Supplementary Information to

Stilbene derivatives promote Ago2-dependent tumour-suppressive microRNA activity

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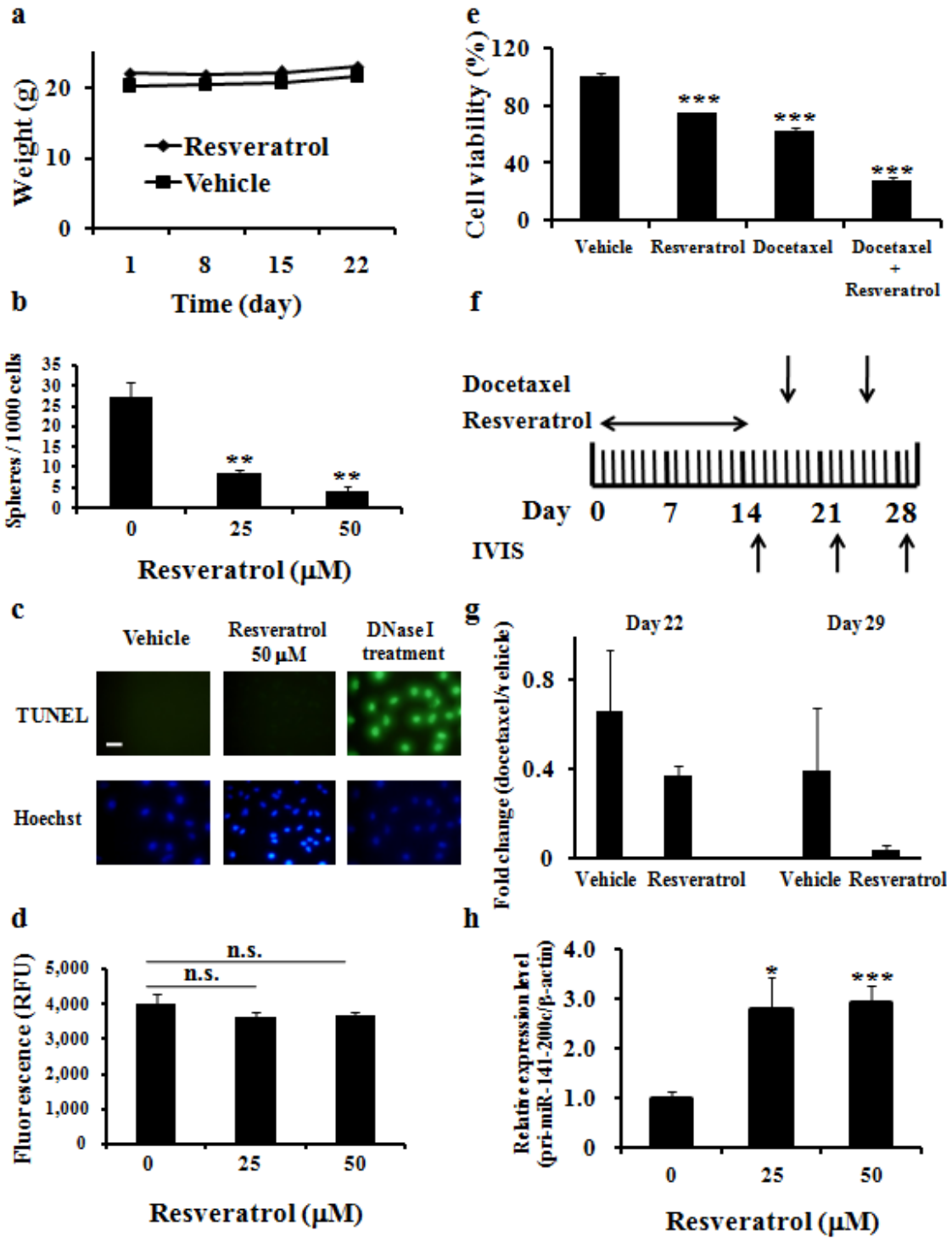
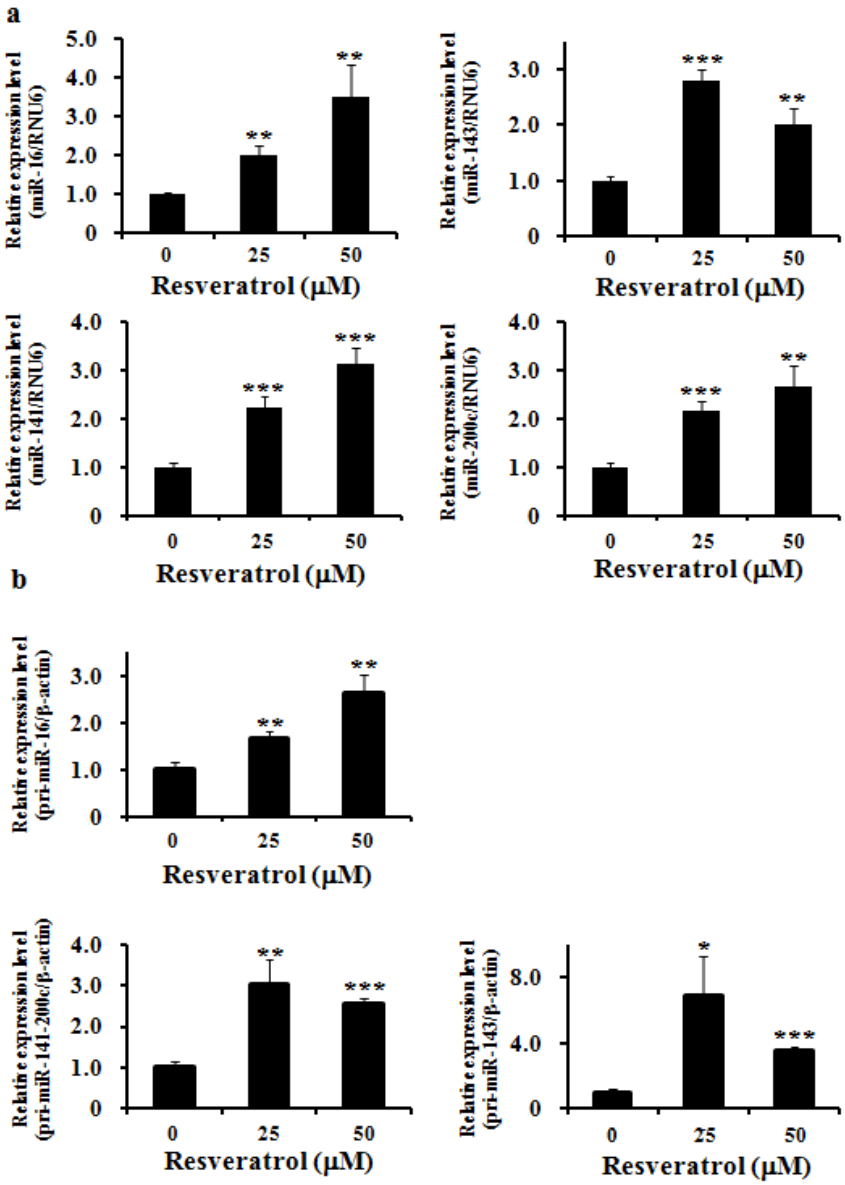
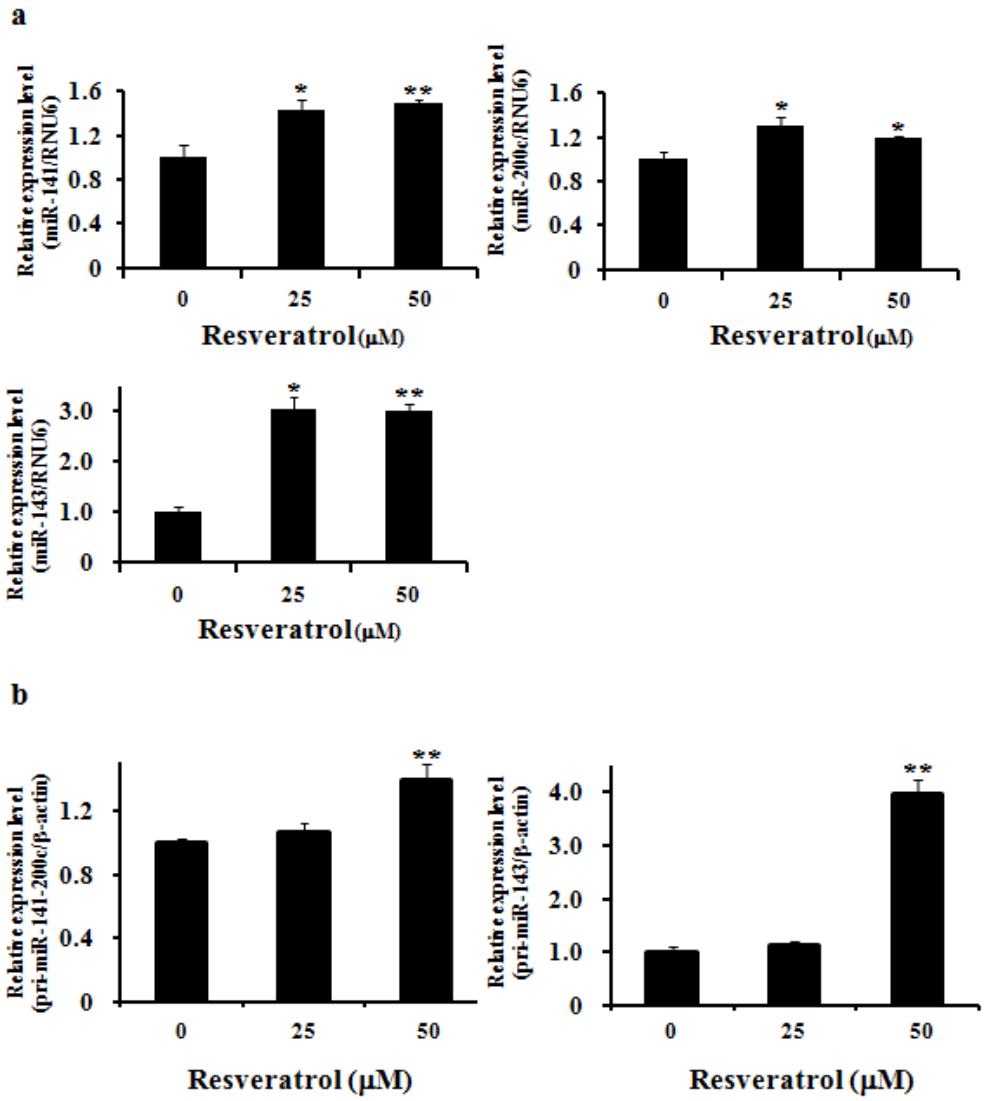


Supplementary Figure 1. Resveratrol had anti-cancer effects in breast cancer cells *in vitro* and *in vivo*. (**a**) The mouse weights in the different groups during the course of

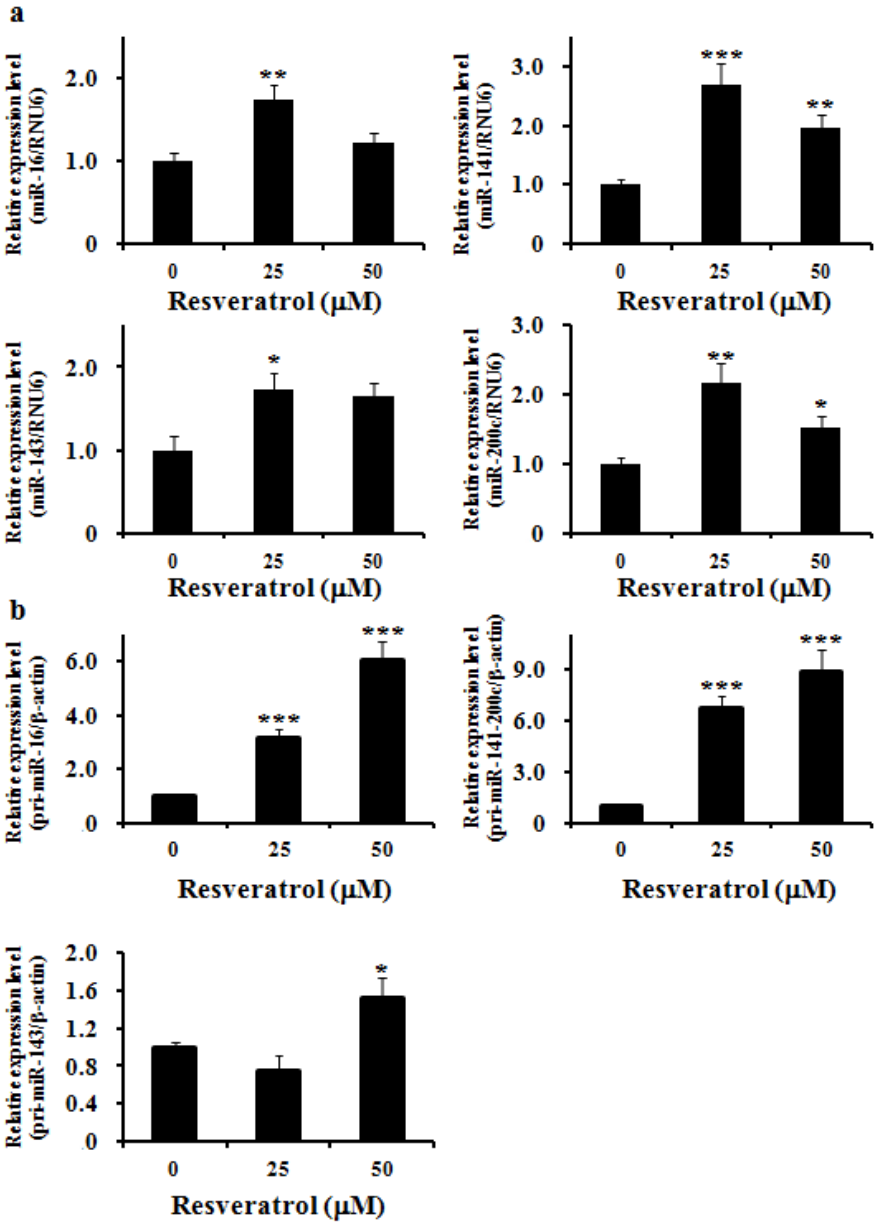
experiment. (**b**) The number of mammospheres/1000 CSC cells after resveratrol treatment. (**c**) Representative images of apoptotic nuclear TUNEL staining visualised with green fluorescent. Hoechst counterstain (blue) highlights all nuclei. Scale bar: 50 μm. (**d**) The Caspase-3/-7 activity was examined in MDA-MB231-luc-D3H2LN cells 72 hours after treatment with resveratrol or DMSO (control). (**e**) MDA-MB231-luc-D3H2LN cells were plated as described above and allowed to attach overnight. The cells were replenished with a fresh medium containing 25 μM resveratrol for 24 hours and then exposed to 2.5 nM of the chemotherapeutic agent docetaxel for an additional 48 hours. The effect of resveratrol pretreatment on cell viability was examined by the MTS assay. (**f**) Schedule of experimental procedures for the drug resistant activity in cancer cells *in vivo*. (**g**) MDA-MB231-luc-D3H2LN cells (2000 cells) were injected into the mammary fat pad of 6-week-old female SCID Hairless Outbred (SHO) mice (n = 3). They were then treated with resveratrol (25 mg/kg) by intraperitoneal injection every day for 2 weeks and then with docetaxel (20 mg/kg) by intraperitoneal injection once per week for 2 weeks. The normalised fold change (day 22 or day 29/ day 15) of bioluminescence emitted from the whole body of mice is shown. Quantified bioluminescence image at day 22 (left) and day 29 (right). (**h**) Expression levels of pri-miR-141-200c in MDA-MB231-luc-D3H2LN cells. The expression levels of the indicated miRNAs were examined in MDA-MB231-luc-D3H2LN cells after 48 hours resveratrol treatment (all data are shown as the mean ± s.e.m., *P<0.05, **P<0.01, ***P<0.001).



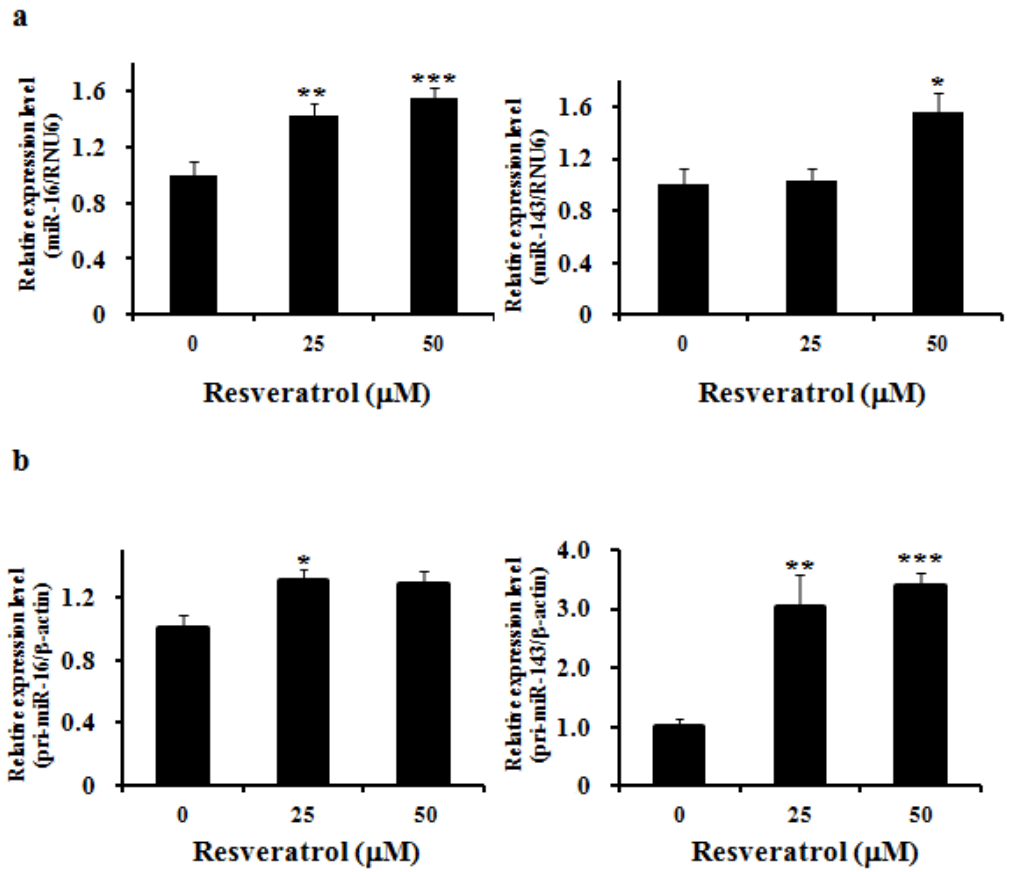
Supplementary Figure 2. Resveratrol up-regulates tumour-suppressive miRNA expression via the induction of primary miRNAs in MCF7-ADR cells. (**a**), (**b**) MCF7-ADR cells were treated with resveratrol or DMSO (control). After 2 days of incubation, the primary and mature miRNA expression levels were compared using qRT-PCR analyses (all data are shown as the mean ± s.e.m., *P<0.05, **P<0.01, ***P<0.001).



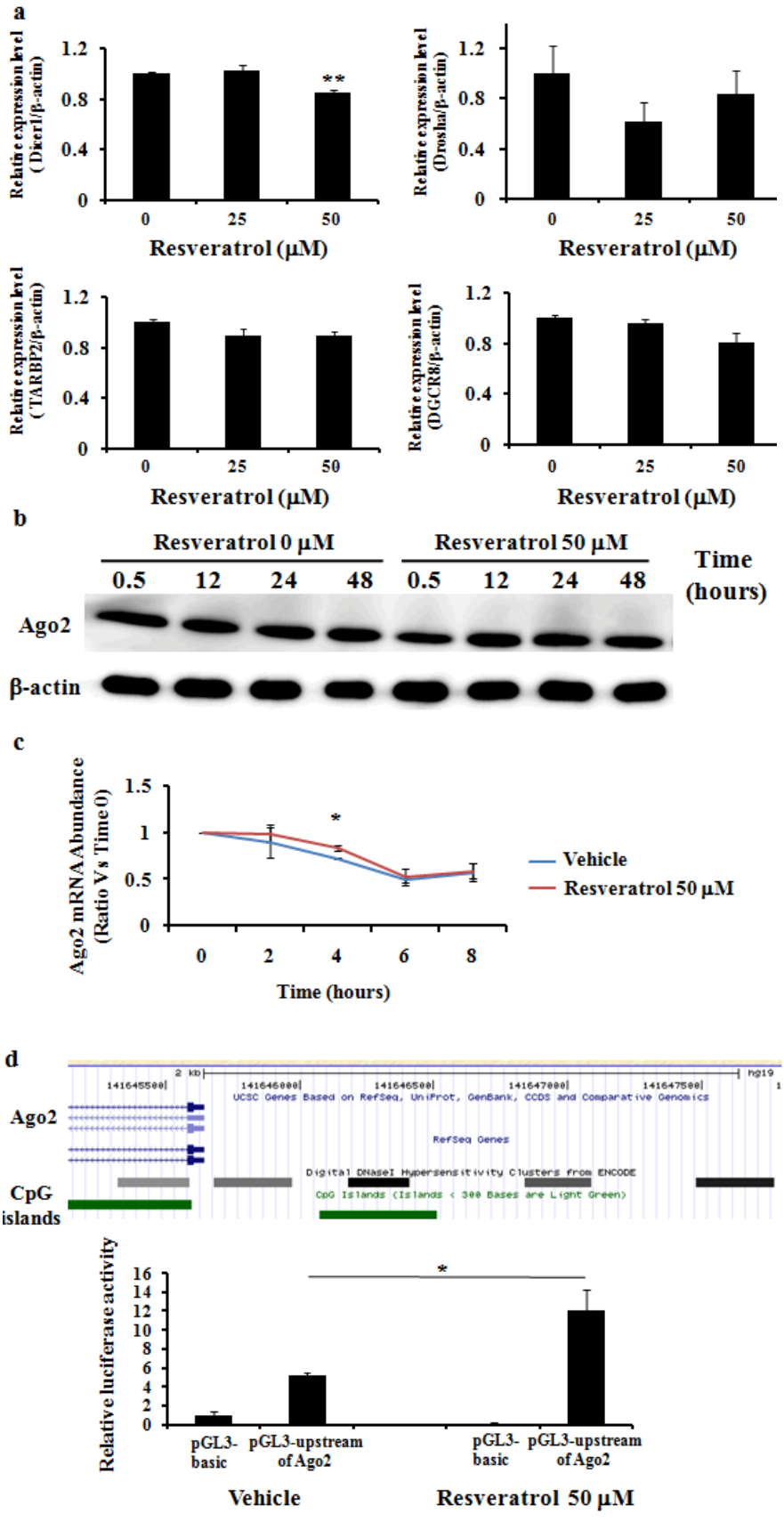
Supplementary Figure 3. Resveratrol up-regulates tumour-suppressive miRNA expression via the induction of primary miRNAs in MCF7 cells. (**a**), (**b**) MCF7 cells were treated with resveratrol or DMSO (control). After 2 days of incubation, the primary and mature miRNA expression levels were compared using qRT-PCR analyses (all data are shown as the mean ± s.e.m., *P<0.05, **P<0.01).

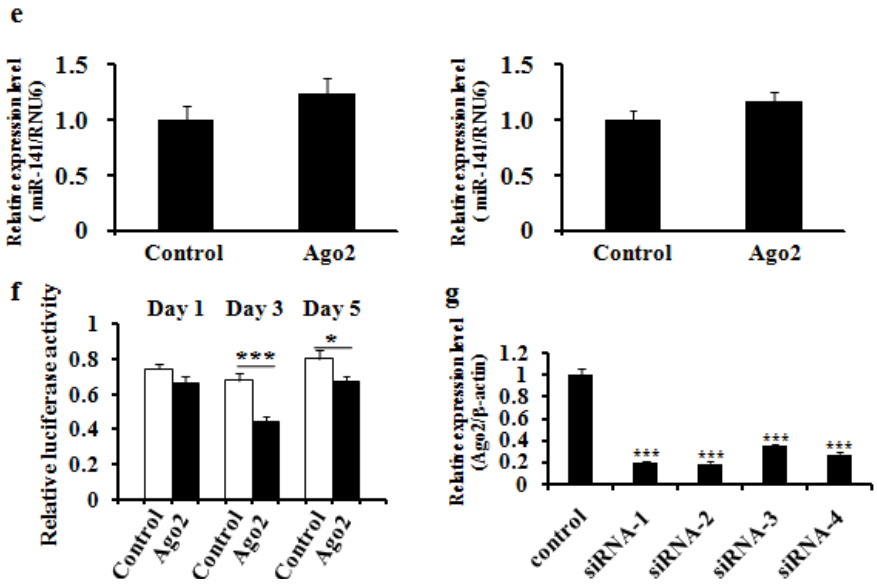


Supplementary Figure 4. Resveratrol up-regulates tumour-suppressive miRNA expression via the induction of primary miRNAs in MCF10A cells. (**a**), (**b**) MCF10A cells were treated with resveratrol or DMSO (control). After 2 days of incubation, the primary and mature miRNA expression levels were compared using qRT-PCR analyses (all data are shown as the mean ± s.e.m., *P<0.05, **P<0.01, ***P<0.001).



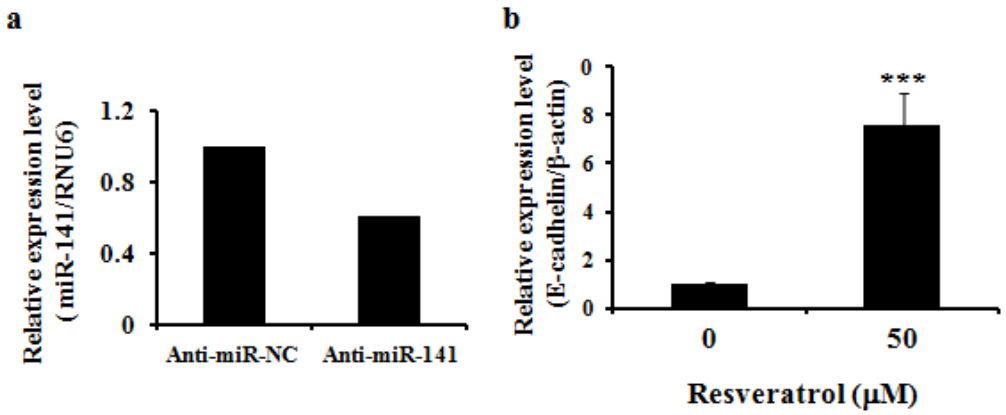
Supplementary Figure 5. Resveratrol up-regulates tumour-suppressive miRNA expression via the induction of primary miRNAs in MDA-MB231-luc-D3H2LN cells. (**a**), (**b**) MDA-MB231-luc-D3H2LN cells were treated with resveratrol or DMSO (control). After 2 days of incubation, the primary and mature miRNA expression levels were compared using qRT-PCR analyses (all data are shown as the mean ± s.e.m., *P<0.05, **P<0.01, ***P<0.001).


a
Relative expression level (Dicer1/β-actin)
1.2
0.8
0.4
0
**
0
25
50
Resveratrol (μM)
Relative expression level (Drosha/β-actin)
Relative expression level (TARBP2/β-actin)
Relative expression level (DGCR8/β-actin)
b
Resveratrol 0 μM
Resveratrol 50 μM
0.5
12
24
48
Time (hours)
Ago2
β-actin
c
Ago2 mRNA Abundance (Ratio Vs Time 0)
1.5
1
0.5
0
*
0
2
4
6
8
Time (hours)
Vehicle
Resveratrol 50 μM
d
Ago2
CpG islands
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
RefSeq Genes
Digital DNaseI Hypersensitivity Clusters from ENCODE
CpG Islands (Islands < 300 Bases are Light Green)
Relative luciferase activity
16
14
12
10
8
6
4
2
0
*
pGL3-basic
pGL3-upstream of Ago2
Vehicle
Resveratrol 50 μM

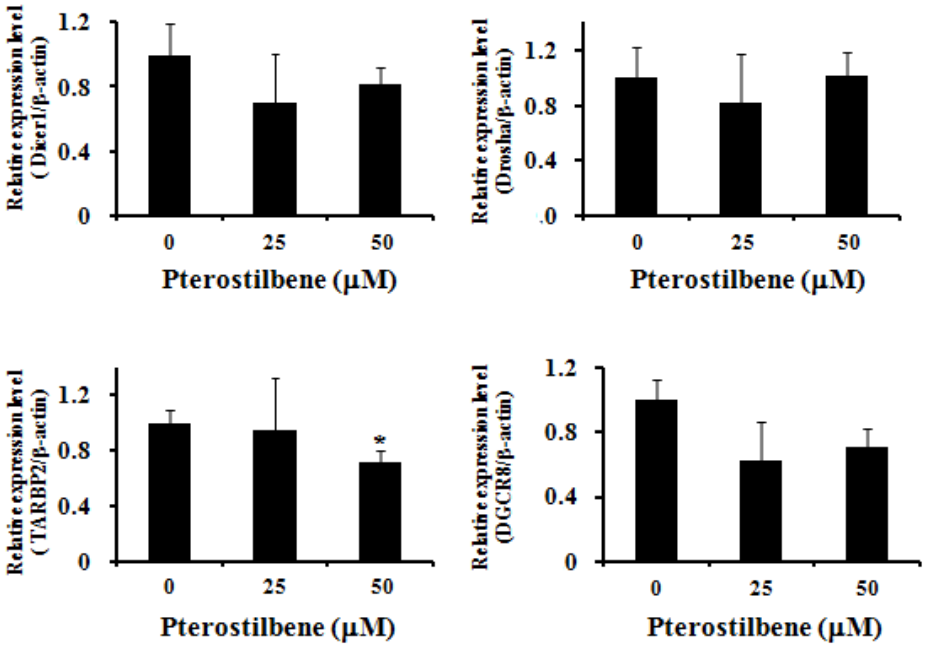


Supplementary Figure 6. Association between resveratrol and miRNA pathway. (**a**) The expression levels of Dicer1, Drosha, TARBP2, and DGCR8 were examined in MDA-MB231-luc-D3H2LN cells. The expression levels of the indicated mRNAs were examined in MDA-MB231-luc-D3H2LN cells after 48 hours treatment with resveratrol. (**b**) MDA-MB-231-luc-D3H2LN cells were treated with 30 μg/ml CHX. The cells were then treated with 50 μM resveratrol and harvested at the indicated time points. The effect of resveratrol on Ago2 stability was examined by immunoblotting**.** (**c**) The Ago2 mRNA abundance was determined in 5,6-dichlorobenzimidazole riboside treated cells at various time-points after resveratrol or DMSO (control) treatment. (**d**) The promoter region of Ago2 has not been defined yet. In this regard, we cloned about 1770 bp region from the upstream of transcriptional initiation site of Ago2 gene. UCSC genome browser view of the human Ago2 promoter sites is shown. From in sillico analysis, this region predicts to contain CpG islands and open chromatin region, which is usually considered to promoter region of the gene (upper panel). MCF7 cells were transfected with 100 ng of pGL3-basic with the insert of promoter region of the human Ago2 gene

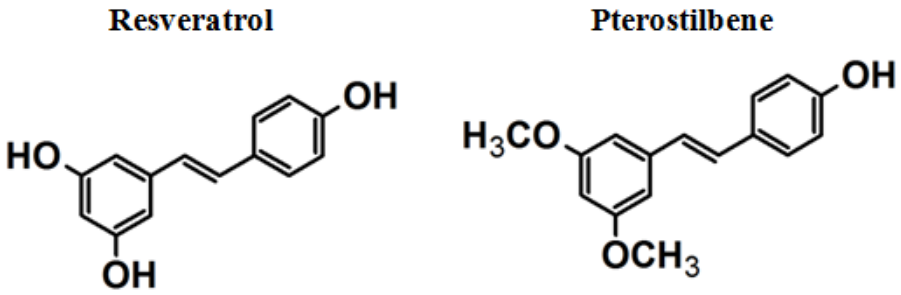
(pGL3-upstream of Ago2) or pGL3-basic control vector. The cells were then treated with 50 μM resveratrol or DMSO (control). After a 1-day culture, the cells were applied to a luciferase reporter assay. The values on the *y-axis* are depicted relative to the luciferase activity of the pGL3-Basic control vector, which is defined as 1 (lower panel). (**e**) MDA-MB231-luc-D3H2LN cells were grown and transiently transfected with Ago2 or EGFP-IRES vectors (control). After 2 days of incubation, the cell extract was analysed by real-time miRNA and mRNA qRT-PCR (**f**) HEK293-luc cells were cotransfected with luciferase siRNA or AllStars Negative Control siRNA (10 nM) and Ago2 or EGFP-IRES vector. After a 1, 3, or 5-day culture, the cells were applied to a luciferase reporter assay. (**g**) MDA-MB231-luc-D3H2LN cells were grown and transiently transfected with Ago2 siRNA or AllStars Negative Control siRNA. After 2 days of culture, the cell extract was subjected to real-time mRNA qRT-PCR (all data are shown as the mean ± s.e.m., *P<0.05, **P<0.01, ***P<0.001).



Supplementary Figure 7. Tumour-suppressive miRNAs induced by resveratrol have multiple anti-cancer effects. (**a**) MDA-MB231-luc-D3H2LN cells were grown and transiently transfected with anti-miR-141 or anti-miR-NC (control). The miR-141 level expression was examined in MDA-MB231-luc-D3H2LN cells 48 h after transfection. (**b**) MDA-MB231 cells were treated with resveratrol or DMSO (control). After 2 days of culture, the cell extract was subjected to real-time mRNA qRT-PCR (all data are shown as the mean ± s.e.m., ***P<0.001).



Supplementary Figure 8. Pterostilbene did not affect the expression of the components associated with the miRNA pathway except for Ago2. The expression levels of Dicer1, Drosha, TARBP2, and DGCR8 were examined in MDA-MB231-luc-D3H2LN cells. The expression levels of the indicated mRNAs were examined in MDA-MB231-luc-D3H2LN cells after treatment for 48 hours with pterostilbene (all data are shown as the mean ± s.e.m., *P<0.05).



Supplementary Figure 9. Structure of resveratrol and pterostilbene.