Supplementary information:

Autocrine Bone Morphogenetic Protein 9 signals via Activin Receptor Like Kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation

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Supplementary Figure legends

Supplementary Figure 1.

IOSE397 cells were transiently transfected with pGL3(BRE)-luciferase reporter gene and EF-LacZ and treated with different concentrations of BMP9 for 15 hours (0.1% FBS). Normalised luciferase activity is shown as fold induction (relative to 0 ng/ml). Data from 2 independent experiments performed in triplicate (mean ± S.E.M.).

Supplementary Figure 2.

(*A, B, C*) IOSE397 cells were transfected with siRNA oligonucleotides in parallel to the experiment shown in Fig. 2*A* and RNA was isolated after 48 hours. The levels of ALK1 (**A**), ALK2 (*B*) and ALK5 (*C*) were determined by qRT-PCR and normalized to β-actin. Data are from 1 representative experiment out 3 with three determinations, expressed relative to mock treated cells (assigned an arbitrary value of 1) and are displayed as mean ± S.D. (*D*) IOSE397 cells were tranfected with ALK2 siRNA oligonucleotides in parallel to the experiment shown in Fig. 2*B* and RNA was isolated

after 48 hours. The levels of ALK2 were determined by qRT-PCR and normalized to β-actin. Data from 1 representative experiment with three determinations, expressed relative to mock sample (assigned an arbitrary value of 1) and are displayed as mean ± S.D. (*E*) IOSE397 cells were transfected with ALK2 siRNA oligonucleotides in parallel to the experiment shown in Supplementary Fig. 2*F* and RNA was isolated after 48 hours. The levels of ALK2 were determined by qRT-PCR and normalized to β-actin. Data are from 3 independent experiments with 3 determinations, expressed relative to mock sample (assigned an arbitrary value of 1) and are displayed as mean ± S.E.M. (*F*) IOSE397 cells were transiently transfected with siRNA oligonucleotides as indicated and treated for 1 hour -/+ 5 ng/ml BMP9 and Id1 levels were analysed by qRT-PCR and normalized to β-actin. Fold changes relative to mock transfected untreated samples are shown (mean ± S.E.M, n=3). (*G, H, I)* IOSE397 cells were transfected with siRNA oligonucleotides in parallel to the experiment shown in Fig. 2*D* and RNA was isolated after 48 hours. The levels of ActRIIA (*G*), ActRIIB (*H*) and BMPRII (*I*) were determined by qRT-PCR and normalized to β-actin. Data are from 4 independent experiments with 3 determinations, expressed relative to mock sample (assigned an arbitrary value of 1) and are displayed as mean ± S.E.M. Statistical analysis was carried out using paired t-test, * = $P< 0.05$.

Supplementary Figure 3.

ALK1 and ALK2 RNA levels of HUVEC, IOSE397, TR175 and OVCA433 cell lines were analysed by qRT-PCR and normalized to β-actin. (*A*) For each cell line, ALK1 RNA levels are expressed relative to ALK2 RNA levels (assigned an arbitrary value of 1). (*B*) ALK1 and ALK2 RNA of IOSE397, TR175 and OVCA433 cell lines

were expressed relative to ALK1 and ALK2 RNA levels of HUVEC cells (assigned an arbitrary value of 1). Data are from 3 determinations and are displayed as mean ± S.D.

Supplementary Figure 4.

Proliferation curve of IOSE397 cells incubated for different periods of time without or with BMP9 (5ng/ml) in media supplemented with 5% FBS. Data are from 3 independent experiments performed in triplicate (mean ± S.E.M.).

Supplementary Figure 5.

(*A-B*) TR175 and OVCA433 cells were transiently transfected with a non silencing siRNA oligonucleotide (N.S.) or with two different ALK2 siRNA oligonucleotides or mock transfected. 24 hours after transfection, cells were cells were serum starved in 0.1% FBS medium and treated without or with BMP9 (5ng/ml) for 1 hour. Whole cell extracts were obtained and fractionated by SDS polyacrylamide gel electrophoresis and western blots were performed with antibodies that recognized phosphorylated Smad1,5,8, and Smad2/3. (*C-D*) TR175 and OVCA433 cells were transfected with ALK2 siRNA oligonucleotides in parallel to the experiment shown in supplementary Fig. 5*A, B.* The levels of ALK2 were determined by qRT-PCR. Data are from 1 representative experiment with 3 determinations, expressed relative to mock sample (assigned an arbitrary value of 1) and are displayed as mean ± S.D.

Supplementary Figure 6.

(*A*) OVCA433 cells were transfected with siRNA oligonucleotides in parallel to the experiment shown in Fig. 3*B, left panel* and RNA was isolated after 48 hours. The

levels of ALK2 were determined by qRT-PCR and normalized to β-actin. Data from 3 determinations, expressed relative to non silencing sample (assigned an arbitrary value of 1), (mean ± S.D.). (*B*) IOSE397 cells were transfected with pGL3(BRE)-luciferase reporter gene and EF-LacZ (internal control). Cells were serum starved in 0.1% FBS media and treated for 15 hours with different concentrations dorsomorphin (Dm) in absence or presence of BMP9 (5ng/ml). Luciferase activity was measured and normalized to β-Gal activity. Data are from 1 representative experiment performed in triplicate, expressed as fold induction relative to untreated samples and are displayed as mean ± S.D. (*C, D*), OVCA433 cells were transfected with siRNA oligonucleotides in parallel to the experiment shown in Fig. 3*C* and RNA was isolated after 48 hours. The levels of ActRIIA (*C*) and BMPRII (*B*) were determined by qRT-PCR and normalized to β-actin. Data are from one representative experiment out of 2 with 3 determinations, expressed relative to N.S. sample (assigned an arbitrary value of 1) and are displayed as mean ± S.D. (*E, F, G*) OVCA433 cells were transfected with siRNA oligonucleotides in parallel to the experiment shown in Fig. 3*D* and RNA was isolated after 48 hours. The levels of Smad1 (*E*), Smad4 (*F*) and Smad3 (*G*) were determined by qRT-PCR and normalized to β-actin. Data from 1 representattive experiment out of 5, with 3 determinations, expressed relative to non silencing sample (assigned an arbitrary value of 1), (mean ± S.D). (*H, I, J*) OVCA433 cells were transfected with siRNA oligonucleotides in the same conditions than the experiment shown in Fig. 3*D*. Whole cell extracts were fractionated by SDS polyacrylamide gel electrophoresis and western blots were performed with antibodies that recognized Smad1 (*H*), Smad4 (*I*) and Smad3 (*J*). β-tubulin was analysed as loading control. The amount of Smad protein relative to the non silencing control

(assigned an arbitrary value of 100) was determined by densitometry and is displayed on the figure.

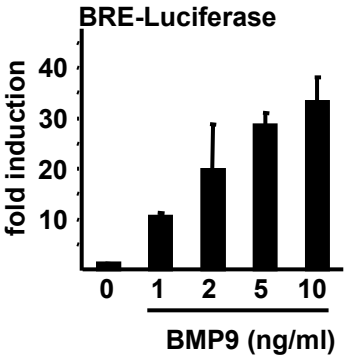
Supplementary Figure 7.

(*A*) IOSE397 cells were transfected with pGL3(BRE)-luciferase reporter gene and EF-LacZ (internal control). Cells were serum starved in 0.1% FBS media and treated for 15 hours with different concentrations of ALK1ecd (fold molar excess concentration, F.M.E.) and in absence or presence of BMP4 (10ng/ml). Luciferase activity was measured and normalized to β-Gal activity. Data are from 1 representative experiment performed in triplicate and are displayed as means ± S.D. (*B*) IOSE397 cells were treated as in (*A*) and then incubated for 15 hours with different concentrations of ALK3ecd (F.M.E.) and in absence or presence of BMP9 (5ng/ml). Luciferase activity was measured and normalized to β-Gal activity. Data are from 1 representative experiment performed in triplicate and are displayed as mean ± S.D. (*C*) IOSE397 cells were incubated for 1 hour in absence or presence of different concentrations ALK3ecd (F.M.E.) and in absence or presence of BMP9 (5ng/ml) for in 5% FBS medium. Whole cell extracts were fractionated by SDS polyacrylamide gel electrophoresis and western blots were performed with antibodies that recognized P-Smad1,5,8 and Smad1. (*D*) IOSE397 cells were treated as in (*A*) and then incubated for 15 hours with different concentrations of ALK1ecd (F.M.E.) and in absence or presence of BMP9 (5ng/ml). Luciferase activity was measured and normalized to β-Gal activity. Data are from one representative experiment performed in triplicate and are displayed as mean ± S.D. (*E*) IOSE397 cells were treated as in (*C*) and then incubated for 1 hour in absence or presence of different concentrations ALK1ecd (F.M.E.) and in absence or presence of BMP9 (5ng/ml) in 5% FBS media. Whole cell

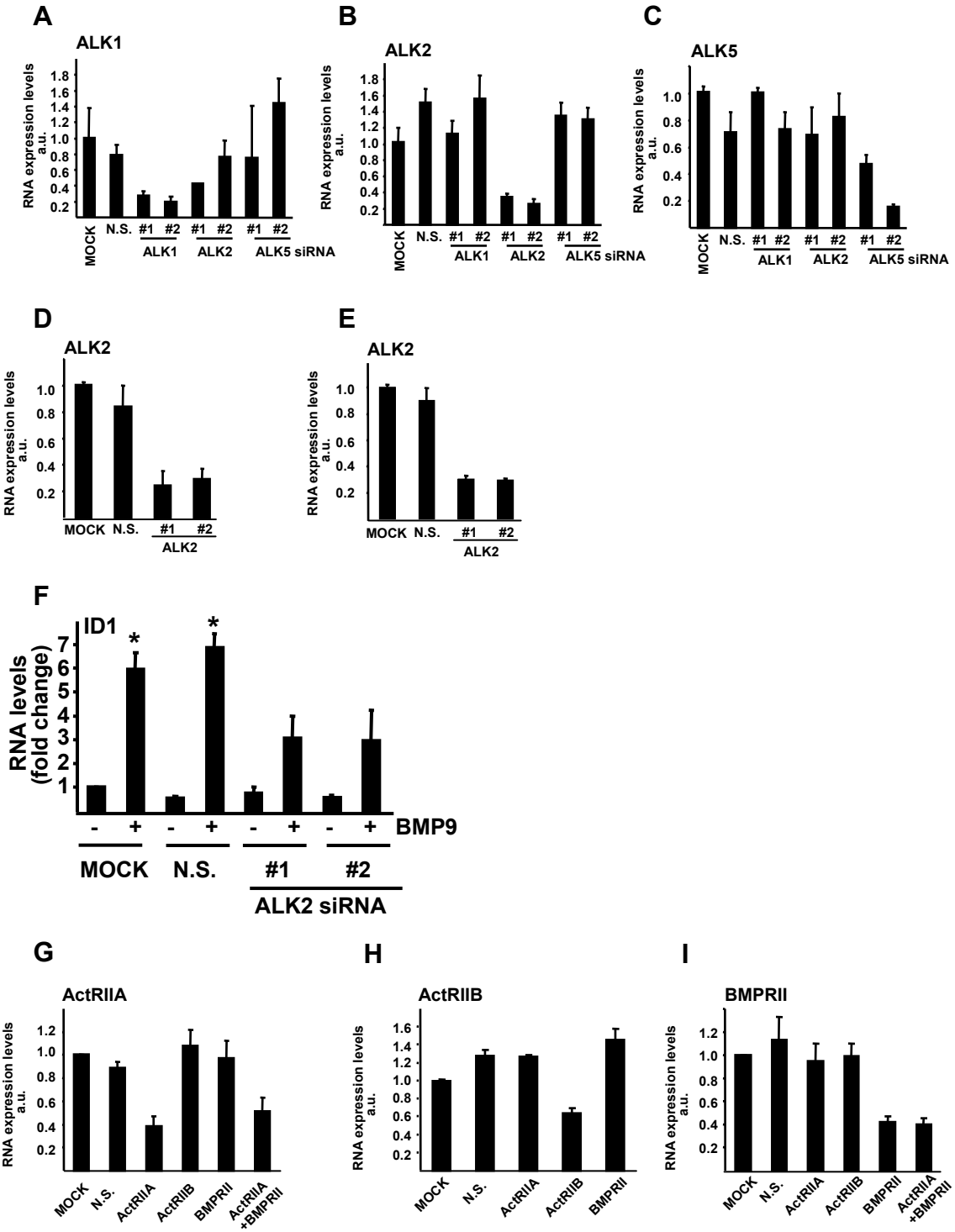
extracts were fractionated by SDS polyacrylamide gel electrophoresis and western blots were performed with antibodies that recognized Id1 and Smad2/3. (*F*) OVCA433 cells were incubated with mouse IgG or mouse monoclonal BMP9 blocking antibody (0.5ng/ml) in 10% FBS medium and were counted at day 6. Data are from one representative experiment performed in triplicate, out of 3 independent experiments and are displayed as percentage of mouse IgG treated cells, mean ± S.D. Statistical analysis was carried out using paired t-test, * = $P< 0.05$.

Supplementary Figure 8.

(*A,B,C*) IOSE397, TR175 and OVCA433 cells were transfected with siRNA oligonucleotides in parallel to the experiment shown in Fig. 5*B right panel* and RNA was isolated after 48 hours. The levels of BMP9 in IOSE397 (*A*), TR175 (*B*) and OVCA433 (*C*) were determined by qRT-PCR and normalized to β-actin. Data expressed relative to non silencing treated cells (assigned an arbitrary value of 1), from three determinations, (mean ± S.D). (*D ,E*) The knockdown levels of BMP9 were determined by qRT-PCR in parallel to the experiments shown in Fig. 5*C* of stable cell lines (*D*) TR175 and (*E*) OVCA433 expressing non-silencing (N.S) and two different BMP9 shRNAs. Data expressed relative to cells expressing N.S. (assigned an arbitrary value of 1), from 3 determinations, (mean ± S.D.). (*F,G*) TR175 and OVCA433 cells were transfected with siRNA oligonucleotides in parallel to the experiment shown in Fig. 5*D* and RNA was isolated after 48 hours. The levels of ALK2 in TR175 (*F*) and OVCA433 (*G*) were determined by qRT-PCR and normalized to β-actin. Data expressed relative to non silencing treated cells (assigned an arbitrary value of 1) from three determinations, (mean ± S.D).

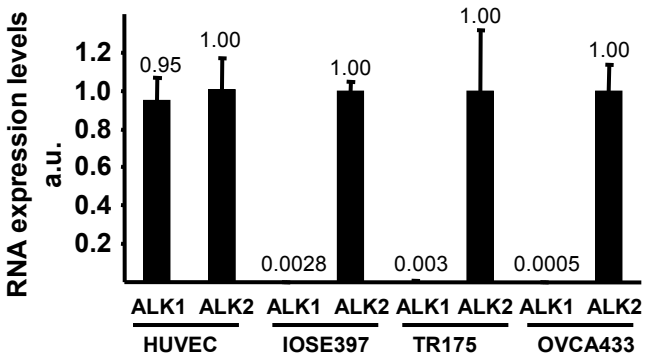


Herrera et al. Supplementary Figure 1



Herrera et al. Supplementary Figure 2

A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0.6
0.4
0.2
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0.0028
1.00
0.003
1.00
0.0005
1.00
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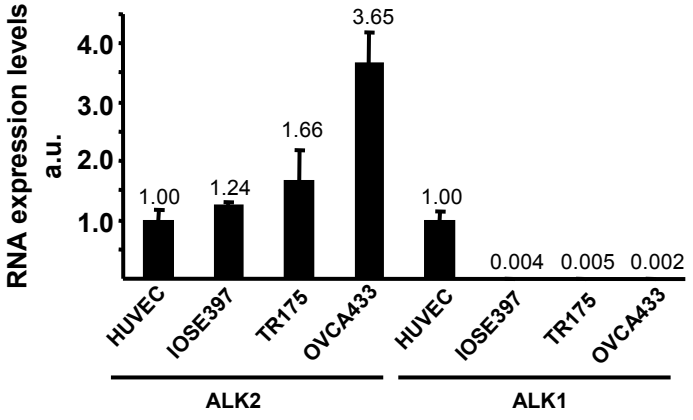
HUVEC
IOSE397
TR175
OVCA433

B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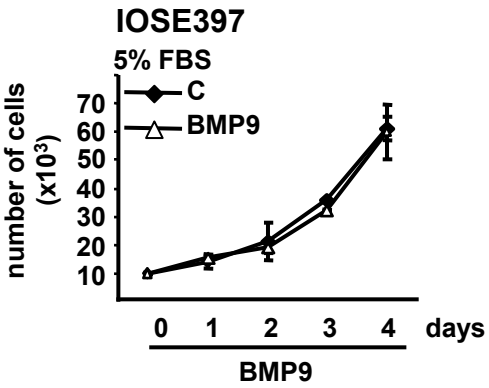
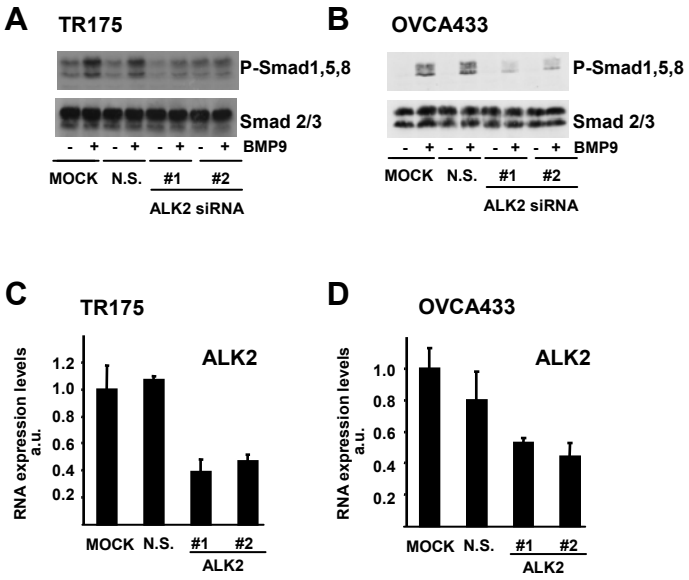
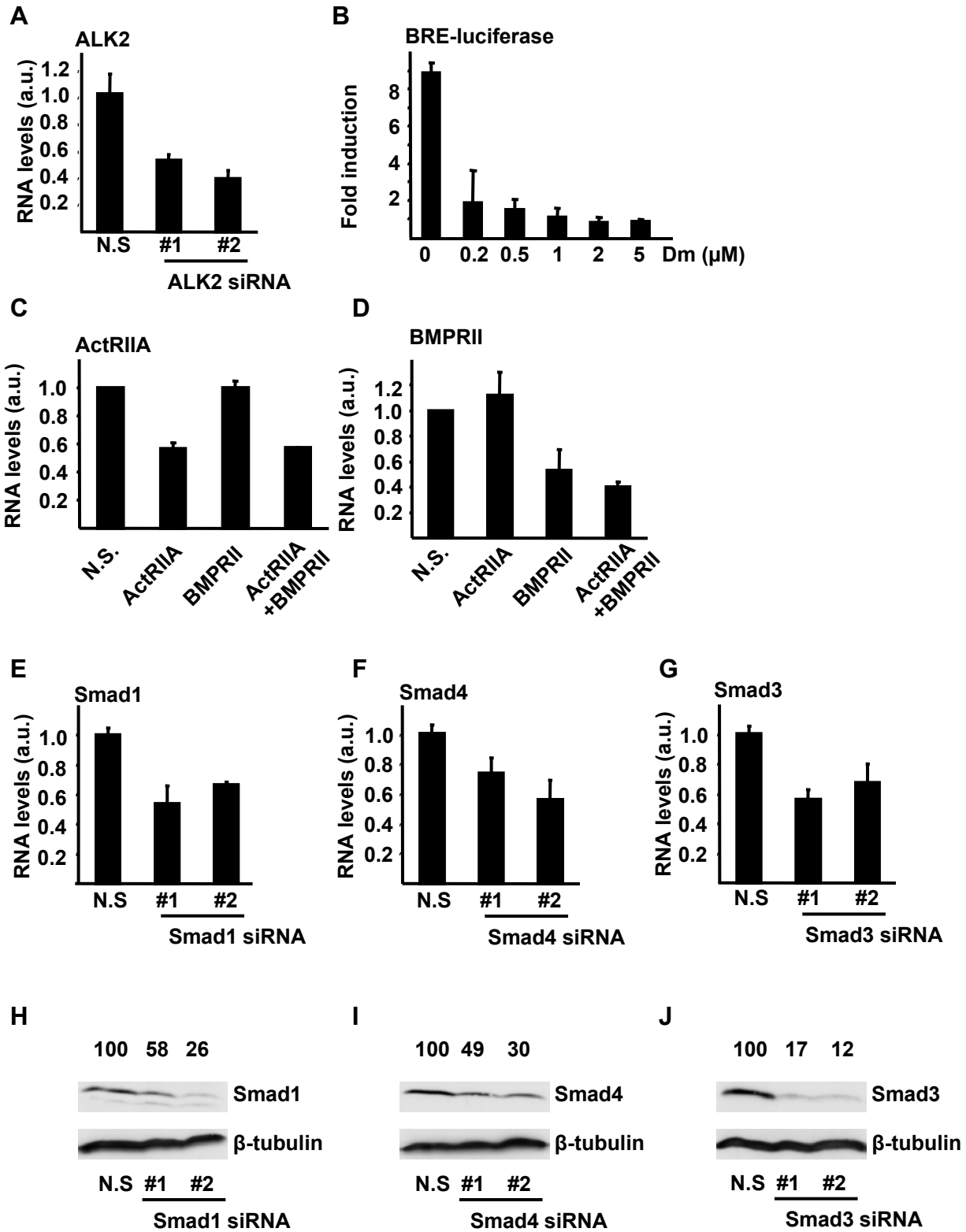
3.0
2.0
1.0
1.00
1.24
1.66
3.65
1.00
0.004
0.005
0.002
HUVEC
IOSE397
TR175
OVCA433
HUVEC
IOSE397
TR175
OVCA433
ALK2
ALK1

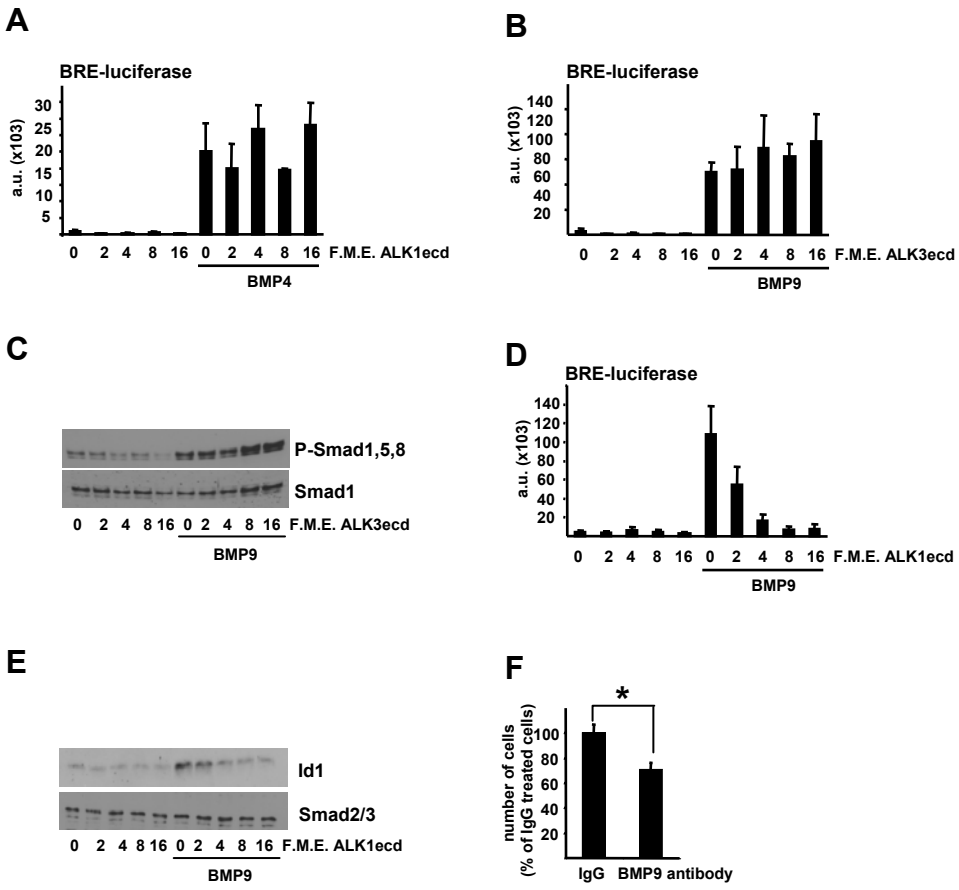

IOSE397
5% FBS
C
BMP9
number of cells
(x10³)
70
60
50
40
30
20
10
0
1
2
3
4
days
BMP9



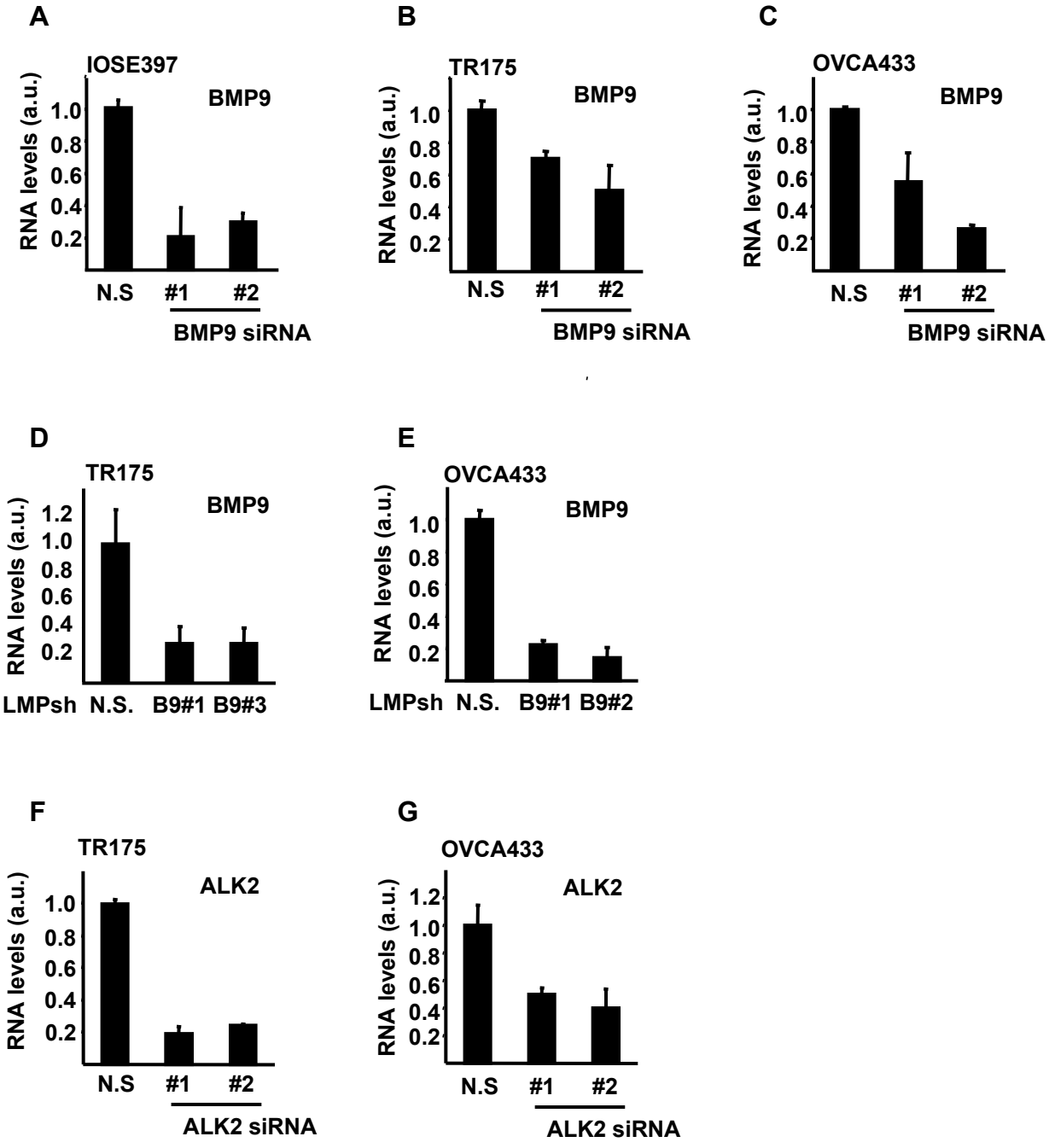
Herrera et al. Supplementary Figure 5



Herrera et al. Supplementary Figure 6



Herrera et al. Supplementary Figure 7



Herrera et al. Supplementary Figure 8

	FORWARD	REVERSE	PB	Annealing T[a]
ALK1	CCTGGACATCGGCAACAA	GGGGTCATTGGGCACCACA	216	60
ALK2	TGAGGAAGGTAGTCTGTGTGGAT	CACTGACTTAATGCCCAGATCTC	517	65
ALK3	CATCATGGCTGACATCTACAGC	TAGAAGTTCCCAGCTTGTATCC	508	66
ALK4	TCTGCTGTGTGCGTGCACCA	CCAGTCTCTGGCGGTTGTGATAGA	395	66
ALK5	TGAATCCTTCAAACGTGCTGA	TCCTTCTGTTCCCTCTCAGTGAGGTA	435	62
ALK6	CCTTATCATGACCTAGTGCCCAGTGAC	TCTCCGTCCTACAAACAGACATGGATC	450	63
ALK7	TGCCTTATTATGACATGGTGCCTT	GCATCACCTAAAGATAGCATGC	670	59
SMAD1	CTACCATCATGGATTTCATCCTACT	TGTCTGACTCATCCATCCTTCAAG	382	53
SMAD2	AGATCAGTGGGATACAACAGG	GGCACTAATACTGGAGGCAA	264	63
SMAD3	GAAGCAAAGATCTGTTTCATTCTT	AGACCTTTCCAACTTTCTTAAA	704	56,5
SMAD4	ACCTGGAGATGCTGTTCA	TGTCTTGGGTAATCCGGTC	285	63
SMAD5	GCAGCATATTTGTACAGAGTAG	TTCTAAGAGTTAATCACATGAAC	546	59,6
SMAD8	AGATCCCCAGCGGCTGCAGCC	TTGACAATATCGCCTCTCAA	548	56,5
β-ACTIN	AAGATCAAGATCATTGCTCCTCCT	TCATAGTCCGCCTAGAAGCA	121	55

Supplementary Table 1: Sense and antisense primers used in RT-PCR analysis

	FORWARD	REVERSE
Non silencing	TCGAGAAGGTATATTGCTGTTGACA GTGAGCGATCTCGCTTGGGCGAGAG TAAGTAGTGAAGCCACAGATGTACT TACTCTCGCCCAAGCGAGAGTGCCT ACTGCCTCGG	AATTCCGAGGCAGTAGGCACTTAC TCTCGCCCAAGCGAGATTACATCT GTGGCTTCACTAACTCTCGCTTGG GCGAGAGTAAGCGCTCACTGTCAA CAGCAATATACCTTC
shBMP9#1	TCGAGAAGGTATATTGCTGTTGACA GTGAGCGAAAGGACTGATTCAATCT GCATTAGTGAAGCCACAGATGTAAT GCAGATTGAATCAGTCCTTGTGCCTA CTGCCTCGG	AATTCCGAGGCAGTAGGCACAAG GACTGATTCAATCTGCATTACATC TGTGGCTTCACTAATGCAGATTGA ATCAGTCCTTTCGCTCACTGTCAA CAGCAATATACCTTC
shBMP9#2	TCGAGAAGGTATATTGCTGTTGACA GTGAGCGAATGGAAGATGCCATCTC CATATAGTGAAGCCACAGATGTATA TGGAGATGGCATCTTCCATGTGCCTA CTGCCTCGG	AATTCCGAGGCAGTAGGCACATG GAAGATGCCATCTCCATATACATC TGTGGCTTCACTATATGGAGATGG CATCTTCCATTCGCTCACTGTCAA CAGCAATATACCTTC
shBMP9#3	TCGAGAAGGTATATTGCTGTTGACA GTGAGCGCCAACAGGTACACGTCCG ATAATAGTGAAGCCACAGATGTATT ATCGGACGTGTACCTGTTGTTGCCTA CTGCCTCGG	AATTCCGAGGCAGTAGGCAACAA CAGGTACACGTCCGATAATACATC TGTGGCTTCACTATTATCGGACGT GTACCTGTTGGCGCTCACTGTCAA CAGCAATATACCTTC

Supplementary Table 2: non silencing oligonucleotides and oligonucleotides targeting human BMP9

	IOSE397	IOSE398	TR175	SKOV3	OVCAR3	OVCA443	IGROV
ALK1	++	++	+	+	+	-/+	+
ALK2	+	++	+	+	++	++	+
ALK3	++	++	+	++	++	++	++
ALK4	++	+	++	++	++	++	++
ALK5	++	++	++	++	++	++	++
ALK6	-	+	+	-	-	+	-
ALK7	+	+	-	-	-	-	-
SMAD1	++	++	++	+	++	++	++
SMAD2	++	++	++	++	++	++	++
SMAD3	++	++	++	++	++	++	++
SMAD4	++	++	++	++	++	++	++
SMAD5	++	++	++	++	++	++	-
SMAD8	+	+	+	+	+	+	-

Supplementary Table 3. Analysis of the expression of different components of TGF-β/BMP signalling pathways in ovarian cell lines**.** RNA from IOSE cells and different ovarian carcinoma cell lines was isolated and the expression of different components of the TGF-β/BMP signalling pathways were analysed by RT-PCR. The intensity of the bands was scored as absent (-), low (-/+), medium (+) and high (++).