Supplementary Materials

Materials and Methods

Tree shrews

A total of 37 artificially propagated female tree shrews (*Tupaia belangeri chinensis*) (6 months to 1 year old) were provided by the Kunming Institute of Zoology, Chinese Academy of Sciences, for use in this study. All animal care and experimental protocols were approved by the Animal Ethics Committee of Kunming Institute of Zoology.

Lentivirus preparation

HEK293T cells (derived from human embryo kidney cells, purchased from ATCC) were seeded at a density of 5×10^6 in a 10 cm Petri dish for 12–16 h and incubated at 37 °C and 5% CO_2. Transfection was carried out using polyethylenimine (PEI, Polysciences, USA, 23966-1) on the second day. The plasmid transfection system was as follows: 10 μg of lentiviral expression vector FU-CGW (*PIK3CA-H1047R*) and auxiliary vectors: 6.52 μg of pMDL, 3.52 μg of VSV-G, and 2.52 μg of pREV. The medium was changed 6 h after transfection, and the virus-containing medium was harvested 48 h after transfection. Lentiviruses were harvested using a high-performance ultracentrifuge (Hitachi, Japan), liquated, and stored at −80 °C. To determine viral titers, we infected 1×10^5 HEK293T cells with the viruses for three days, and green fluorescent protein (GFP)-positive cells were counted by flow cytometry (BD, USA, Accuri C6). Viral titer (TU/mL) = 1×10^5 × percentage (of GFP^+ cells) / V (volume of virus).

Intraductal injection

Injection of the breast duct has been described in detail previously (Siwko et al., 2008). Tree shrews have three pairs of mammary glands, and we chose the third pair as the site of injection. The third pair of breast glands are located close to the chest wall in the groin area and are relatively easy to access and dissect. After the tree shrews were anesthetized by intraperitoneal injection of ketamine, the mammary ducts were exposed by removing the nipples, and the concentrated virus (10 μL/5 μL) was injected directly into the third pair of mammary duct lumens with a 33-gauge needle (Hamilton, Reno, NV, USA). The same procedure was performed on the fourth pair of mammary glands in FVB background mice (sensitive to Friend leukemia virus B) to induce mammary tumor formation. Additional details of the viruses, such as viral titer and amount, are provided in the text and Table 1. Trypan blue was added to track the distribution of the viral solution. The left and right glands were injected with the lentivirus. The incubation period to palpable breast tumors was recorded, and the tumors were surgically removed 12 weeks after lentivirus injection.

Histological and immunohistochemical (IHC) staining

After dissection, tumors were surgically removed, fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The IHC protocol was as described in our previous study (Xia et al., 2012). The IHC antibodies included anti-ERα (kit-0012, Maixin, 1:50), anti-PR (RMA-0502, Maixin, 1:100), anti-HER2 (kit-0043, Maixin, ready-to-use), anti-Ki-67 (kit-0005, Maixin, 1:200), anti-p-AKT (Cat# 4060S, Cell Signaling, 1:100), and anti-p-ERK (Cat# sc-7383, Santa Cruz, 1:100). All samples were observed and assessed by an experienced pathologist (Yang, CY).

Western blot (WB) analysis

After 48 h of lentivirus infection, MCF-10A cells (normal breast epithelial, purchased from

ATCC) were harvested in RIPA lysis buffer (1 M Tris-HCl (pH 8.8) 10 mL, NaCl 1.75 g, Triton X-100 2 mL, Na-deoxycholate 1 g, sodium dodecyl sulfate (SDS) 0.2 g, ddH2O to 198 mL, pH 7.5, and protease inhibitor mixture PI-8340 (Sigma, St Louis, USA, 1:100)) on ice for WB analysis. The WB protocol was performed according to our previous study (Wu et al., 2016). The GAPDH protein was used as a loading control. The following antibodies were used for WB analysis: anti-*PIK3CA* (4255S, CST, USA, 1:1 000), anti-pAKT (S473, 4051S, 4051S, CST, USA, 1:1 000), anti-AKT (4685, 4051S, CST, USA, 1:1 000), anti-pERK (sc-7383, Santa Cruz, USA, 1:1 000), and anti-ERK (c-154, Santa Cruz, USA, 1:1 000).

Tumor transplantation experiment

The tree shrew tumors were surgically dissected, cut into the smallest possible fractions, and washed with sterilized phosphate-buffered saline (PBS). The tumor tissues were digested with 8 mL of medium, 1 mL of 2 000 U/mL collagenase II, and 1 mL of 1 000 U/mL hyaluronidase in a 37 °C water bath for 40 min. After digestion, the tumor cells were filtered using a nylon filter, centrifuged with 500 g at 4 ℃ for 20 min, and resuspended in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) to obtain single tumor cells. The tumor cells were resuspended in PBS and mixed with Matrigel on ice. Non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice were anesthetized and fixed for surgery. To ensure tumor transplantation *in situ*, the abdominal skin of the mice was cut with scissors to expose the breast fat pads, and the tumor cell suspension was injected into the fourth mammary fat pad. The wound was then sutured by needles after the injection. Needles were removed 5–7 days after the operation.

Alpelisib treatment

The NOD-SCID mice carrying *PIK3CA-H1047R*-induced tree shrew breast tumors were given vehicle (5% dimethyl sulfoxide (DMSO), 40% PEG300, 5% Tween 80, and 50% saline) and alpelisib (25 and 50 mg/kg), as figure 1C depicted. Tumor size was measured with a caliper twice a week, and tumor volume was calculated using the standard formula $L \times W^2 \times 0.5$ (L: length, W: width). After 21 days, the tumors were harvested from the mice euthanized by cervical dislocation and weighted.

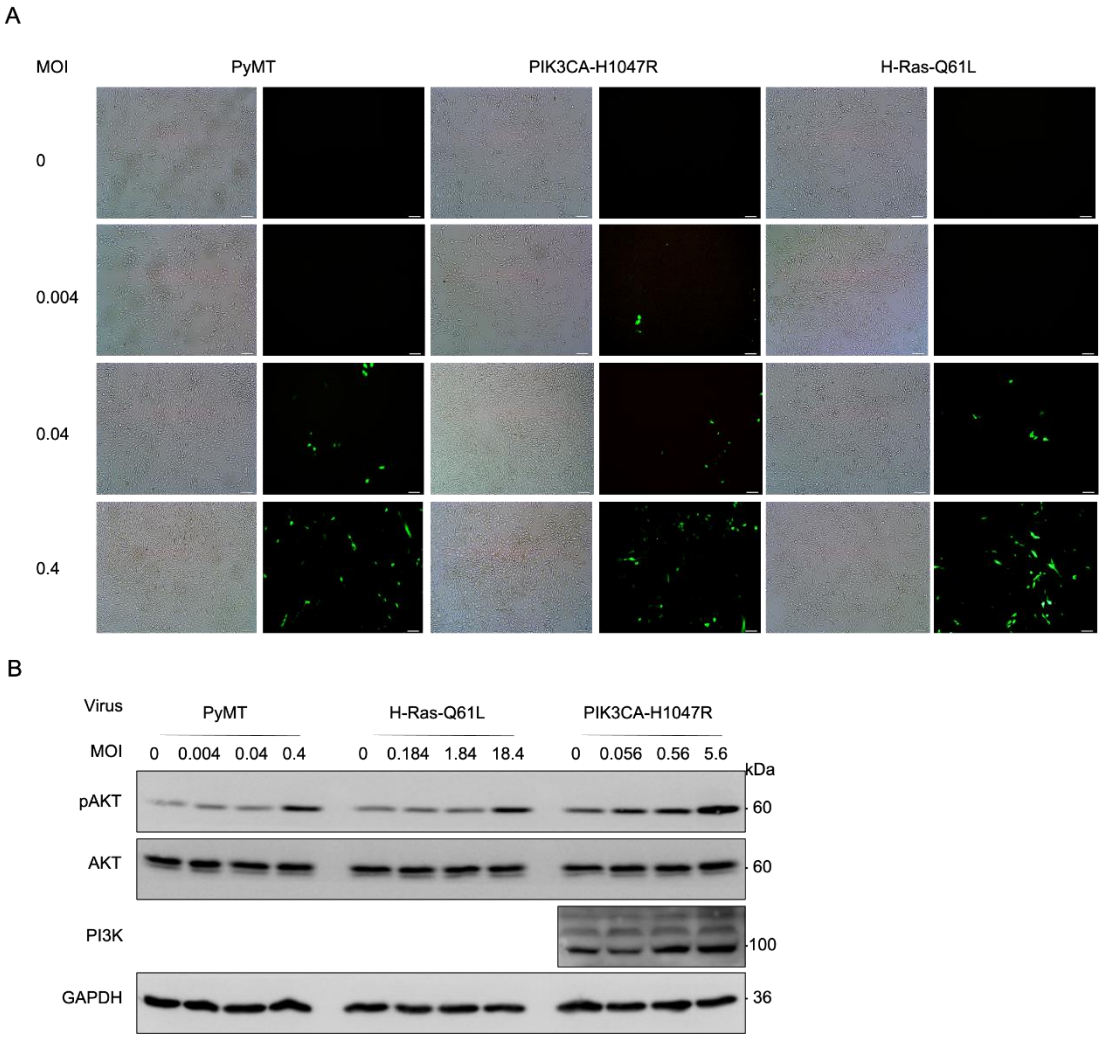
Statistical analysis

Data are presented as mean±standard deviation (SD). Statistical analyses were performed using an unpaired two-tailed *t*-test in GraphPad Prism v9.0 (GraphPad Software), with $P<0.05$ considered statistically significant.

REFERENCES

Siwko SK, BU W, Gutierrez C, Lewis B, Jechlinger M, Schaffhausen B, et al. 2008. Lentivirus-mediated oncogene introduction into mammary cells *in vivo* induces tumors. *Neoplasia*, **10**(7):653–662.

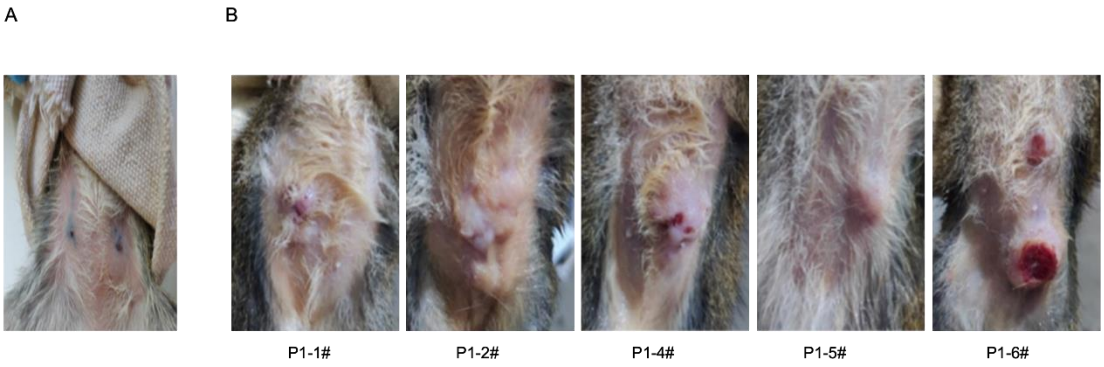
Wu J, Ding Y, Chen CH, Zhou Z, Ding C, Chen H, et al. 2016. A new oridonin analog suppresses triple-negative breast cancer cells and tumor growth via the induction of death receptor 5. *Cancer Letters,* **380**(2): 393–402.



Supplementary Figure S1 *PIK3CA-H1047R* and *H-RAS-Q61L* lentiviruses activated AKT in MCF-10A cells

A: MCF-10A cells were infected with lentiviruses expressing GFP and *PIK3CA-H1047R*, *H-RAS-Q61L*, or *PyMT* at different multiplicities of infection (MOIs) (0, 0.004, 0.04, and 0.4). Scale bar, 200 μm.

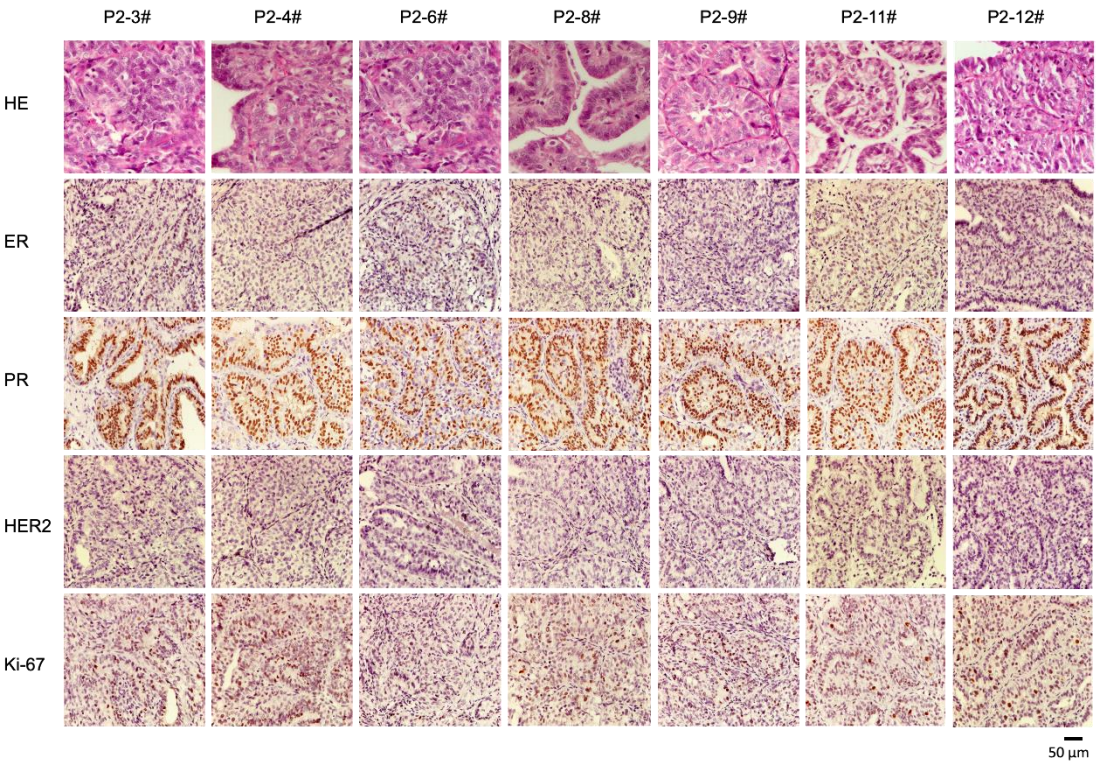
B: *PyMT, PIK3CA-H1047R*, and *H-RAS-Q61L* activated p-AKT at different MOIs in MCF-10A cells. Activation of AKT was examined by WB. GAPDH served as a loading control.



Supplementary Figure S2 *PIK3CA-H1047R* lentivirus induced tree shrew breast tumors

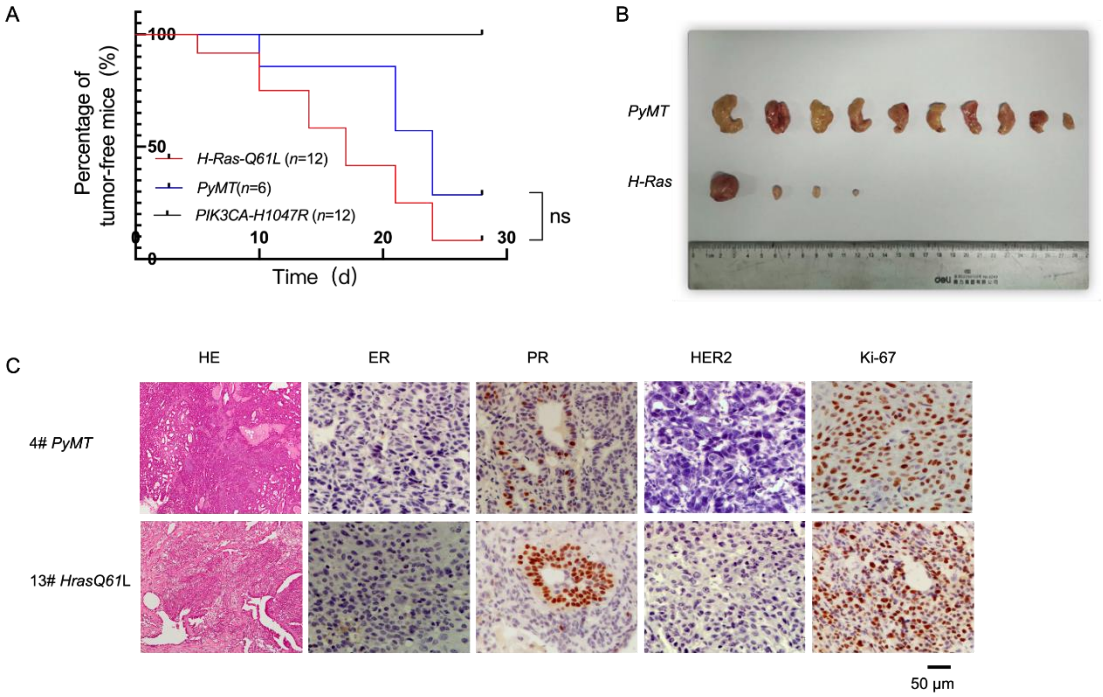
A: Nipple injection of 10 μL of *PIK3CA-H1047R* lentivirus (3×10^7 /mL) into the third pair of mammary glands of tree shrews. Trypan blue was used as a tracer to ensure successful viral injection.

B: *PIK3CA-H1047R* lentivirus successfully induced breast tumors in tree shrews 5 weeks after lentivirus infection. Five animals with tumors are shown.



Supplementary Figure S3 Pathological characteristics of tree shrew breast tumors induced by *PIK3CA-H1047R*

Pathological characteristics of breast tumors induced by *PIK3CA-H1047R* for the second time. Scale bar, 50 μm.



Supplementary Figure S4 *H-RAS-Q61L*, but not *PIK3CA-H1047R*, induced mammary tumors in FVB mice

A: *PIK3CA-H1047R, H-RAS-Q61L*, and *PyMT* lentiviruses were injected into fourth pair of mammary glands in FVB mice. *H-RAS-Q61L* and *PyMT* successfully induced breast tumors over time. No tumors were observed in the *PIK3CA-H1047R* group.

B: Carcinogenic ability of *PyMT* was stronger than that of *H-RAS-Q61L* in FVB mice under the same titer (4×10^4 /mL).

C: Pathological characteristics of breast tumors induced by *H-RAS-Q61L* and *PyMT* lentiviruses. Scale bar, 50 μm.

Supplementary Table S1 Two batches of *PIK3CA-H1047* lentivirus injections

Experiment	Virus titer (TU/mL)	Virus amount at each point	Injection volume (μl)	Incubation period (w)	Incidence
1	3×10^7	3×10^5	10	5	41.7%（5/12）
2	3×10^7	1.5×10^5	5	3	58.3%（7/12）

Supplementary Table S2 Pathological characterization of breast tumors induced by *PIK3CA-H1047R* in tree shrews

Experiments	No.	Tumor type	ER*	PR*	HER-2*	Ki67(%) *
1	P1-1#	IP*	-	+++	-	90%
	P1-2#	IP	+	+++	-	30%
	P1-4#	IP	+	+++	-	90%
	P1-5#	IDC*	+	+	-	30%
	P1-6#	IP	-	+++	-	80%
2	P2-3#	IP with AH*	+	+++	-	50%
	P2-4#	IP with AH	+	+++	-	30%
	P2-6#	IP with AH	+	+++	-	5%
	P2-8#	IP with AH	-	+++	-	30%
	P2-9#	IP with AH	-	+++	-	40%
	P2-11#	IP with AH	+	+++	-	50%
	P2-12#	IP with AH	+	+++	-	30%

***** ER: estrogen receptor. PR: progesterone receptor. HER-2: human epidermal growth receptor-2. Ki67: a nuclear protein encoded by *MKI-67* gene, a proliferation marker protein. IP: intraductal papilloma. IDC: invasive ductal carcinoma. AH: atypical hyperplasia

Supplementary Table S3 *PIK3CA-H1047R*, *H-RAS-Q61L*, and *PyMT* lentiviruses induced tumor formation in FVB mice

Experiments	Virus	Virus titer (TU/mL)	Virus amount at each point	Injection volume (μl)	Incubation period (d)	period (d)	Incidence
1	*PyMT*	4×10^6	4×10^4	10	10	21	66.7%（4/6）
	H-RAS-Q61L	1.84×10^8	1.4×10^6	10	5	21	91.7%（11/12）
	PIK3CA-H1047R	5.6×10^7	3×10^5	10	-	-	-
2	*PyMT*	4×10^6	4×10^4	10	10	16	100% (10/10)
	H-RAS-Q61L	1.84×10^8	4×10^4	10	16	16	20% (2/10)

Supplementary Table S4 Pathological characterization of breast tumors induced by *H-RAS-Q61L* and *PyMT* in FVB mice

Virus	No.	Tumor type	ER	PR	HER2	Ki67
PyMT	1#	IDC	-	+	-	30%
	2#	IDC	-	-	-	0%
	4#	IDC	-	+	-	20%
	5#	IDC	-	+	-	5%
H-RAS-Q61L	13#	IDC	-	+	-	10%
	14#	IDC	-	+	-	5%