

Titration of ligands for the activation of ALK2 and ALK5 in MDA-MB-231 Reporter Cells (using Western Blot and Dual Luciferase Assay)

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Background:

Stable MDA-MB-231 breast cancer cell lines with constructs expressing firefly luciferase under the control of BMP response element (BRE) or CAGA box (CAGA) were previously obtained from Caroline Hill's lab. BRE and CAGA are activated downstream of ALK2 and ALK5 signalling respectively. Renilla firefly is constitutively expressed in these cell lines as internal loading control. Stable expression of those reporter constructs will, in theory reduce the variability in between assays. Titration with ligands that activates ALK2 and ALK5 was performed to determine the optimal concentration that activated the receptors. Experimentally, ligand titration will be performed in both Western blot and DLA. For Western blot analysis, the cells will be serum starved and stimulated with increasing concentrations of different ligands. Cells will then be lysed and analysed in Western blot to detect phosphorylation of ALK2 and ALK5 substrates. SMAD1 and SMAD5 are phosphorylated by activated ALK2 while SMAD2 is phosphorylated by activated ALK5. For DLA, the cells will be stimulated overnight in different concentrations of ligands and lysed for luciferase measurements.

Experimental details:

Western Blot Protocol

- 1) 1×10^5 of MDA-MB-231 BRE and MDA-MB-231 CAGA cells were seeded into each well of 6-well plates in DMEM with 10% serum and 10 μ g/ml Gentamicin.
- 2) Cells were allowed to attach overnight
- 3) Wells were rinsed twice with PBS
- 4) Cells were starved in 2ml plain OptiMEM for 8 hours
- 5) Cells were stimulated with different ligand concentrations for 1 hour
- 6) Wells were washed once with PBS and aspirated (considerable amount of cells were lost when starved in OptiMEM -FBS)
- 7) Cells were lysed on the plate by addition of 150ul of lysis buffer for 15 minutes on ice and collected using cell scraper
- 8) Lysates were clarified by centrifuge at max speed for 5 minutes
- 9) Unable to measure the concentration with nano-drop. Assuming the protein concentration among the samples to be the same since same number of cells were seeded.
- 10) Clarified lysates were boiled with 4X loading dye for 15 minutes and 15ul was loaded into each well

Composition of cell lysis buffer

150mM NaCl

20mM Tris HCl pH7.5

1% Triton X-100

25mM NaF

25mM Sodium beta-glycerolphosphate

4mM Sodium pyrophosphate

2mM Sodium orthovanadate

mini-proteinase inhibitor (Roche) 1 tablet in 10ml

Western Blot conditions

1) 1 hour 100V wet transfer onto 0.2um pore-sized PVDF membrane

2) 1 hour blocking in PBS-0.1%Tween-20 3%BSA

3) Overnight 4 degree incubation in primary antibodies 1:1000 diluted in PBS-0.1%Tween-20 with 3% BSA (Phospho-SMAD1/5 (Ser463/465) #9516, Phospho-SMAD2 (S465/467) #3108, SMAD1 #9743, SMAD2 #5339, SMAD5 #9517, beta-Tubulin T5201 Sigma)

4) Washed 3X 10 minutes with PBS-0.1%Tween-20

5) 1 hour room temperature incubation in secondary antibodies conjugated to HRP (1:2000) diluted in PBS-0.1%Tween-20 with 3% skimmed milk

6) Washed 3X 10 minutes with PBS-0.1%Tween-20

7) Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Catalog no.: 32209). Images were captured using LAS-4000

Dual Luciferase Assay (DLA) Protocol

1) 4,000 MDA-MB-231 BRE or MDA-MB-231 CAGA cells were seeded per well of 96-wells flat bottom culture plates and allowed to attach overnight

2) Wells were washed once with 200ul of PBS

3) Stimulation was done with different concentrations of ligands in DMEM with 1% serum for 18 hours

4) DLA was performed (Promega catalog no.: E1910)

5) Washed once with PBS

6) Lysed cells in 20ul PLB

7) Transferred to white 96-wells plate and measured in GloMax plate reader (Promega)

- 8) 100ul of LARII was added for Firefly luciferase signal 10s integration
- 9) 100ul of Stop & Glo was added for Renilla luciferase signal 10s integration
- 10) To obtain relative fold change in transcription activities downstream of ALK2 and ALK5, Firefly luciferase signal was divided by Renilla luciferase signal and further normalised to control wells without ligand stimulation

Results

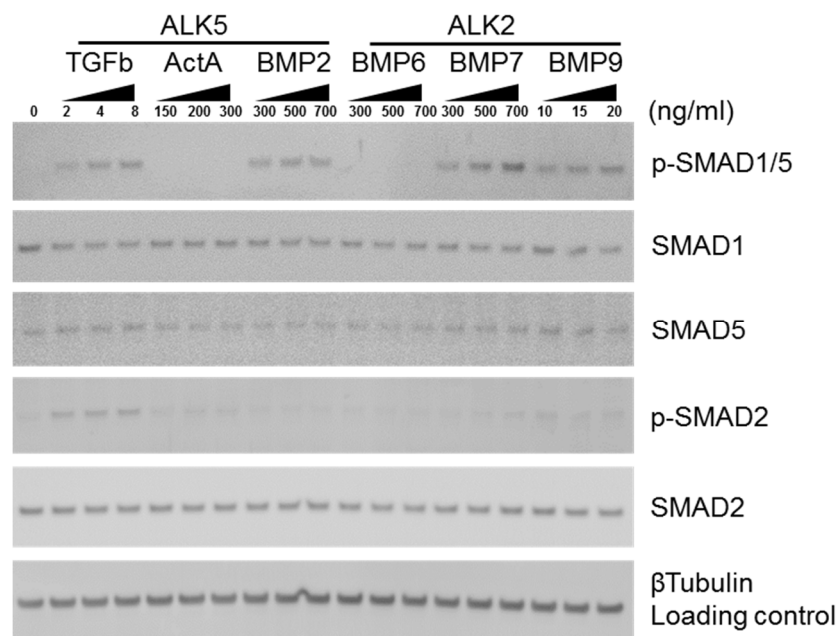


Figure 1 Western blot

Table 1 Raw DLA values

Firefly Luciferase	BRE			CAGA		
Concentrations:	1	2	3	4	5	6
TGFb	83.4003	86.8004	82.1003	210.802	212.402	322.705
Activin A	68.4002	77.8003	73.8003	71.3002	109.601	256.903
BMP2	103.501	107.601	175.001	74.0003	70.6002	71.6002
BMP4	68.6002	78.3003	75.5003	67.1002	67.2002	71.4002
BMP6	70.0002	427.309	355.806	66.6002	63.4002	61.7002
BMP7	272.904	582.416	576.116	66.2002	60.5002	77.2003
BMP9	78.7003	125.101	118.601	57.0002	61.4002	74.1003
No ligand	62.9002	63.9002	66.6002	56.5002	59.8002	57.4002
Renilla Luciferase	BRE			CAGA		
Concentrations:	1	2	3	4	5	6
TGFb	142.901	161.701	141.401	137.501	158.401	150.101
Activin A	138.101	135.101	136.701	129.401	137.501	132.201
BMP2	142.601	144.101	122.301	139.101	147.001	160.301
BMP4	144.601	142.101	129.001	153.501	126.801	166.301
BMP6	143.501	136.701	138.901	125.201	119.401	140.701
BMP7	154.801	156.201	123.301	157.301	122.301	133.801
BMP9	151.701	152.001	139.801	115.801	121.301	119.201
No ligand	151.801	162.001	127.001	118.501	153.101	122.901
Firefly/Renilla	BRE			CAGA		
Concentrations:	1	2	3	4	5	6
TGFb	0.583623	0.536796	0.58062	1.533094	1.340913	2.149919
Activin A	0.495291	0.575868	0.539867	0.551002	0.797092	1.943276
BMP2	0.725808	0.746705	1.430904	0.53199	0.48027	0.446661
BMP4	0.47441	0.551019	0.585269	0.437132	0.529966	0.429343
BMP6	0.487803	3.125866	2.56158	0.531946	0.530986	0.43852
BMP7	1.762934	3.728632	4.672436	0.42085	0.494683	0.576978
BMP9	0.518786	0.823027	0.848356	0.492225	0.50618	0.621642
No ligand	0.41436	0.394443	0.524407	0.476791	0.390593	0.467044

Table 2 Processed relative changes in transcription downstream of ALK2 and ALK5

Activates	Ligands	Concentrations/Fold change				
		0	2	4	8	ng/ml
ALK5	TGFb	1	1.313273	1.207902	1.306517	BRE
		1	3.446632	3.014579	4.833349	CAGA
		0	150	200	300	ng/ml
ALK5	Activin A	1	1.114508	1.295822	1.214812	BRE
		1	1.238737	1.791986	4.368783	CAGA
		0	300	500	700	ng/ml
ALK5	BMP2	1	1.63322	1.680243	3.219833	BRE
		1	1.195995	1.079721	1.004163	CAGA
		0	10	20	30	ng/ml
ALK2	BMP4	1	1.067522	1.239907	1.316978	BRE
		1	0.98274	1.191445	0.96523	CAGA
		0	300	500	700	ng/ml
ALK2	BMP6	1	1.097658	7.03385	5.764089	BRE
		1	1.195897	1.193737	0.98586	CAGA
		0	300	500	700	ng/ml
ALK2	BMP7	1	3.96697	8.390199	10.51396	BRE
		1	0.946137	1.112123	1.297136	CAGA
		0	10	15	20	ng/ml
ALK2	BMP9	1	1.167376	1.851984	1.908978	BRE
		1	1.106599	1.137972	1.397546	CAGA

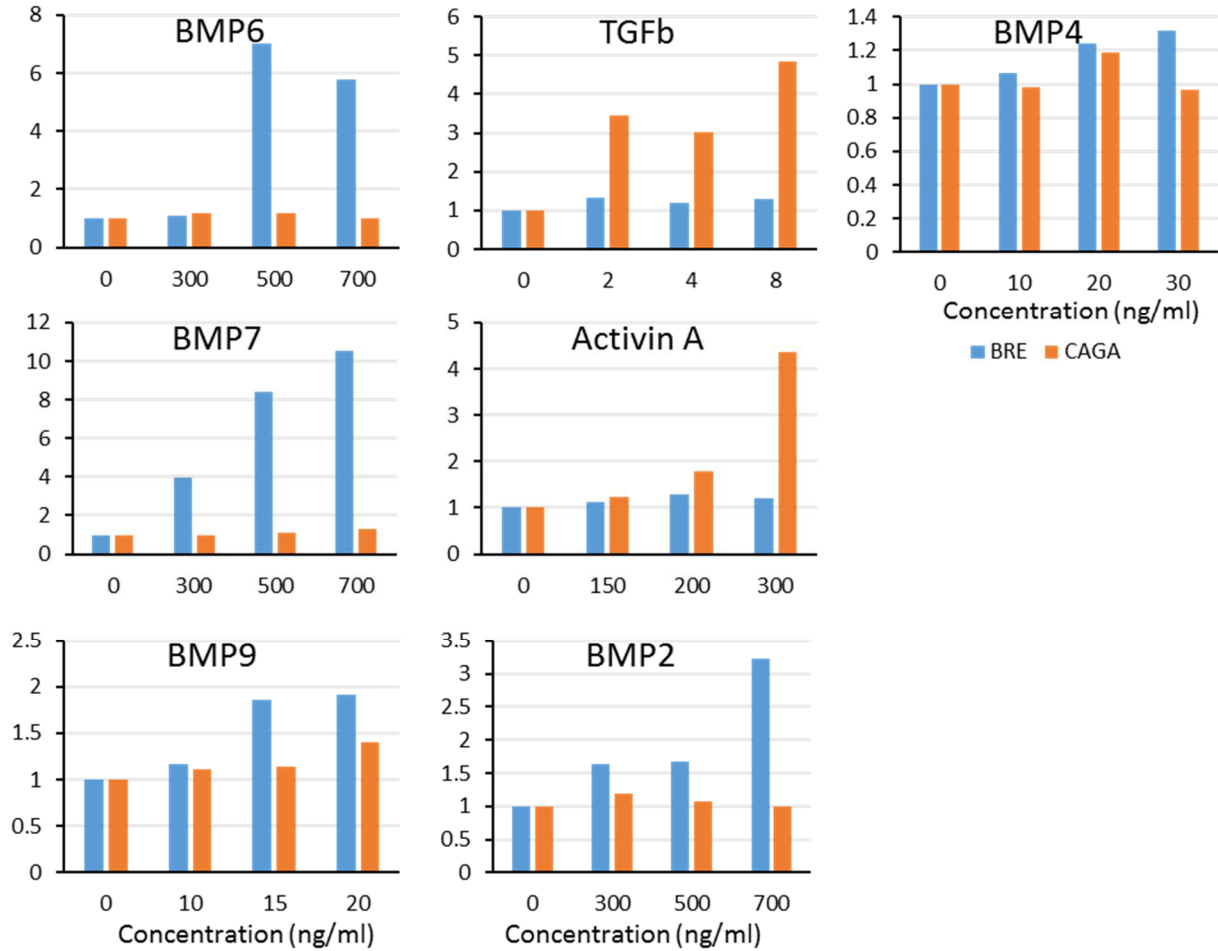


Figure 2 DLA results represented in bar graphs

Conclusions:

Something went wrong with the Western blot of BMP6-stimulated cells. No phospho-SMAD1/5 is detected.

Both Western blot and DLA results showed that high ligand concentrations are required for optimal phosphorylation of SMAD1/5 or SMAD2 and transcription of firefly luciferase under the control of BRE or CAGA.

Based on DLA results, 500ng/ml of BMP6 and 500ng/ml of BMP7 will be used for subsequent stimulation of MDA-MB-231 BRE cells (reporter for the transcription activities downstream of ALK2) and 8ng/ml of TGFβ will be used for stimulation of MDA-MB-231 CAGA (reporter for the transcription activities downstream of ALK5).