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Supplemental Material to:

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Estrogen receptor α is a novel target of the Von Hippel-Lindau protein and is responsible for the proliferation of VHL-deficient cells under hypoxic conditions

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Figure S1.

(A) Si-VHL suppresses pVHL. C2V cells were transfceted with si-VHL or si-control for 24 hr and measured the expression of pVHL. Actin was used for loading control.

(B) Si-VHL suppresses the VHL transcription level. At the same condition, we performed the RT-PCR and observed the reduction of VHL expression. GAPDH was used for loading control and BK indicated blank PCR reaction without cDNA.

(C) Si-pVHL promotes ER-α expression in the C2V cell line. C2V cells were transfected with si-cont or si-pVHL for 24 hr and treated with estrogen (Est; 600 μg/ml). Immunoblot analysis was performed using the indicated antibodies. Actin was used as a loading control.

(D) pVHL is not related to androgen receptor (AR) expression.

(E) ER-α also suppresses pVHL expression. C2V cells were transfected with ER-α for 24 hr and measured the expression of pVHL.

(F) pVHL increases the nuclear expression of ER-α. MCF7 cells were transfected with the indicated si-RNAs for 24 hr. After transfection, the cells were incubated in SF or CM for 6 hr, followed by fixation with Me-OH. Fixed cells were stained with anti-ER-α (green) and DAPI (blue).

Figure S2.

(A) pVHL is retained in the nucleus in the presence of a proteasome inhibitor. 293 cells were transfected with pVHL for 24 hr and treated with ALLN (50 μg/ml) or MG132 (10 μM) for 6 hr. After treatment, cells were fixed with Me-OH and stained with anti-pVHL (red) and DAPI (blue).

(B) The localization of ER-α is not altered by the presence of a proteasome inhibitor. 293 cells were transfected with ER-α for 24 hr and treated with ALLN or MG132 for 6 hr. After treatment, cells were fixed with Me-OH and stained with anti-ER-α (green) and DAPI (blue).

(C) ER-α-induced pVHL suppression is achieved by proteasome pathway. To examine the effect of proteasome inhibitor on ER-α-induced pVHL suppression, ACHN cells were transfected with ER-α for 24 hr. and treated with MG132 for 6 hr.

(D) p53-Snail binding blocker induces the expression of p53. After transfection with the indicated si-

RNAs or Faslodex treatment for 24 hr, cells were treated with GN25 (10 μM) or GN29 (10 μM) for 4 hr. Immunoblot analysis was performed using anti-p53 and anti-actin.

(E) Comparison of cell growth between C2V and Faslodex-treated C2 cells.

Figure S3.

(A) HIF-1α increases ER-α transcriptional activity. Normalized luciferase values for 293 cells transfected with an ER-α-responsive luciferase reporter plasmid and plasmids encoding HIF-α, ER-α and pVHL.

(B) ER-α increases HIF-1α transcriptional activity.

(C) In the absence of pVHL, the transcriptional activity of ER-α is not altered by the transfection of HIF-1α.

(D) pVHL is required for the alteration of HIF-1α activity through ER-α.

Figure S4.

(A-C) Under hypoxic conditions, pVHL translocates from the cytoplasm to the nucleus. The indicated vectors or si-RNAs were co-transfected into VHL-transfected 293 cells. After 24 hr, the cells were stained with anti-pVHL (green) and DAPI (blue).

Figure S5.

Under hypoxic conditions, pVHL is retained in the nucleus by si-HIF-1α. HIF-1α or si-HIF-1α was co-transfected into VHL-transfected 293 cells. After transfection, the cells were stained with anti-pVHL (green) and DAPI (blue).

Figure S6.

(A and B) ER-α does not induce iNOS-Luc activity in a VHL-deficient cell line. The indicated vectors were co-transfected into C2 and A498 cells with an iNOS reporter. Twenty-four hours later, cells were treated with $CoCl_2$ for 12 hr followed by analysis by a luciferase assay.

Figure S7.

(A and B) pVHL is required for the hypoxia-induced suppression of ER-α activity. Normalized luciferase values are given for each cell line transfected with an ER-α-responsive luciferase reporter plasmid and plasmids encoding HIF-1α, ER-α and pVHL. Cells were treated with $CoCl_2$ for 12 hr.

Figure S8

(A) Reduction of ERE-Luc activity in hypoxia condition. 293 cells transfected with ERE-Luc were incubated in hypoxic chamber for 12 hr. A clear reduction in ERE-Luc activity in response to hypoxia was detected.

(B) The resistance of hypoxia-induced ERE-Luc suppression in VHL-deficient cells. Under similar conditions to those described above, a reduction in ERE-Luc activity in response to hypoxia condition in C2 cells was not observed. Instead, pVHL transfection could restore sensitivity to hypoxia.

(C) ER-α promotes HIF-1α transcription activity. 293 cells were co-transfected with an iNOS-Luc reporter containing a HIF-1a response element and the indicated vectors for 24 hr. In addition, cells were incubated in hypoxia chamber for 12 hr to induce the hypoxic conditions.

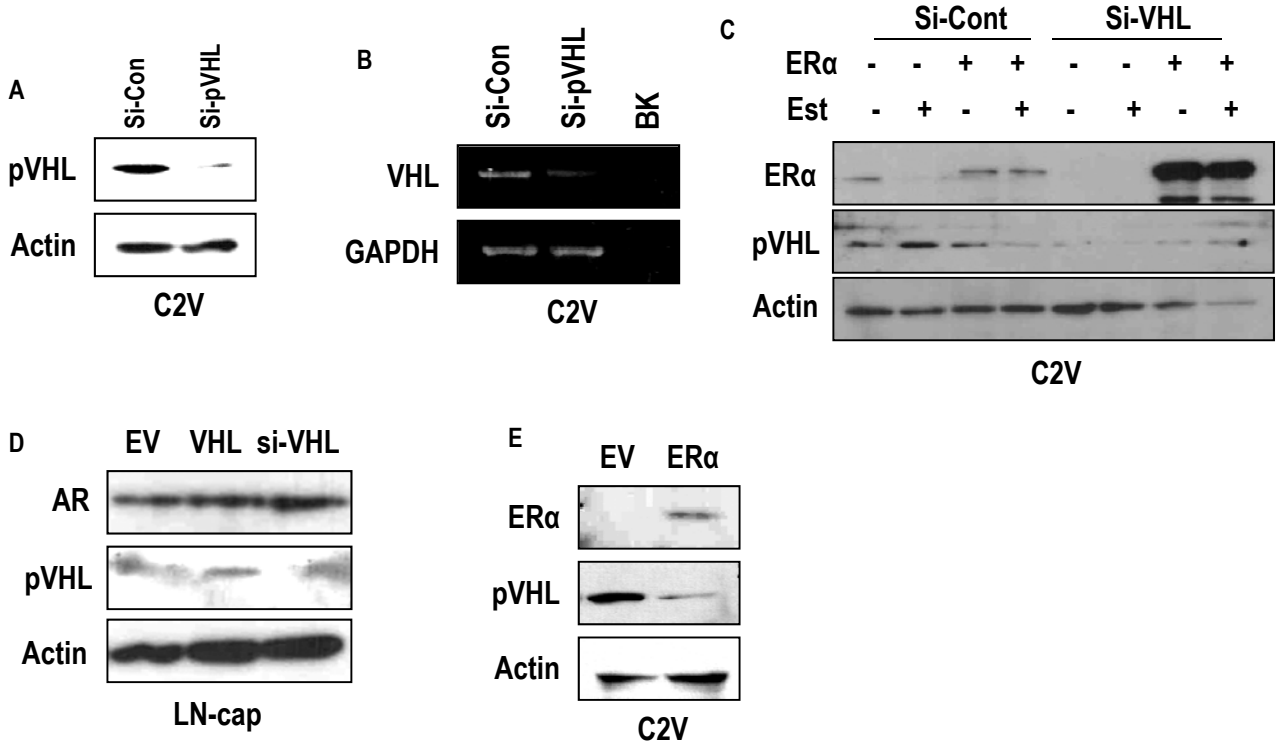
Figure S9.

(A) Comparison of the growth of C2 and C2V cells. Differentially from C2 cells, where hypoxia did not notably suppress cell growth, C2V cells showed the sensitivity to hypoxia-induced growth suppression. In contrast, the growth of C2 cells was reduced by Faslodex. Cells were incubated with Faslodex-containing medium for 3 days and fixed with PFA. Cells were visualized by staining with trypan blue.

(B) pVHL is a critical factor for the determination of hypoxia- or Faslodex-induced growth suppression. Compared to C2V cells, in which Est or Faslodex did not alter cell viability, C2 cells were affected by Faslodex and Est. The opposite effect was observed in hypoxia condition. Cells were incubated with the indicated chemicals for 3 days. Cell viability was determined by a MTT assay.

(C and D) Under hypoxic conditions, cell proliferation is reduced due to the suppression ER-α activity by pVHL. MCF7 cells were treated with Faslodex and/or $CoCl_2$. Cells were maintained for 4 days, and the cell numbers were calculated daily. In addition, we stained the cells using trypan blue after 4 days culture (D)

Fig. S1. pVHL suppresses ER



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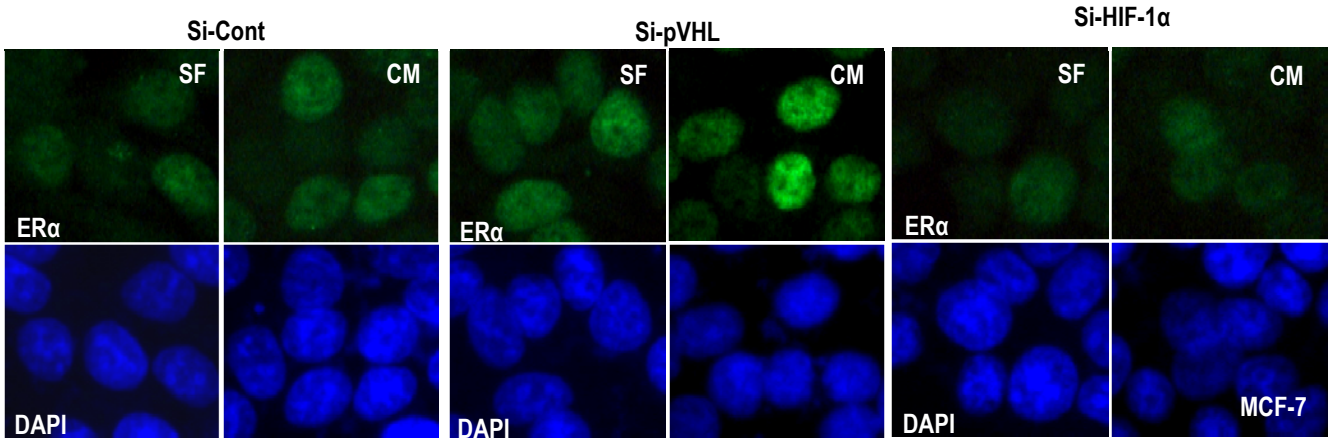


Fig. S2.

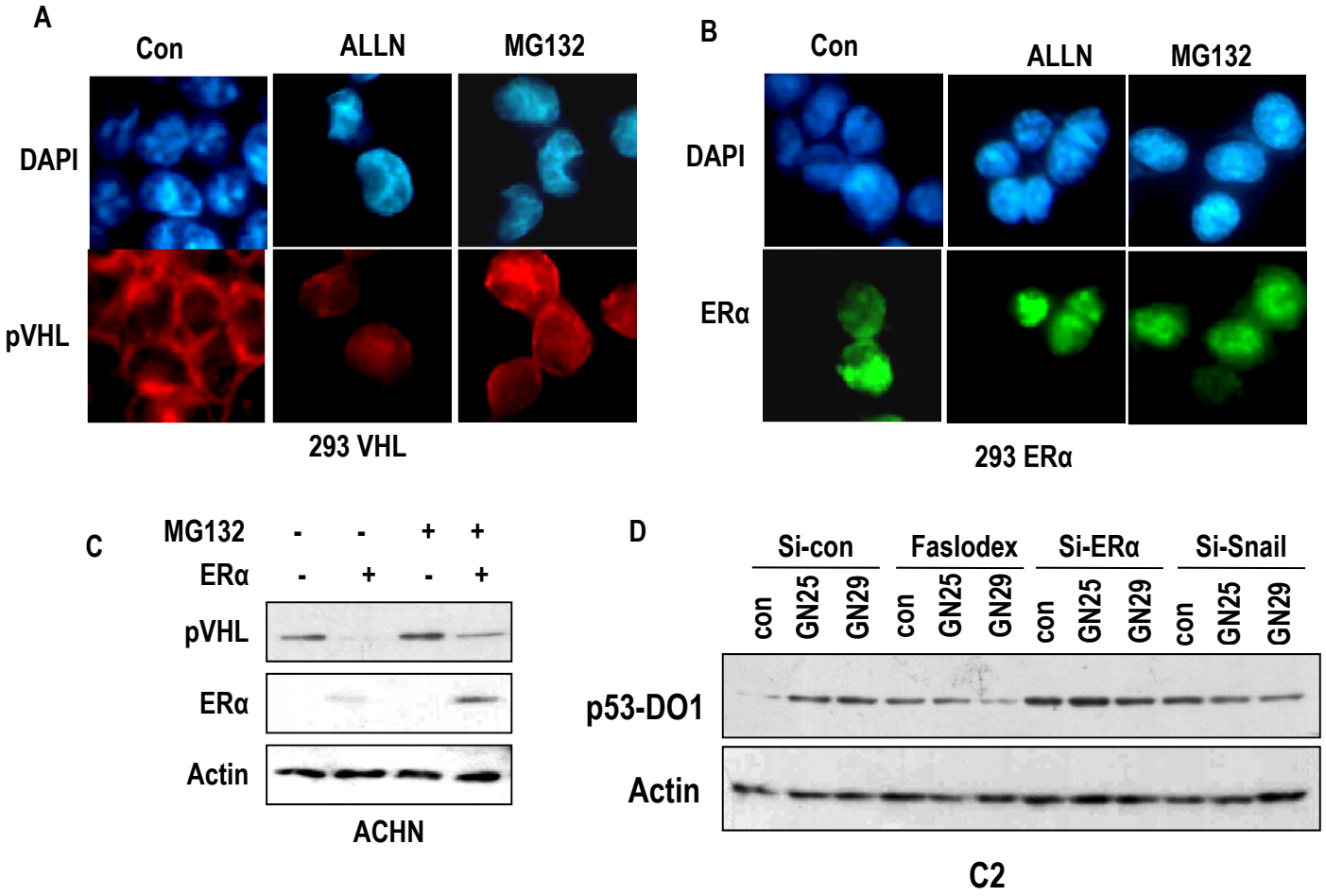

A
Con
ALLN
MG132
DAPI
pVHL
293 VHL
B
Con
ALLN
MG132
DAPI
ERα
293 ERα
C
MG132 - - + +
ERα - + - +
pVHL
ERα
Actin
ACHN
D
Si-con
Faslodex
Si-ERα
Si-Snail
con GN25 GN29 con GN25 GN29 con GN25 GN29 con GN25 GN29
p53-DO1
Actin
C2
E
Cell Number (x10⁴)
12
10
8
6
4
2
0
C2-Faslodex
C2V
0 day
1day
2day
3day

Fig. S3.

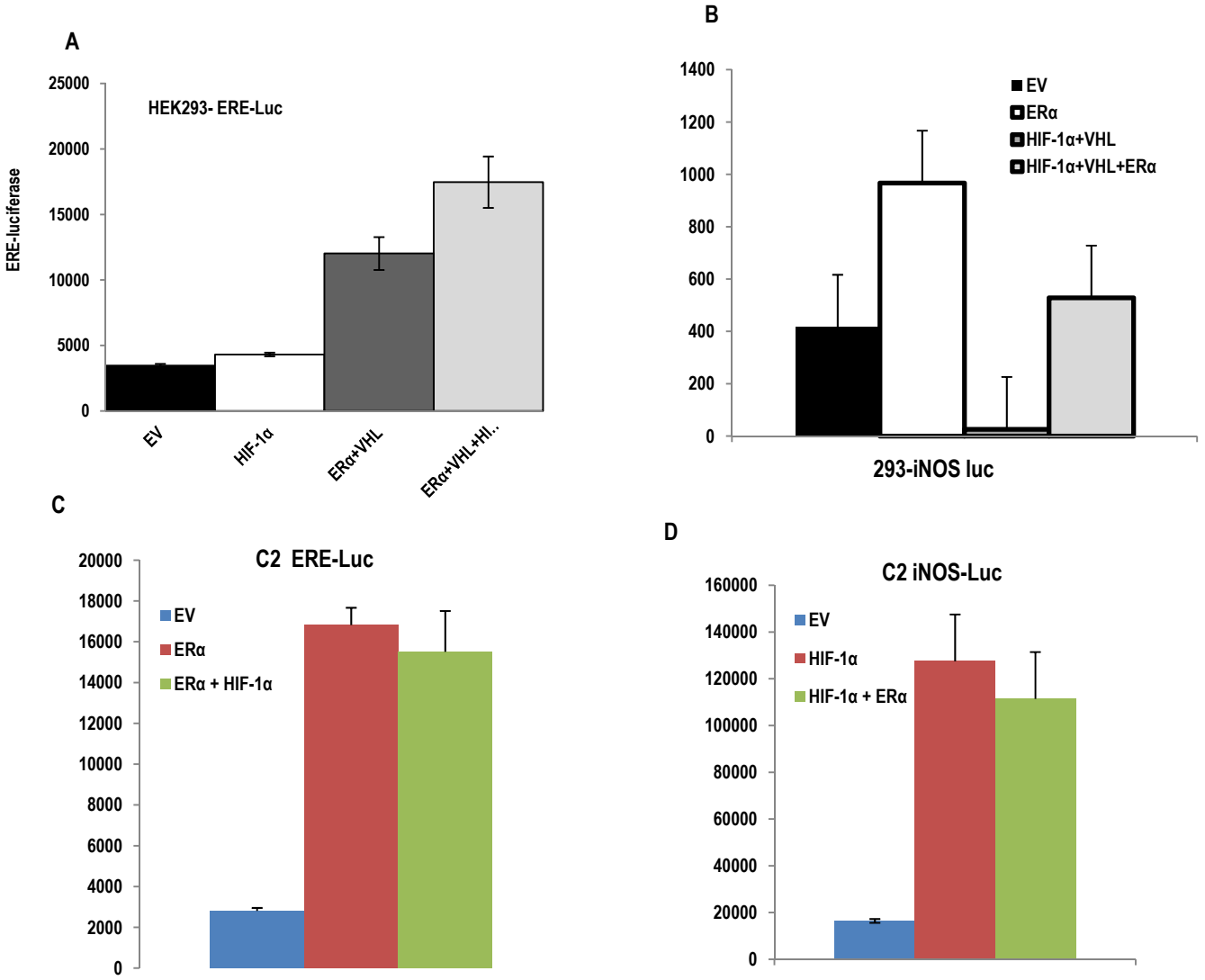

A
HEK293- ERE-Luc
ERE-luciferase
25000
20000
15000
10000
5000
0
EV
HIF-1α
ERα+VHL
ERα+VHL+HI..
B
1400
1200
1000
800
600
400
200
0
EV
ERα
HIF-1α+VHL
HIF-1α+VHL+ERα
293-iNOS luc
C
C2 ERE-Luc
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
EV
ERα
ERα + HIF-1α
D
C2 iNOS-Luc
160000
140000
120000
100000
80000
60000
40000
20000
0
EV
HIF-1α
HIF-1α + ERα

Fig. S4.

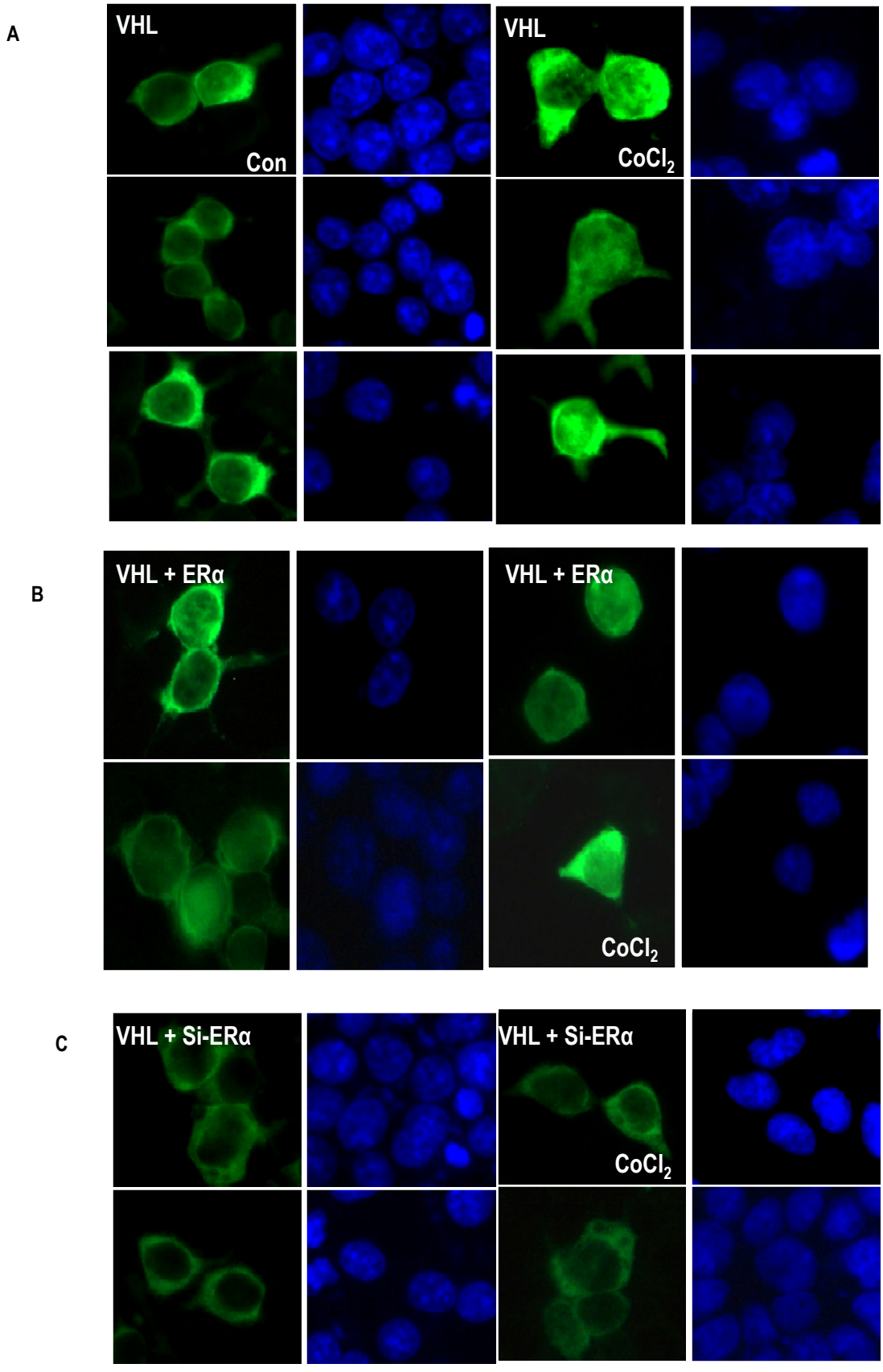

A
VHL
Con
VHL
CoCl2
B
VHL + ERα
VHL + ERα
CoCl2
C
VHL + Si-ERα
VHL + Si-ERα
CoCl2

Fig. S5.

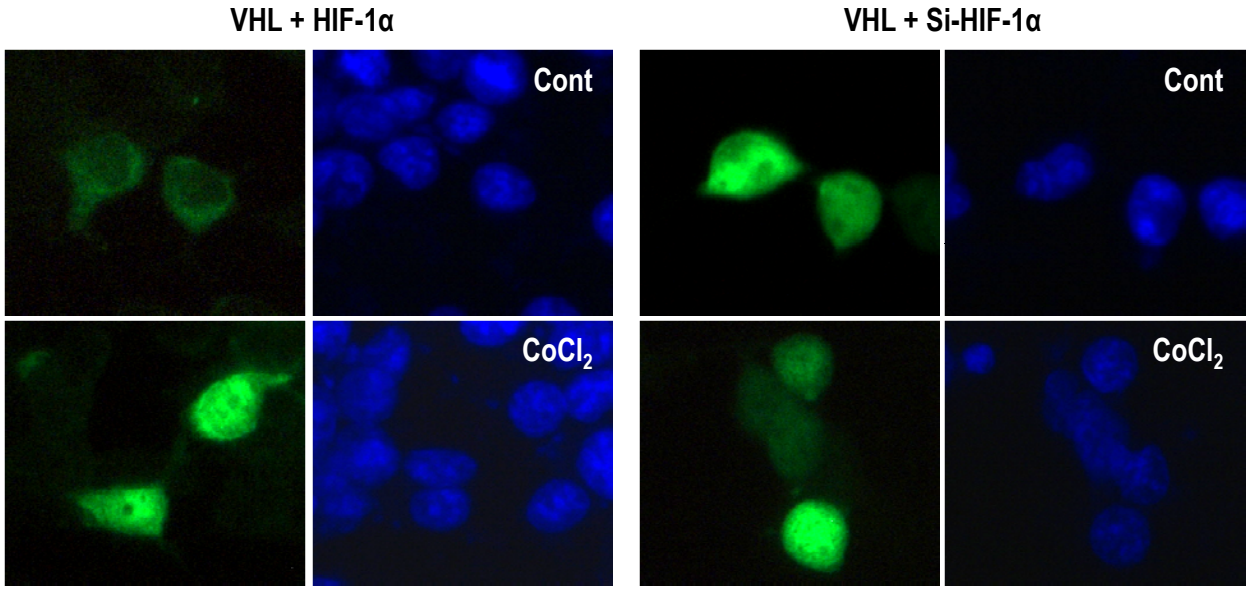
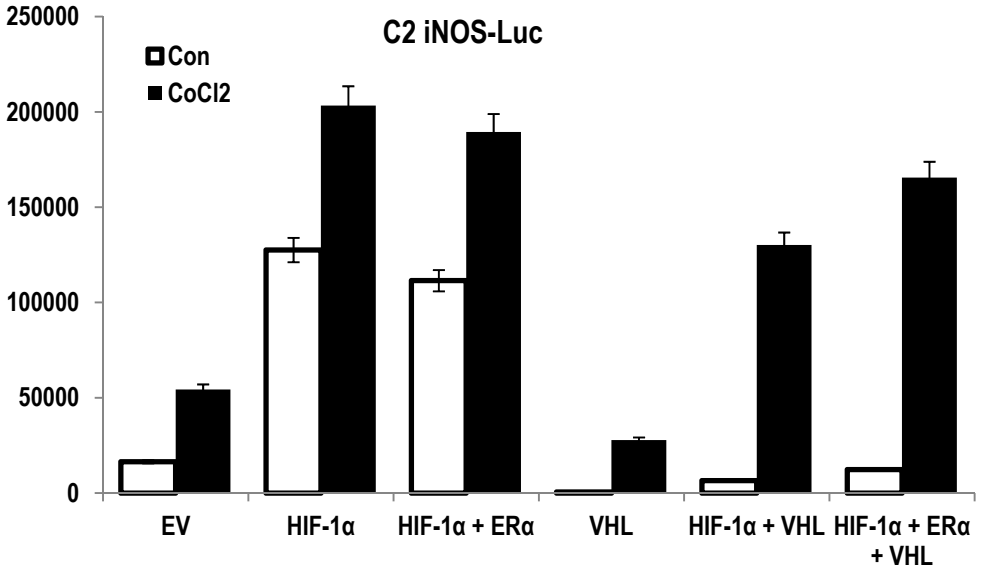


Fig. S6.

A


C2 iNOS-Luc
Con
CoCl2
250000
200000
150000
100000
50000
0
EV
HIF-1α
HIF-1α + ERα
VHL
HIF-1α + VHL
HIF-1α + ERα + VHL

B

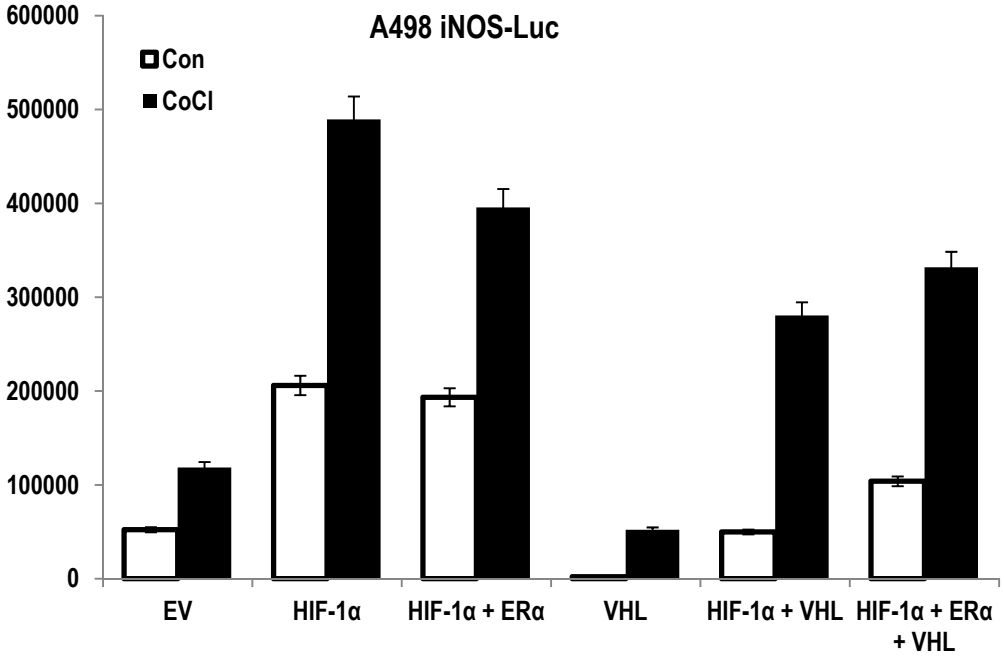

A498 iNOS-Luc
Con
CoCl
600000
500000
400000
300000
200000
100000
0
EV
HIF-1α
HIF-1α + ERα
VHL
HIF-1α + VHL
HIF-1α + ERα + VHL

Fig. S7.

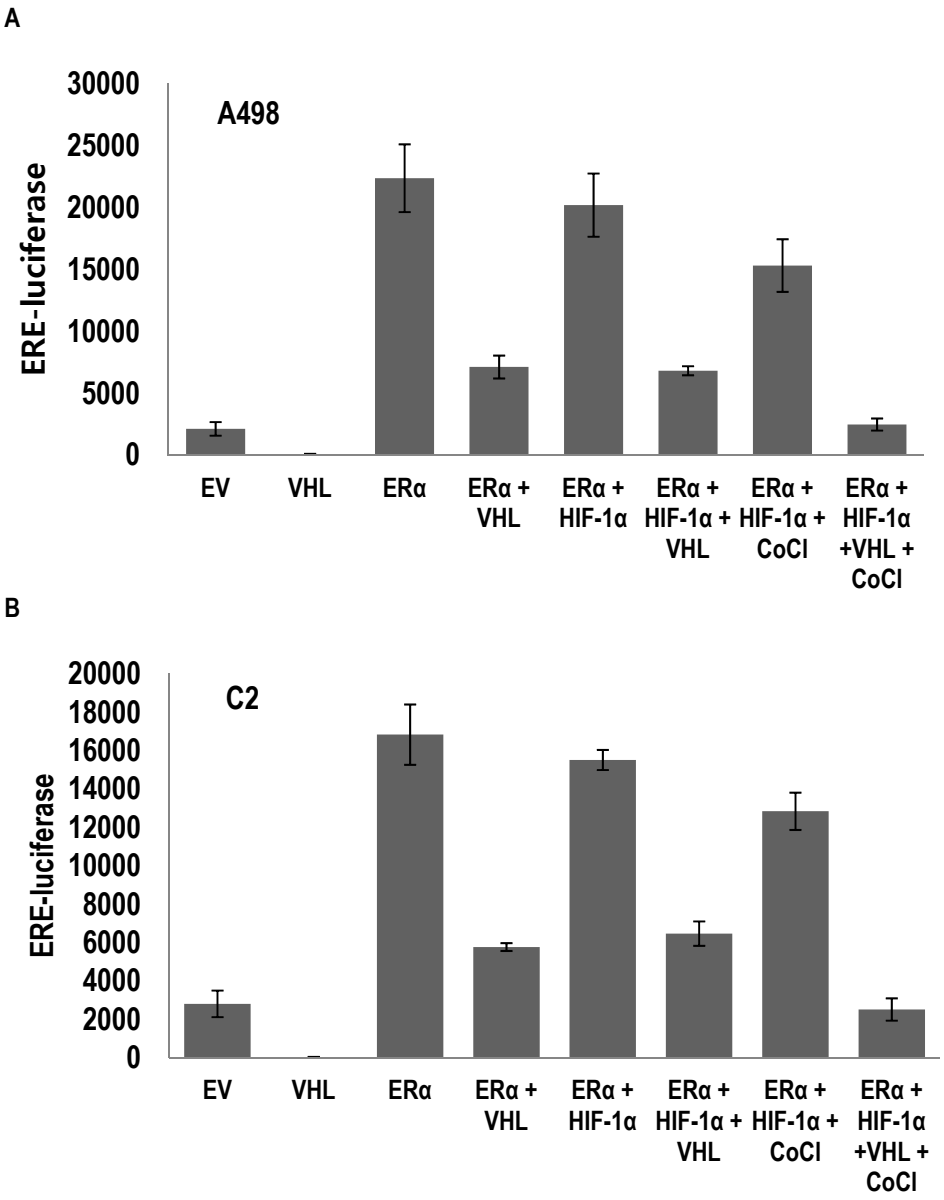
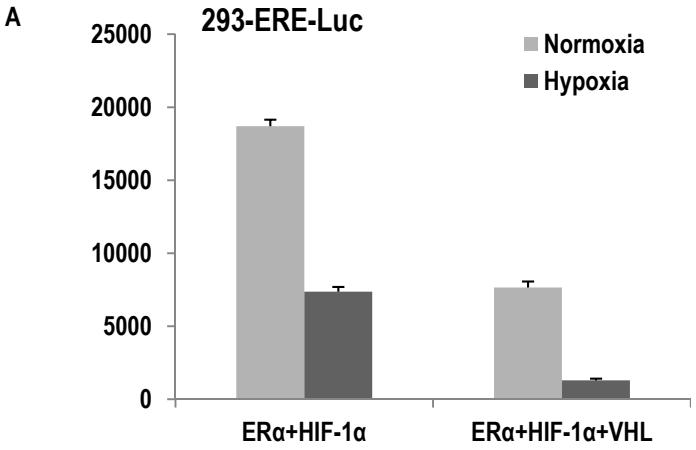
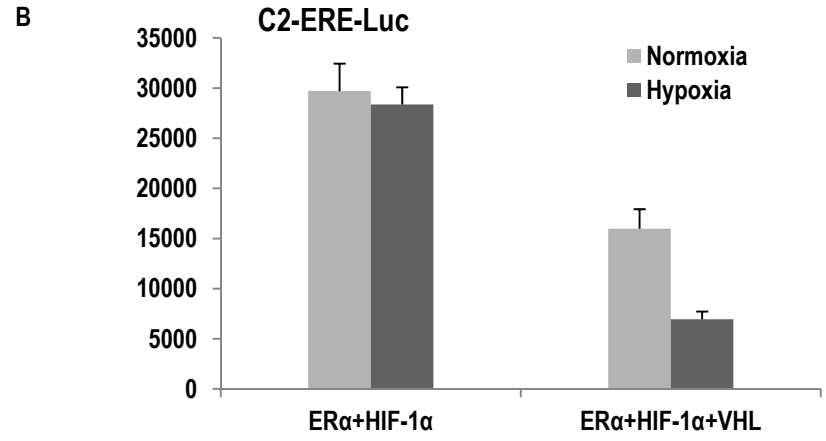

A
A498
ERE-luciferase
30000
25000
20000
15000
10000
5000
0
EV
VHL
ERα
ERα + VHL
ERα + HIF-1α
ERα + HIF-1α + VHL
ERα + HIF-1α + CoCl
ERα + HIF-1α +VHL + CoCl
B
C2
ERE-luciferase
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
EV
VHL
ERα
ERα + VHL
ERα + HIF-1α
ERα + HIF-1α + VHL
ERα + HIF-1α + CoCl
ERα + HIF-1α +VHL + CoCl

Fig. S8.


A
293-ERE-Luc
Normoxia
Hypoxia
25000
20000
15000
10000
5000
0
ERα+HIF-1α
ERα+HIF-1α+VHL


B
C2-ERE-Luc
Normoxia
Hypoxia
35000
30000
25000
20000
15000
10000
5000
0
ERα+HIF-1α
ERα+HIF-1α+VHL

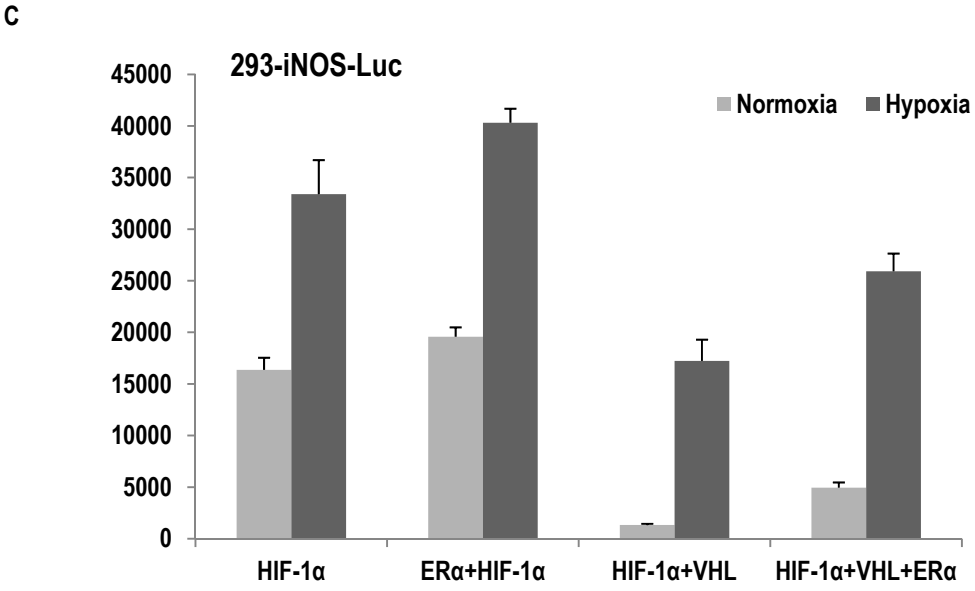
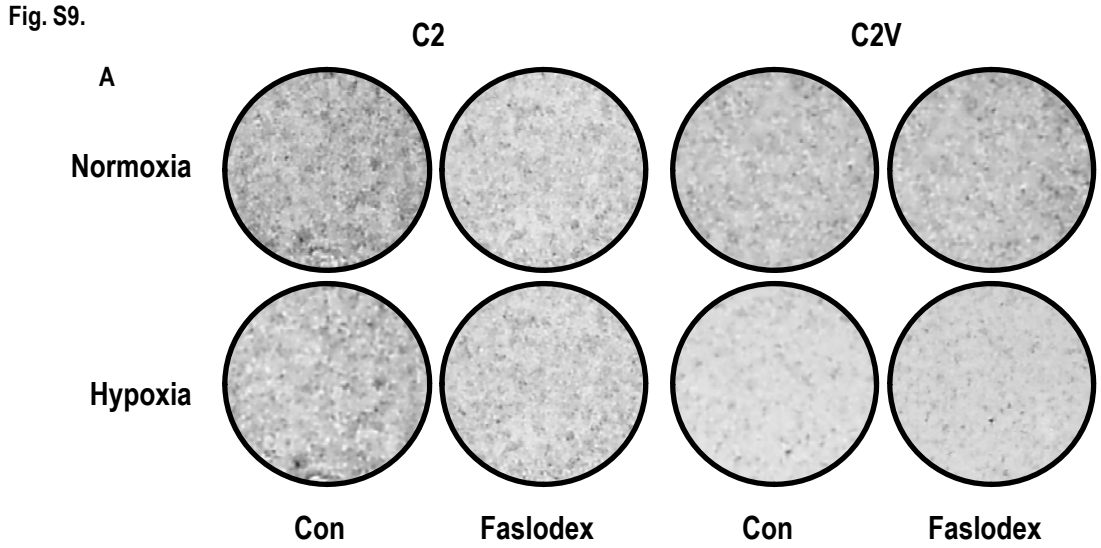
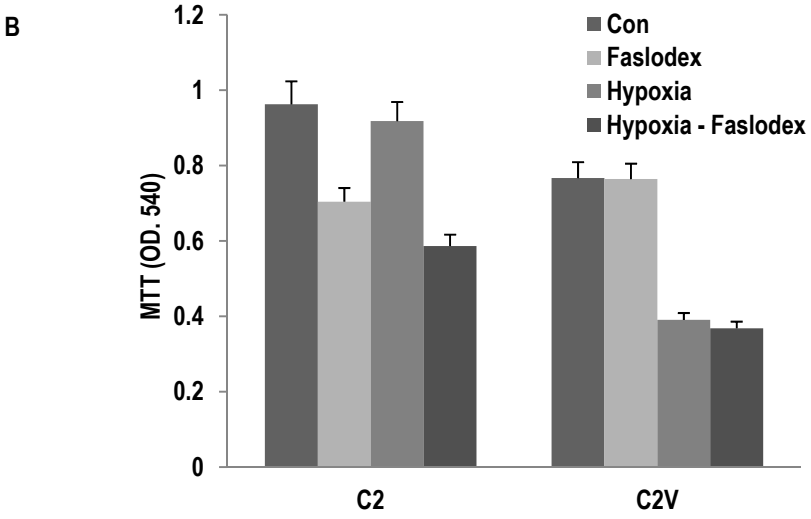
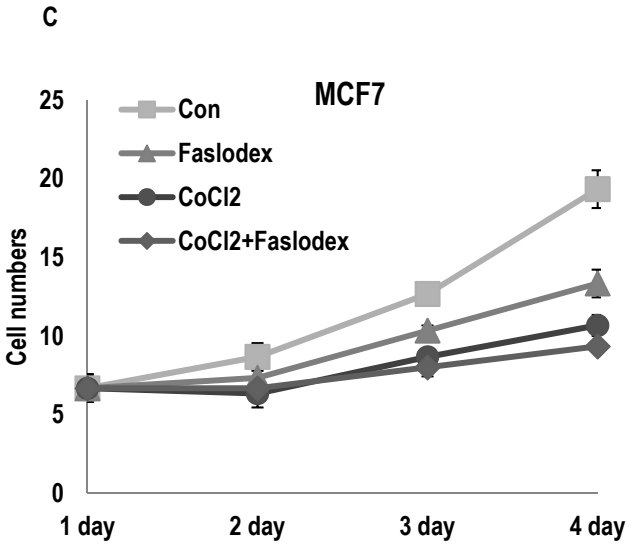
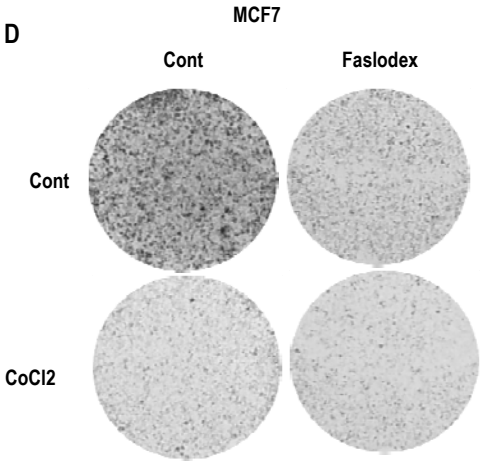

C
293-iNOS-Luc
Normoxia
Hypoxia
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
HIF-1α
ERα+HIF-1α
HIF-1α+VHL
HIF-1α+VHL+ERα

Fig. S9.


A
C2
C2V
Normoxia
Hypoxia
Con
Faslodex
Con
Faslodex


B
MTT (OD. 540)
1.2
1
0.8
0.6
0.4
0.2
0
Con
Faslodex
Hypoxia
Hypoxia - Faslodex
C2
C2V


C
MCF7
Cell numbers
25
20
15
10
5
0
Con
Faslodex
CoCl2
CoCl2+Faslodex
1 day
2 day
3 day
4 day


D
MCF7
Cont
Faslodex
Cont
CoCl2