhibiting mitogen activated protein kinase and its downstream oncogenes. *Biochem Biophys Res Commun* **212**, 767–775 (1995).
20. Yao, S. L. *et al.* Selective radiosensitization of p53-deficient cells by caffeine-mediated activation of p34cdc2 kinase. *Nat Med* **2**, 1140–1143 (1996).
21. Chan, L. P. *et al.* Apigenin induces apoptosis via tumor necrosis factor receptor- and Bcl-2-mediated pathway and enhances susceptibility of head and neck squamous cell carcinoma to 5-fluorouracil and cisplatin. *Biochim Biophys Acta* **1820**, 1081–1091 (2012).
22. Mafuvadze, B., Liang, Y., Besch-Williford, C., Zhang, X. & Hyder, S. M. Apigenin induces apoptosis and blocks growth of medroxyprogesterone acetate-dependent BT-474 xenograft tumors. *Horm Cancer* **3**, 160–171 (2012).
23. Nicholas, C. *et al.* Apigenin blocks lipopolysaccharide-induced lethality in vivo and proinflammatory cytokines expression by inactivating NF- κ B through the suppression of p65 phosphorylation. *J Immunol* **179**, 7121–7127 (2007).
24. Landolfi, R., Mower, R. L. & Steiner, M. Modification of platelet function and arachidonic acid metabolism by bioflavonoids. Structure-activity relations. *Biochem Pharmacol* **33**, 1525–1530 (1984).
25. Hsu, S. H. *et al.* Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J Clin Invest* **122**, 2871–2883 (2012).
26. Lanford, R. E. *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* **327**, 198–201 (2010).
27. Elmén, J. *et al.* LNA-mediated microRNA silencing in non-human primates. *Nature* **452**, 896–899 (2008).
28. Esau, C. *et al.* miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* **3**, 87–98 (2006).
29. Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. & Sarnow, P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* **309**, 1577–1581 (2005).
30. Lindow, M. & Kauppinen, S. Discovering the first microRNA-targeted drug. *J Cell Biol* **199**, 407–412 (2012).
31. Ku, B. M. *et al.* Caffeine inhibits cell proliferation and regulates PKA/GSK3 β pathways in U87MG human glioma cells. *Mol Cells* **31**, 275–279 (2011).
32. Nishizuka, T. *et al.* Procyonidins are potent inhibitors of LOX-1: a new player in the French Paradox. *Proc Jpn Acad Ser B Phys Biol Sci* **87**, 104–113 (2011).
33. Teraoka, M. *et al.* Cytoprotective effect of chlorogenic acid against α -synuclein-related toxicity in catecholaminergic PC12 cells. *J Clin Biochem Nutr* **51**, 122–127 (2012).
34. Shukla, S. & Gupta, S. Apigenin: a promising molecule for cancer prevention. *Pharm Res* **27**, 962–978 (2010).
35. Takata, A. *et al.* MicroRNA-22 and microRNA-140 suppress NF- κ B activity by regulating the expression of NF- κ B coactivators. *Biochem Biophys Res Commun* **411**, 826–831 (2011).
36. Kubota, Y., O’Grady, P., Saito, H. & Takekawa, M. Oncogenic Ras abrogates MEK SUMOylation that suppresses the ERK pathway and cell transformation. *Nat Cell Biol* **13**, 282–291 (2011).
37. Suzuki, H. I. *et al.* Modulation of microRNA processing by p53. *Nature* **460**, 529–533 (2009).

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Author contributions

M. Ohno, C.S. and M. Otsuka planned the research and wrote the paper. M. Ohno, C.S., T.K., T.Y., A.T., K. Kojima, and M. Otsuka performed the majority of the experiments. M.A. and H.Y. contributed materials. Y.K. supported several experiments. K. Koike supervised the entire project.

Additional information

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