Supplementary information

Supplementary methods

Treatment regimens

During our preliminary experiments, we evaluated three different doses of the MK2206 + chloroquine (CQ) combination based on previous mouse studies, pharmacodynamics studies, and our in vitro results:

1. CQ (60 mg/kg/day) + MK2206 (50 mg/kg/day) (n=3): We stopped the experiments after the third administration, because the mice experienced significant weight loss.
2. CQ (60 mg/kg/day) + MK2206 (50 mg/kg, every other day) (n=3): After six administrations, the mice started losing weight.
3. CQ (30 mg/kg/day) + MK2206 (50 mg/kg, every other day) (n=3). We selected these doses for the present study because no animals lost weight and all animals were healthy during and after treatment.

Supplementary Figure Legends

Supplementary Figure S1:

A, B: Beclin-1 protein expression in DES (n=6) after transfection with either Beclin-1 siRNAs or siRNA negative controls.

A: Relative expression level of Beclin-1 protein.

*: $p<0.05$: versus siRNA negative controls

§: $p<0.05$: Si-2 versus Si-1 or Si-3

Numerical values are presented as the mean + SD.

C1: siRNA negative control, low GC duplex for Si-1 and Si-2

C2: siRNA negative control, medium GC duplex for Si-3

Si-1: Beclin-1 siRNA-1 (HSS112742)

Si-2: Beclin-1 siRNA-2 (HSS 189498)

Si-3: Beclin-1 siRNA-3 (HSS112741)

B: Representative photomicrograph of western blot analysis for Beclin-1 protein.

C, D: ATG12-ATG5 protein expression in DES (n=6) after transfection with either ATG12 siRNAs or siRNA negative control.

C: Relative expression level of ATG12-ATG5 protein.

*: $p<0.05$: versus siRNA negative control

Numerical values are presented as the mean + SD.

C1: siRNA negative control, low GC duplex

Si-1: ATG12 siRNA-1 (HSS113235)

Si-2: ATG12 siRNA-2 (HSS113534)

Si-3: ATG12 siRNA-3 (HSS 189932)

D: Representative photomicrograph of western blot analysis for ATG12 protein.

E, F: ATG13 protein expression in DES (n=5) after transfection with either ATG13 siRNAs or siRNA negative control.

E: Relative expression level of ATG13 protein.

*: $p<0.05$: versus siRNA negative control

§: $p<0.05$: Si-1 versus Si-2 or Si-3

Numerical values are presented as the mean + SD.

C1: siRNA negative control, low GC duplex

Si-1: ATG13 siRNA-1 (HSS114712)

Si-2: ATG13 siRNA-2 (HSS114713)

Si-3: ATG13 siRNA-3 (HSS114714)

F: Representative photomicrograph of western blot analysis for ATG13 protein.

Supplemental Figure S2: A, B**:** Experimental design for the mouse experiment.

Supplementary Figure S3:

Effects of treatment with either CQ (50 μM) alone, MK2206 (9 μM) alone, or the combination of MK2206 (9 μM) + CQ (50 μM) on Annexin V-positive cells of DES and EES within the same patients (n=6).

Numerical values are presented as box and whisker plots showing medians and the smallest and largest data points ≤1.5 × IQR from the 25th and 75th percentiles, respectively.

*: $p<0.05$ versus C.

C: Control (vehicle alone)
CQ: chloroquine
MK: MK2206
MK+CQ: MK2206+ chloroquine

Supplementary Figure S4:

Effects of treatment with either vehicle alone (n=10), CQ alone (n=10), MK2206 alone (n=10), or MK2206 + CQ (n=10) on endometriotic implants in a mouse model of endometriosis.

C: Control (vehicle alone)
CQ: chloroquine
MK: MK2206
MK+CQ: MK2206+ chloroquine

A: Representative photomicrographs of immunofluorescence staining for pAKT, LC3 and p62.
Scale bar, 50 μm.

B: Expression levels for pAKT, LC3 or P62
*: $p<0.05$: versus control (vehicle alone)

§: $p<0.05$: versus MK alone and MK+CQ

¶: $p<0.05$: versus CQ alone and MK alone

Supplementary Figure S5:

Effects of ATG13, Beclin-1, and ATG12 knockdown on light chain 3 (LC3)-II and p62 protein expression in DES and EES within same patients (n=6) after treatment with vehicle alone, CQ alone, BafA1 alone, or MK2206 alone.

A-H: Representative photomicrograph of western blot analysis for LC3-II and p62 protein in DES (A, C, E, G) and EES (B, D, F, H) after treatment with vehicle alone (A, B), CQ alone (C, D), BafA1 alone (E, F) or MK2206 alone (G; H)

I-L: Relative expression level of LC3-II (I, K) and p62 (J, L) in DES (I, J) and EES (K, L)
*: $p<0.05$: versus siRNA negative control

V: Control (vehicle alone)
CQ: chloroquine
MK: MK2206
Baf: bafilomycin A1

SiNC: SiRNA negative control
SiBeclin-1: siRNA Beclin1
SiATG13: siRNA ATG13
SiATG12: siRNA ATG12

Supplementary Figure S6:
Effects of Beclin-1 knockdown on cell proliferation after treatment with CQ alone, MK2206 alone, or MK2206 + CQ in DES and EES within the same patients (n=12).
*: $p<0.05$: siBeclin-1 versus siControl

SiControl: siRNA negative control
SiBeclin-1: Beclin-1 siRNA

Cells were transfected with either small interfering RNA (siRNA) targeting Beclin-1 (siBeclin-1) or siRNA negative control (siControl)
Numerical values are presented as the mean ± SD.

C: Control (vehicle alone)
CQ: chloroquine
MK: MK2206
MK+CQ: MK2206+ chloroquine

Supplementary Figure S7:

Beclin-1, ATG12-ATG5 or ATG13 protein expression in DES (A, C, E) and EES (B, D, F) within the same patients (n=6) after transfection with either Beclin-1 siRNA, ATG12 siRNA, ATG13 siRNA or siRNA negative control.

A-F: Relative expression level of Beclin-1 (A, B), ATG12-ATG5 (C, D) and ATG 13 (E, F) protein after the 48h treatment with vehicle alone (48h) and 72-h drug (vehicle alone) discontinuation (48h+72h) in DES and EES within the same patients (n=6).

*: $p<0.05$: versus siRNA negative controls at 48h

§: $p<0.05$: versus siRNA negative controls at 48h +72h.

Numerical values are presented as the mean + SD.

SiC: SiRNA negative control (low GC duplex)

SiBec: SiRNA Beclin-1 (HSS 189498)

Si12: SiRNA ATG12 (HSS113235)

Si13: SiRNA ATG13 (HSS114713)

Representative photomicrograph of western blot analysis for Beclin-1 (A, B), ATG12-ATG5 (C, D) and ATG13 (E, F) protein.

Supplementary Figure S8:

Effects of Beclin-1, ATG12 or ATG13 knockdown on cell growth in DES and in EES within the same patients (n=6) after treatment with vehicle alone.

Percent cell proliferation was calculated as percent of vehicle control after the 48-h treatments
Numerical values are presented as the mean + SD.

SiC: siRNA negative control
SiBeclin-1: Beclin-1 siRNA
SiATG12: ATG12 siRNA
SiATG13: ATG13 siRNA

Supplementary Figure S9:

Effects of Beclin, ATG12, or ATG13 knockdown on cell growth after treatment with CQ alone, MK2206 alone or MK2206 + CQ in endometrial stromal cells of patients without endometriosis (NEES) (n=5).

SiControl: siRNA negative control
SiBeclin-1: Beclin-1 siRNA

CQ: chloroquine
MK: MK2206
MK+ CQ: MK2206 + chloroquine

Cells were transfected with either small interfering RNA (siRNA) targeting Beclin-1 (siBeclin-1) , ATG12 (siATG12), ATG13 (siATG13) or siRNA negative control (siControl)
Numerical values are presented as the mean ± SD.

Supplementary Figure S10:

Effects of Beclin-1, ATG12, or ATG13 knockdown on cell regrowth after drug discontinuation with either vehicle alone, CQ alone, MK2206 alone or MK2206 + CQ in NEES (n=5).

C: Control (vehicle alone)
CQ: chloroquine
MK: MK2206 (9 μM)
MK+ CQ: MK2206(9 μM) + chloroquine (50 μM)

Cells were transfected with either small interfering RNA (siRNA) targeting Beclin-1 (siBeclin-1), ATG12 (siATG12), ATG13 (siATG13) or siRNA negative control (siControl)
Percent cell proliferation was calculated as percent of vehicle control after the 48-h treatments (at 0h)
Numerical values are presented as the mean ± SD.

*: $p<0.05$: 0h (after the 48 h treatments) versus 72h after drug discontinuation

Supplementary Figure S11:

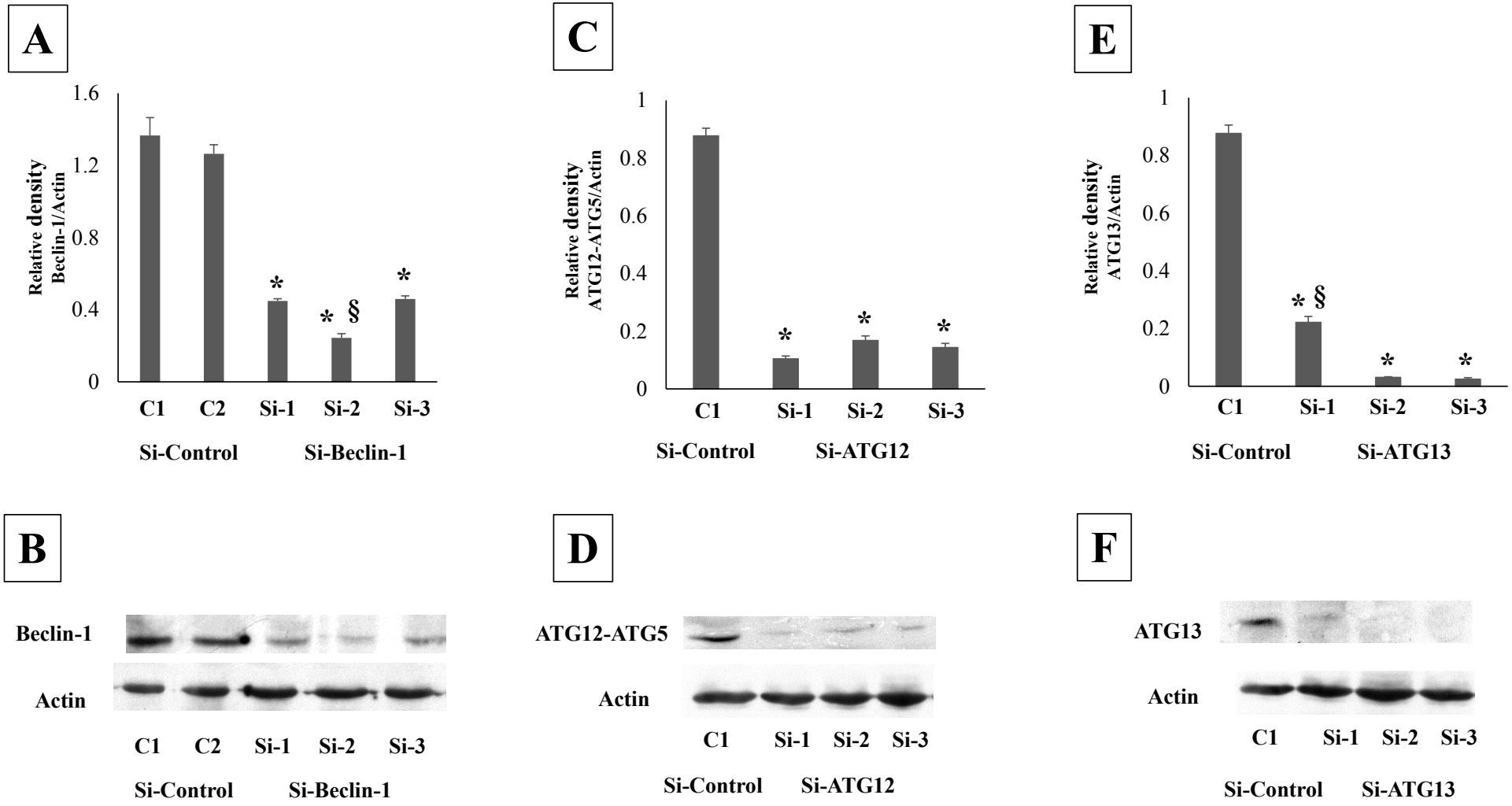
Effects of CQ alone, MK2206 alone, or MK2206 + CQ on Beclin-1 (A-D) and ATG12-ATG5 (E-H) protein expression in DES (A, B, E, F) and EES (C, D, G, H) within the same patients (n=6)

A, C, E, G: Relative expression level of Beclin-1 (A, C) and ATG12-ATG5 (E, G) protein.

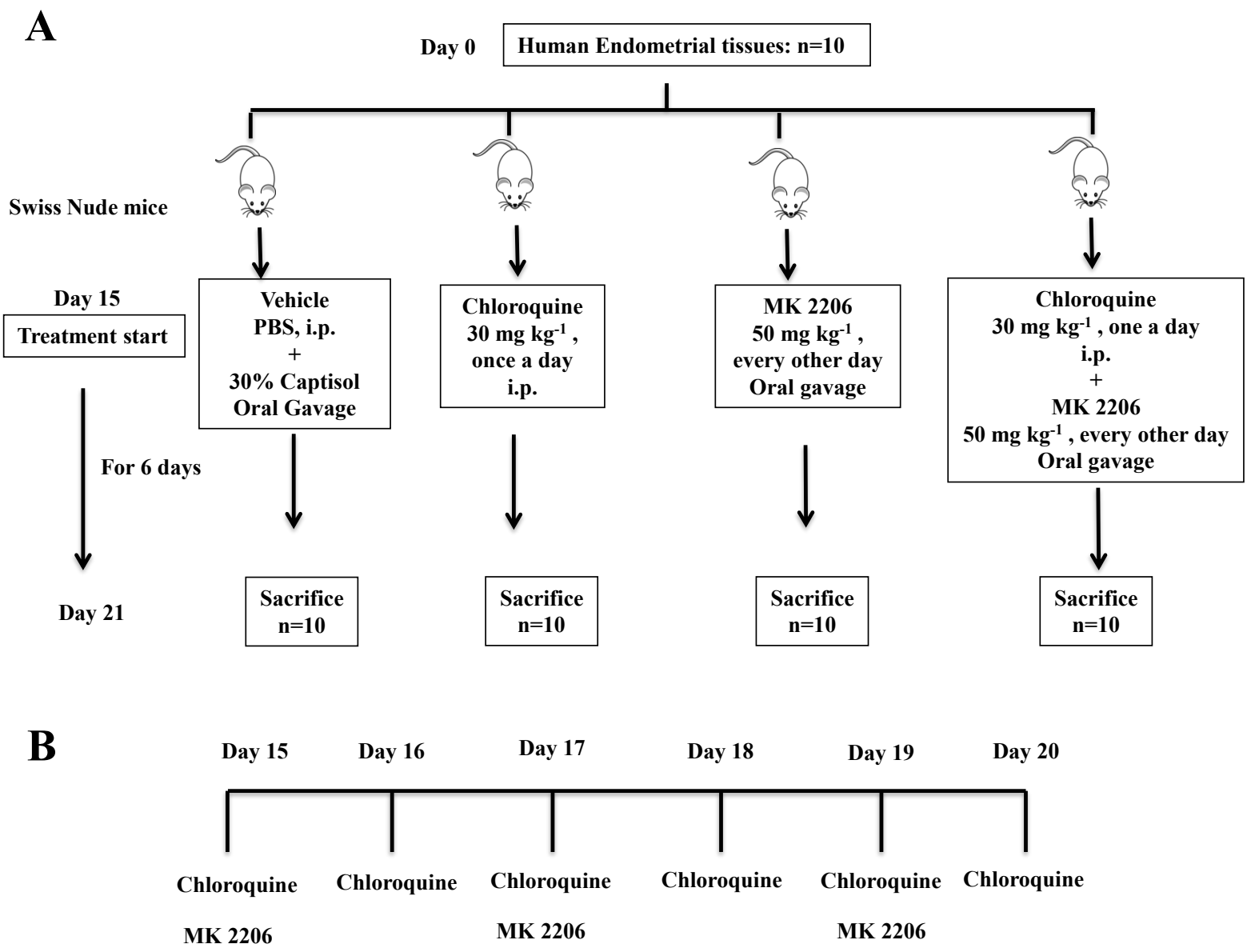
*: $p<0.05$: versus control (vehicle alone) and CQ alone

B, D, F, H: Representative photomicrograph of western blot analysis for Beclin-1(B, D) and ATG12-ATG5 (F, H) protein.

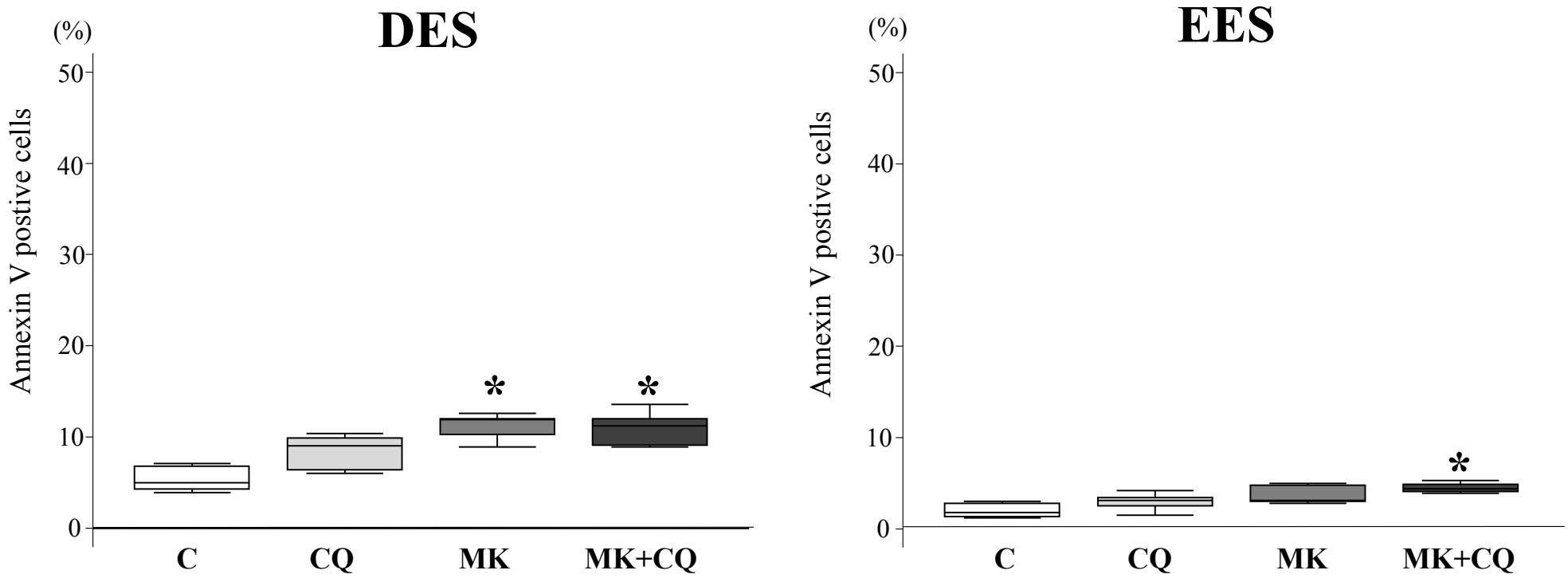
Supplementary Figure 1



Supplementary Figure 2

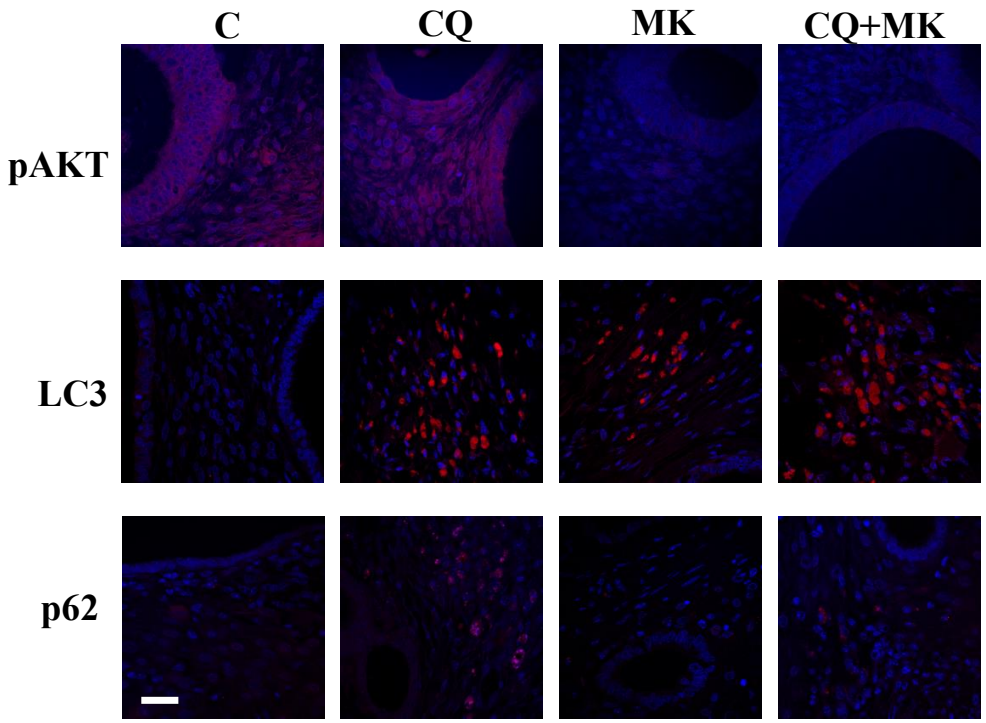


Supplementary Figure 3



Supplementary Figure 4

A



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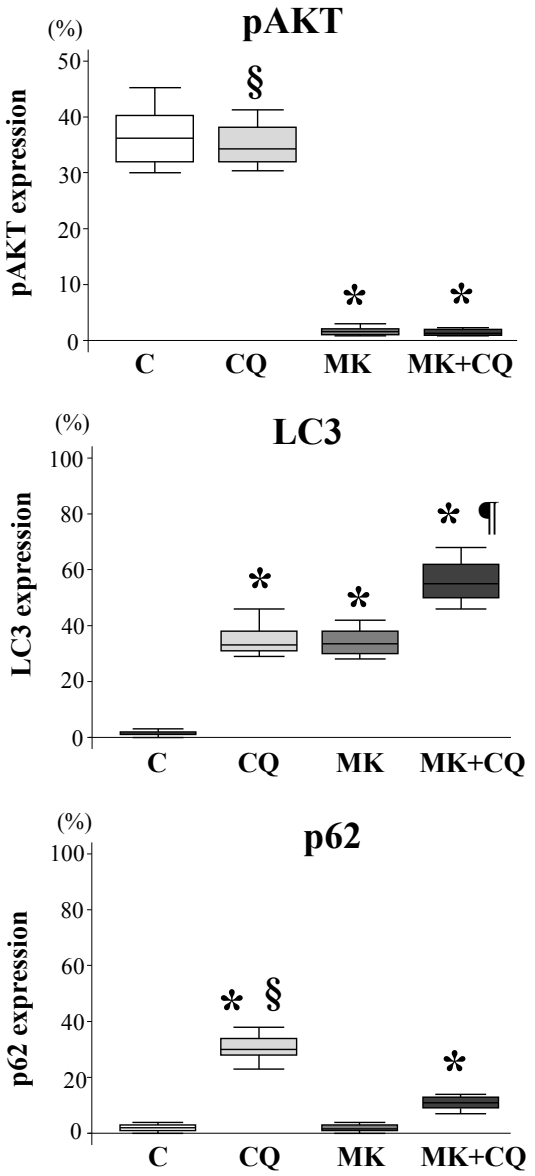

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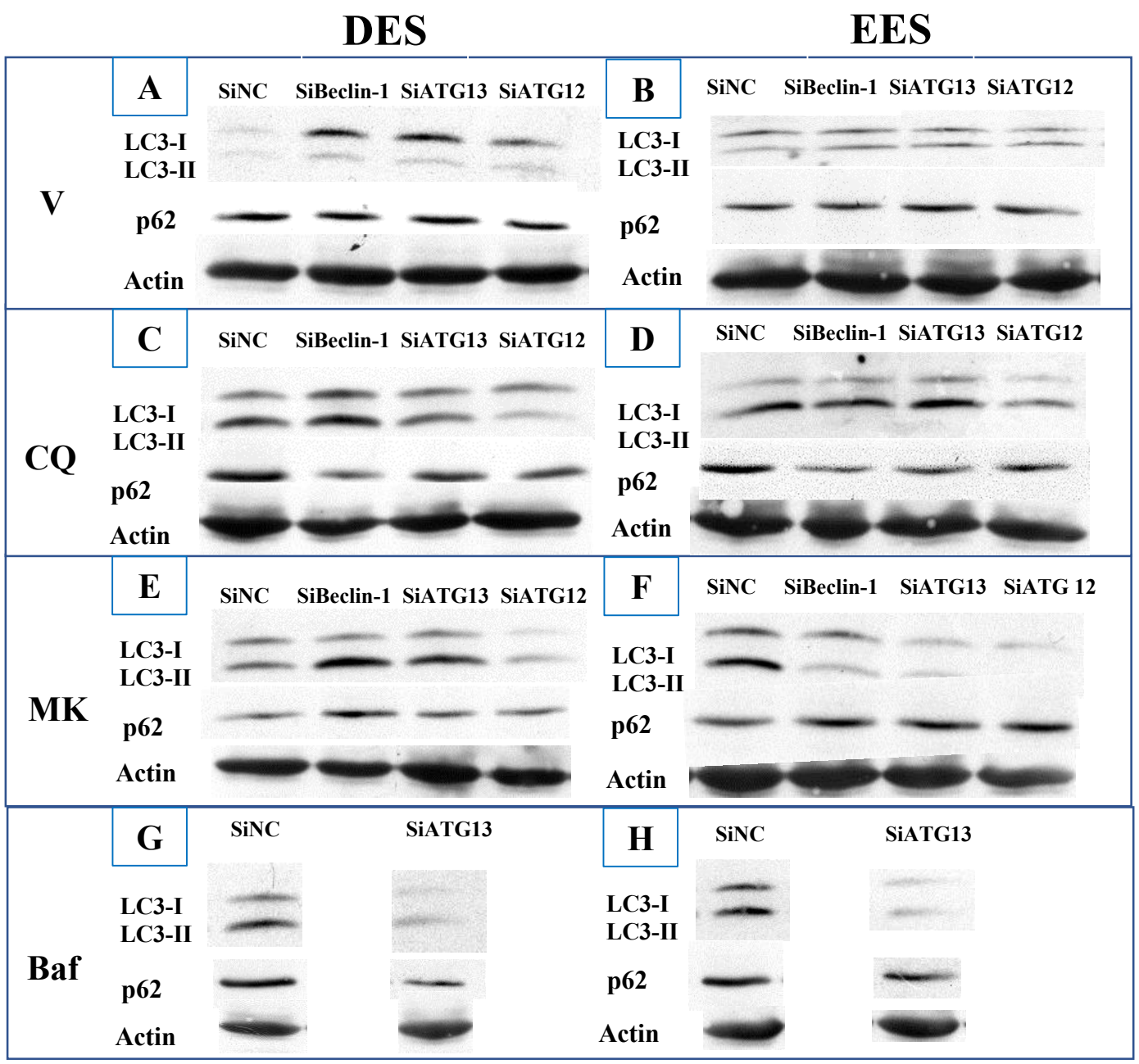

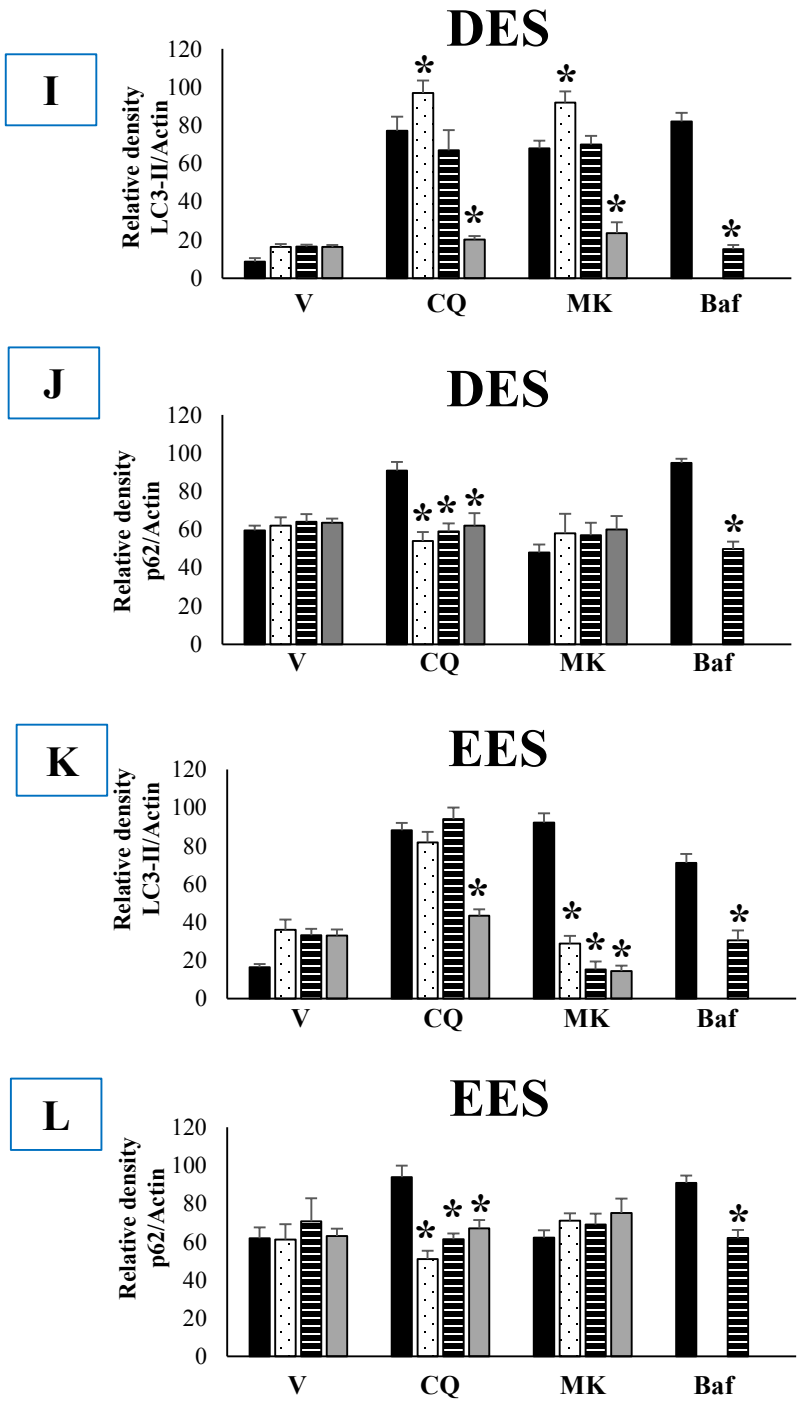

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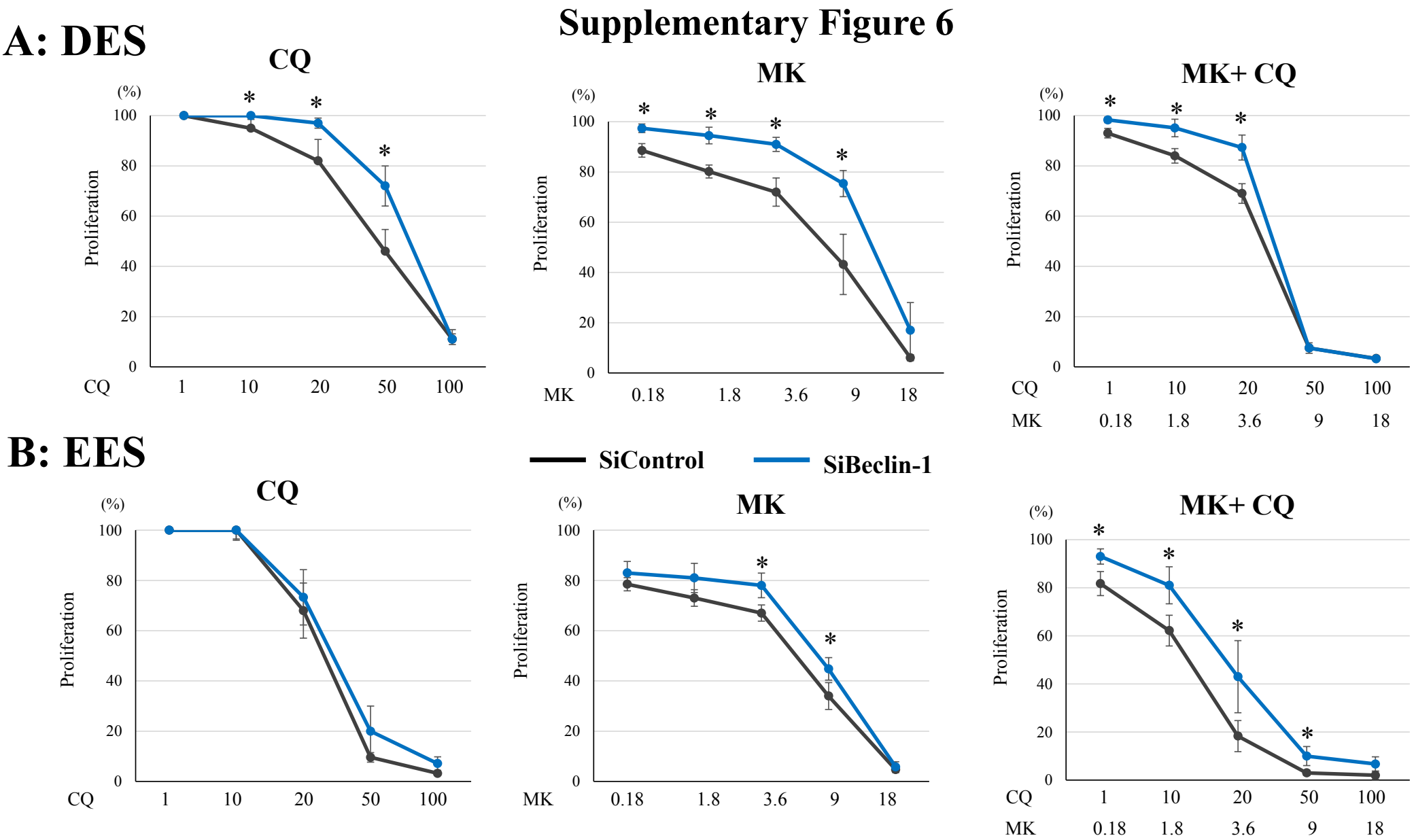
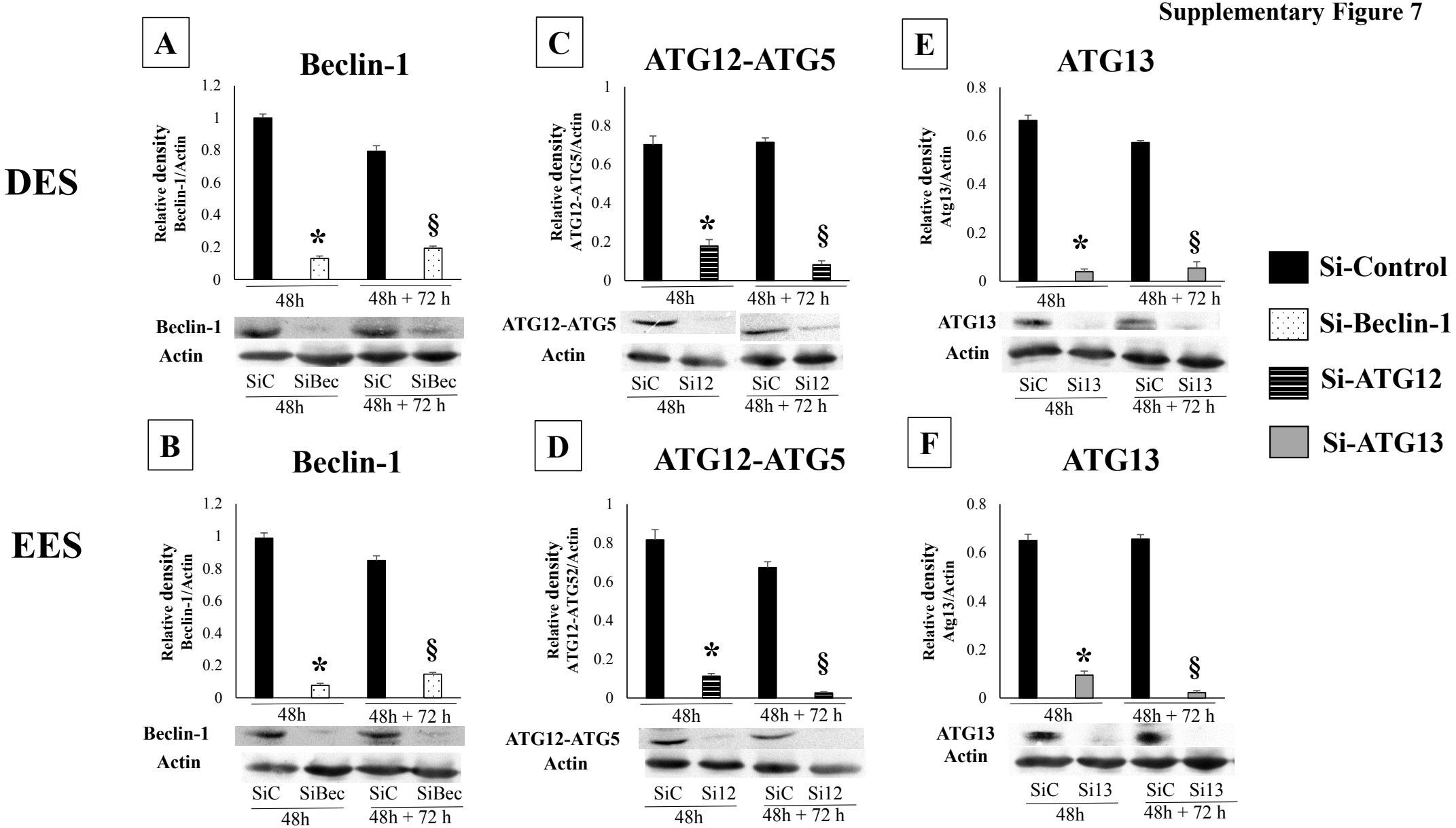
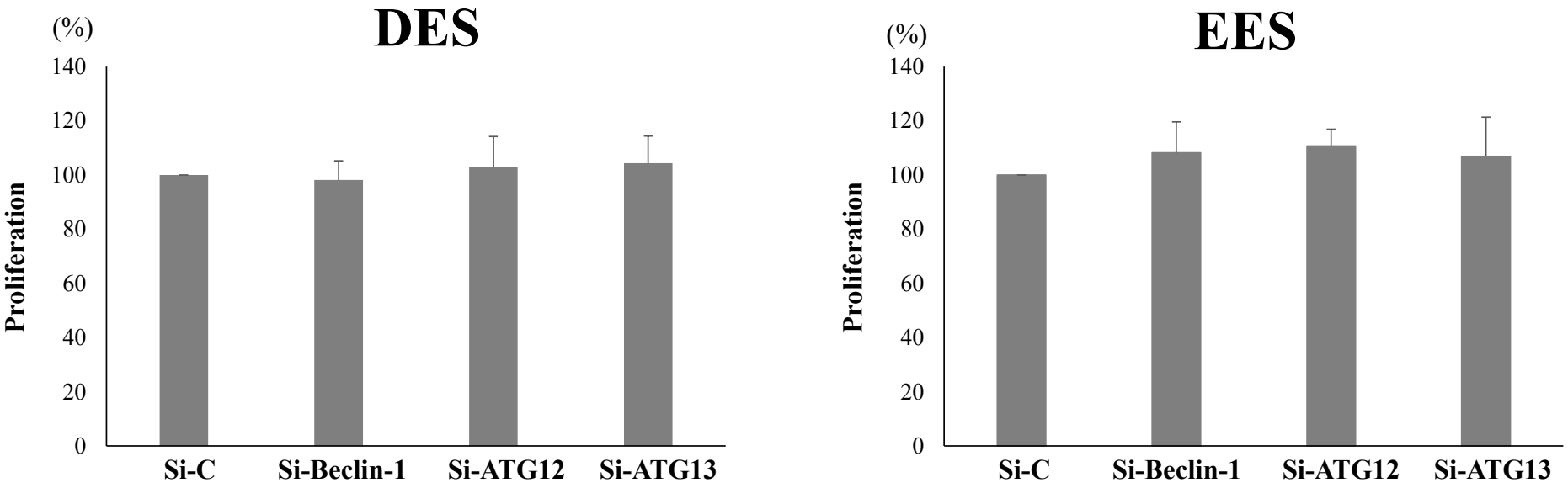


Supplementary Figure 7



Supplementary Figure 8



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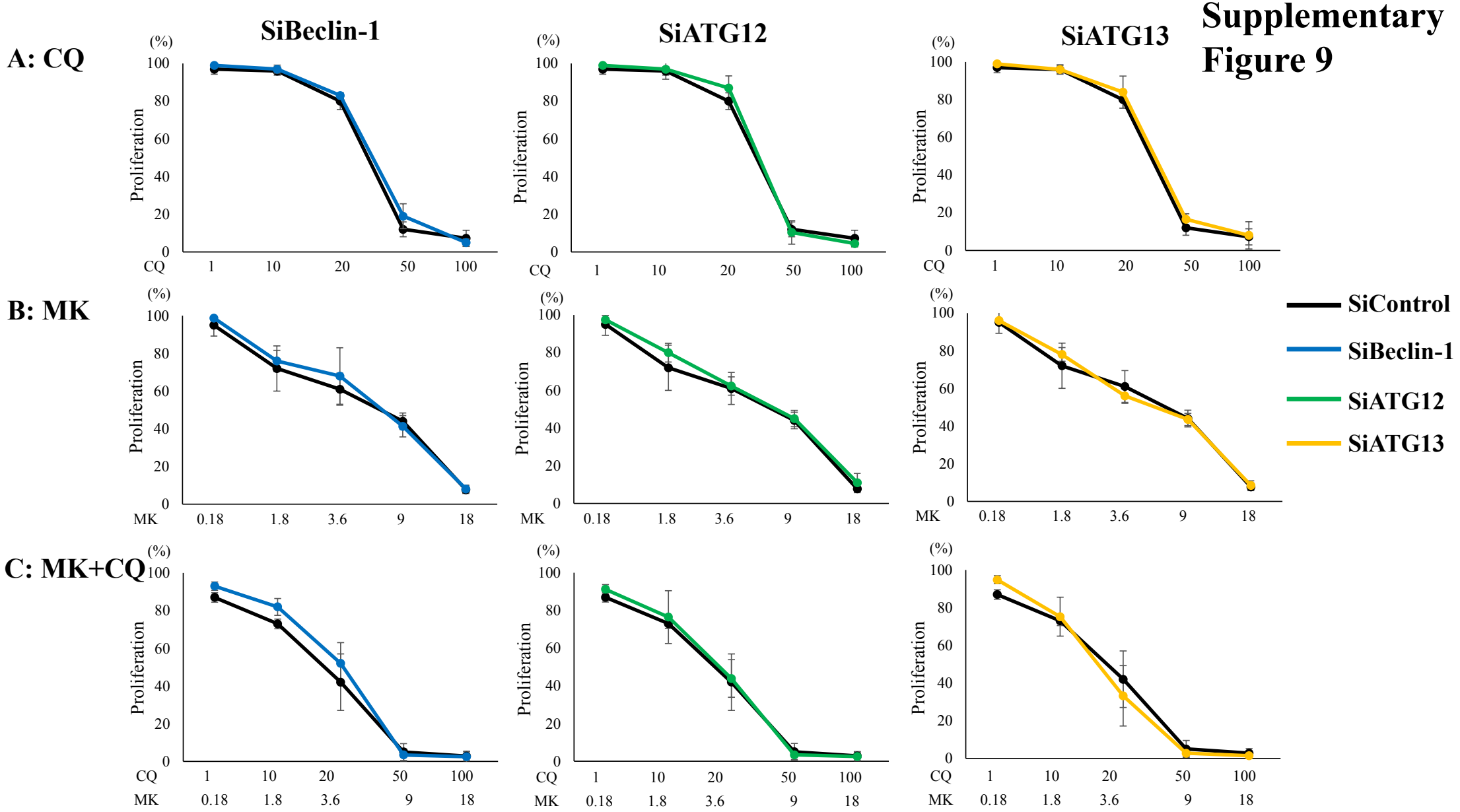
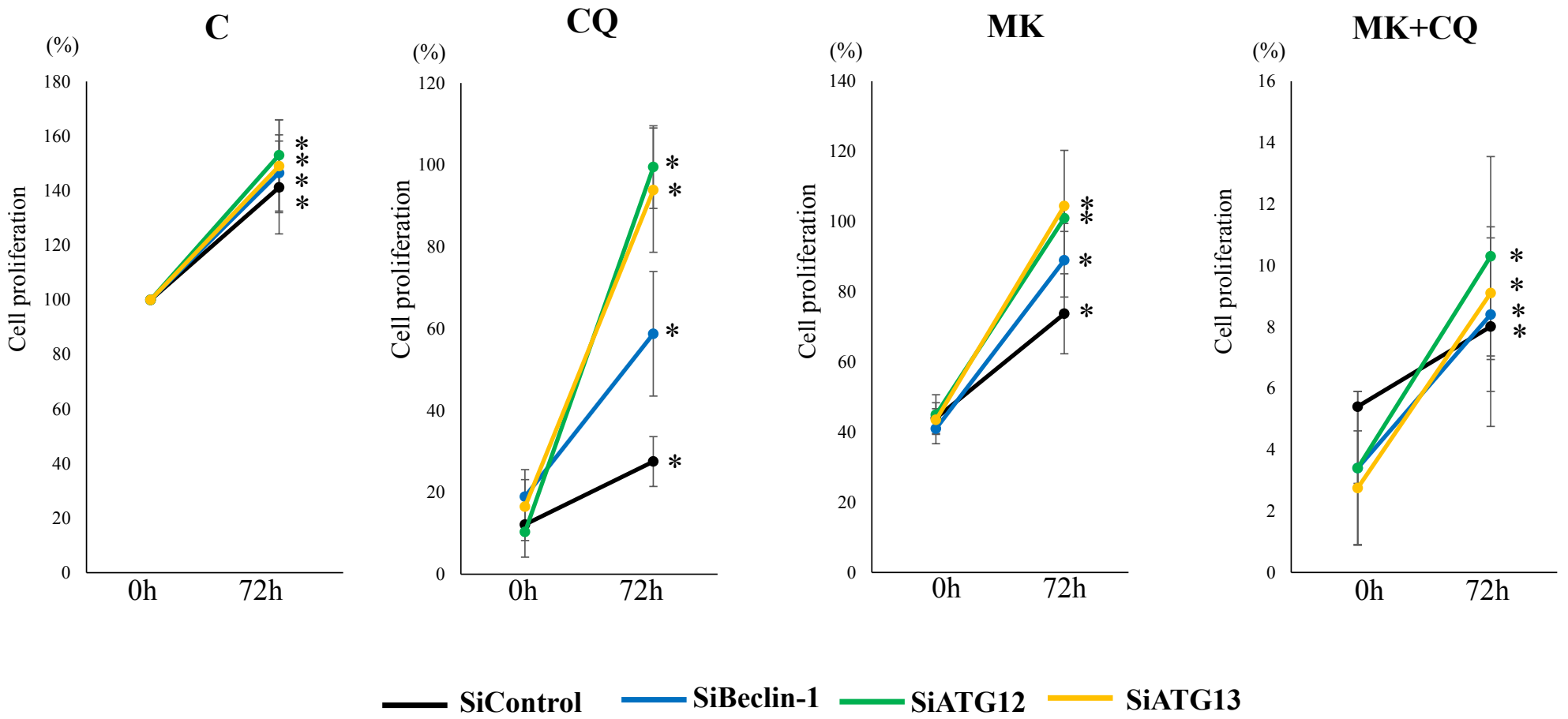
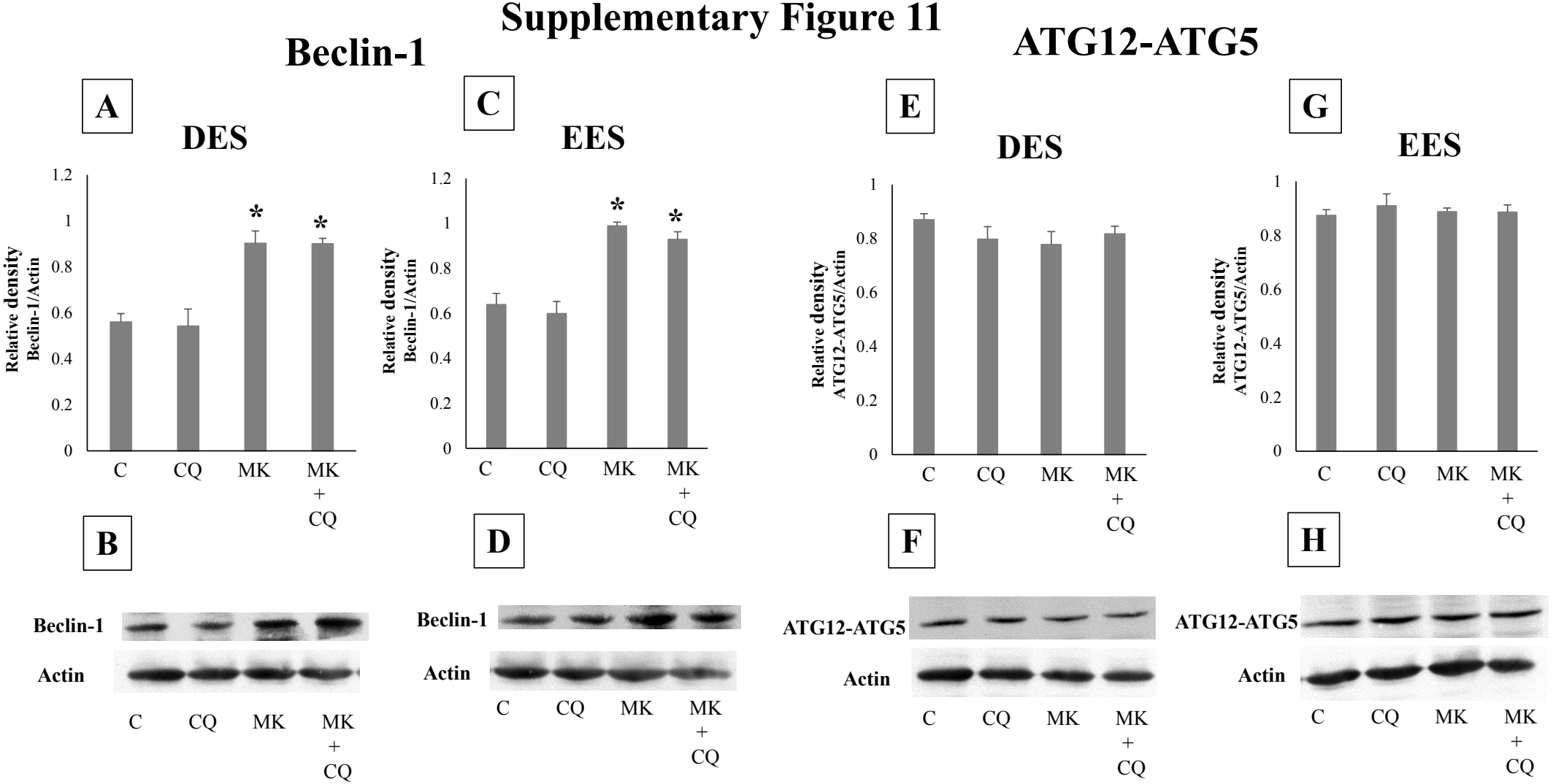


Supplementary Figure 10



Supplementary Figure 11



Supplemental Table S1. Clinical characteristics of patients

	Endometriosis	Tubal infertility
No of cases	60	15
Age [a]	32.0 (20-37)	30.0 (21-34)
Parity [a]	0 (0-1)	0 (0-1)
rASRM stage[b]		
I	20	
II	13	
III	12	
IV	15	

[a] Median (range)

[b] Revised American Society for Reproductive Medicine classification (rASRM) (American Society for Reproductive Medicine, 1997).

Supplemental Table S2: The numbers of samples of DES, EES, and/or NEES used for each experiment

	Samples			
	DES	EES	EEE	NEES
Effects of CQ alone, MK2206 + CQ, and U0126 + MK2206 + CQ on cell growth and regrowth	12	16		
Effects of CQ alone, MK2206 alone and MK2206 + CQ on Annexin V expression	6	6		
Clonogenic assay	10	10		
Mouse model of endometriosis		10		
Effects of MK2206 + CQ/MK2206+BafA1 on LC3-II and p62 protein expression/LC3B and p62 staining	6	6		
Effects of combination treatment of MK2206 with pharmacologic autophagy inhibitors (CQ, BafA1 or 3-MA)	12	12		10
Effects of Beclin-1 knockdown on cell growth	12	12		

Effects of Beclin-1, Atg12 and Atg13 knockdown on cell growth and regrowth	6	6		5
IC_{50} for CQ alone, MK2206 alone or MK2206 + CQ in EEE versus EES		6	6	

DES: deep infiltrating endometriotic stromal cells
EES: endometrial stromal cells of patients with endometriosis
EEE: endometrial epithelial cells of patients with endometriosis
NEES: endometrial stromal cells of patients without endometriosis
CQ: chloroquine
MK: MK2206
BafA1: bafilomycin A1
3-MA: 3-methyalanine
LC3-II protein: light chain 3-II protein

Supplementary Table S3: IC_{50} for CQ alone, BafA1 alone, MK2206 alone, MK2206 + CQ, or MK2206 + BafA1 in DES versus EES within the same patients (n=12).

	IC_{50}		
	DES	EES	p-value
CQ (μM) alone	44.7 (63.8)	35.4 (20.6)	<0.05
MK (μM) alone	14.4 (12.1)	3.8 (3.7)	<0.05
MK+CQ			
CQ (μM)	20.3 (4.4)	5.4 (3.6)	<0.05
MK (μM)	3.6 (0.79)	1.0 (0.7)	<0.05
BafA1 (nM) alone	46.6 (16.2)	26.0 (23.4)	<0.05
MK+BAfA1			
BafA1 (nM)	15.5 (12.4)	2.6 (2.5)	<0.05
MK (μM)	14.0 (14.7)	2.4 (2.2)	<0.05

CQ: chloroquine
MK: MK2206
BafA1: bafilomycin A1
DES: deep endometriotic stromal cells
EES: endometrial stromal cells of patients with endometriosis

Numerical values are presented as the median (interquartile range [IQR])

Supplementary Table S4: IC_{50} for CQ alone, MK2206 alone or MK2206 + CQ in EEE versus EES within the same patients (n=6).

	IC_{50}		
	EEE	EES	p-value
CQ (μM) alone	58 (24.2)	41 (20.0)	<0.05
MK (μM) alone	3.0 (0.9)	3.6 (2.2)	0.17
MK+CQ			
CQ (μM)	3.1 (1.5)	6.8 (1.3)	<0.05
MK (μM)	0.8 (0.3)	1.7 (0.9)	<0.05

CQ: chloroquine

MK: MK2206

EEE: endometrial epithelial cells of patients with endometriosis derived from the proliferative phase

EES: endometrial stromal cells of patients with endometriosis derived from the proliferative phase

Numerical values are presented as the median (interquartile range [IQR])