Supplemental Data

TGF-β-mediated miR-181a expression promotes breast cancer metastasis by targeting Bim.

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Supplemental Table 1

miRs upregulated by TGF-β in 67NR cells

Compliant	Fold Upregulation	P-value
mmu-miR-181a	**1.87**	**0.00009**
mmu-miR-181b	**1.79**	**0.00028**
mmu-miR-106a	1.06	0.00374
mmu-miR-186*	1.25	0.00026
mmu-miR-21	1.28	0.00367
mmu-miR-181d	1.34	0.00443
mmu-miR-27a*	1.11	0.00152
mmu-miR-34a	1.07	0.07899
mmu-miR-125b*	1.15	0.00196
mmu-miR-183*	1.05	0.01789
mmu-miR-101b	1.07	0.02083
mmu-miR-379	1.05	0.04343
mmu-miR-292-3p	1.13	0.00422
mmu-miR-345-3p	1.13	0.00936
mmu-miR-425*	1.07	0.01164
mmu-miR-710	1.06	0.01074
mmu-miR-879	1.11	0.01951
mmu-miR-350	1.18	0.07207
mmu-miR-138	1.16	0.01365
mmu-miR-299	1.20	0.01704
mmu-miR-708*	1.08	0.01513
mmu-miR-679	1.09	0.04203
mmu-miR-615-3p	1.22	0.02645
mmu-miR-470*	1.09	0.05316
mmu-miR-199b*	1.18	0.0178
mmu-miR-412	1.15	0.02719
mmu-miR-143	1.17	0.02144
Rigid	**Fold Upregulation**	**P-value**
mmu-miR-181b	**1.79**	**0.0003**
mmu-miR-181d	**1.36**	**0.00082**
mmu-miR-181a	**1.68**	**0.00201**

Supplemental Table 2

miRs upregulated by TGF-β in 4T07 cells

Compliant	Fold Upregulation	P-value
mmu-miR-181a	**2.04**	**0.00012**
mmu-miR-181b	**2.10**	**0.00028**
mmu-miR-494	1.24	0.00027
mmu-miR-149	1.27	0.0015
mmu-miR-500	1.12	0.00363
Rigid	**Fold Upregulation**	**P-value**
mmu-miR-181b	**1.94**	**0.00007**
mmu-miR-181a	**1.80**	**0.00043**
mmu-miR-181d	**1.35**	**0.00292**
mmu-miR-22	1.51	0.00162
mmu-miR-292-5p	1.10	0.01134
mmu-miR-181a-2*	1.12	0.00711
mmu-miR-882	1.13	0.00547
mmu-miR-362-3p	1.20	0.00615
mmu-miR-34a	1.34	0.00658
mmu-miR-140*	1.58	0.00902
mmu-miR-99b	1.12	0.03687

Supplemental Table 3

miRs upregulated by TGF-β in 4T1 cells

Compliant	Fold Upregulation	P-value
mmu-miR-22	2.28	0.00022
mmu-miR-146a	2.82	0.00028
mmu-miR-146b	2.39	0.00049
mmu-miR-362-5p	1.09	0.00114
mmu-miR-149	1.58	0.00132
mmu-miR-181b	**1.94**	**0.00371**
mmu-miR-181a	**1.91**	**0.00684**
mmu-let-7i	1.99	0.00214
mmu-miR-155	1.26	0.02205
mmu-miR-34a	1.72	0.00908
mmu-miR-183	1.20	0.01577
mmu-miR-181d	1.27	0.06512
Rigid	**Fold Upregulation**	**P-value**
mmu-miR-290-5p	1.49	0.00015
mmu-miR-181b	**1.95**	**0.00028**
mmu-miR-146b	1.79	0.00038
mmu-miR-146a	2.18	0.00042
mmu-miR-132	1.67	0.0005
mmu-miR-181a	**1.82**	**0.0015**
mmu-miR-22	2.39	0.00256
mmu-miR-34a	1.59	0.00535
mmu-let-7i	1.56	0.00637
mmu-miR-181d	**1.33**	**0.02755**

Supplementary Table 4
Real-time PCR primer pairs

Target	Application	Sequence (5' to 3')
miR-181a	PCR-Sense	5'-AACATTCAACGCTGTCGGTGAGT
miR-181b	PCR-Sense	5'-AACATTCATTGCTGTCGGTGGGT
miR-181c	PCR-Sense	5'-AACATTCAACCTGTCGGTGAGT
miR-181d	PCR-Sense	5' AACATTCAACCTGTCGGTGAGT
U6	PCR-Sense	5'-GTGCTCGCTTCGGCAGCACAT
Bim	PCR-Sense	5'-TCTGAGTGTGACAGAGAAGGTGGAC
Bim	PCR-Antisense	5'-CAGCTCGGTGTGCAATCCGTATC
GAPDH	PCR-Sense	5'-CAACTTTGGCATTGTGGAAGGGCTC
GAPDH	PCR-Antisense	5'-GCAGGGATGATGTTCTGGGCAGC

Shown are the sense and antisense primers used to amplify the indicated target gene.

Supplemental Table 5
Immunoblotting antibodies

Antibody	Dilution	Supplier (catalog #)
Phospho-Smad 2	1:1000	Cell Signaling (#3101)
Phospho-Smad 3	1:500	Cell Signaling (#9520)
Total Smad 2/3	1:1000	Cell Signaling (#3102)
Phospho-Src	1:500	Cell Signaling (#2113)
Phospho-Erk1/2	1:1000	Cell Signaling (#9101)
Phospho-Akt	1:500	Cell Signaling (#4060)
Total Src	1:1000	Cell Signaling (#2108)
Total Erk1/2	1:1000	Cell Signaling (#4695)
Total Akt	1:500	Cell Signaling (#9272)
E-Cadherin	1:5000	BD Biosciences (#610182)
Caspase-3	1:1000	Cell Signaling (#9662)
β-Actin	1:1000	Santa Cruz (#sc-1616)

Shown are the antibodies and dilutions used to visualize the indicated proteins. Also provided are the vendors where these reagents were obtained.

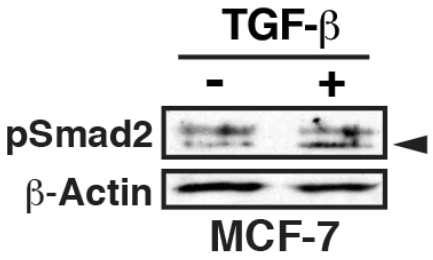
Supplementary Table 6

Pharmacological inhibitors

Name	Target	Concentration	Supplier
TβR-I Inh II	TβR-I	3.5 μM	Calbiochem
U0126	MEK1/2	10 μM	Promega
Akt Inh VIII	Akt	1 μM	Calbiochem

Shown are the pharmacological antagonists and final concentrations used inhibit the indicated protein targets. Also provided are the vendors where these reagents were obtained.

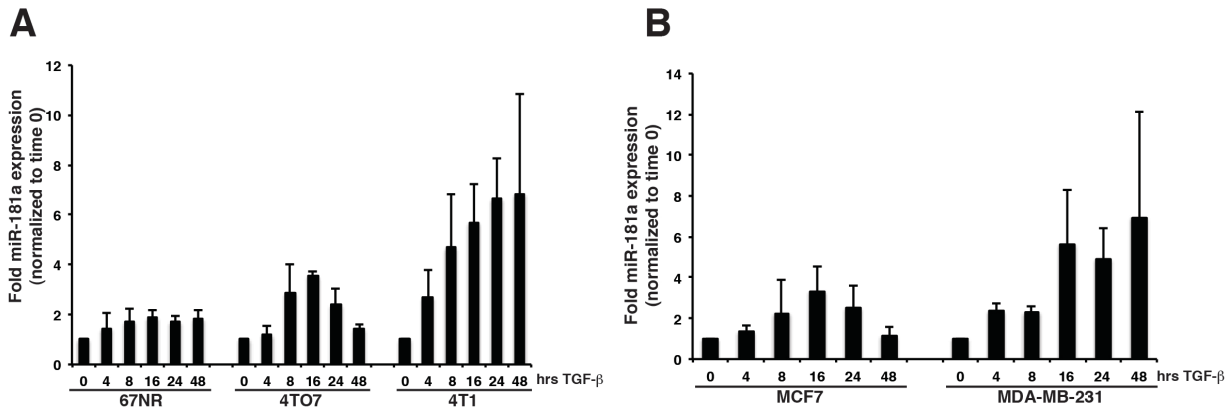
Supplemental Figure 1: Taylor *et al*



Supplemental Figure 1

MCF-7 cells were stimulated with TGF-β1 (5 ng/ml) for 30 min. Afterward, the phosphorylation of Smad2 (arrowhead) was measured by immunoblotting, and differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Images are representative of 2 similar experiments.

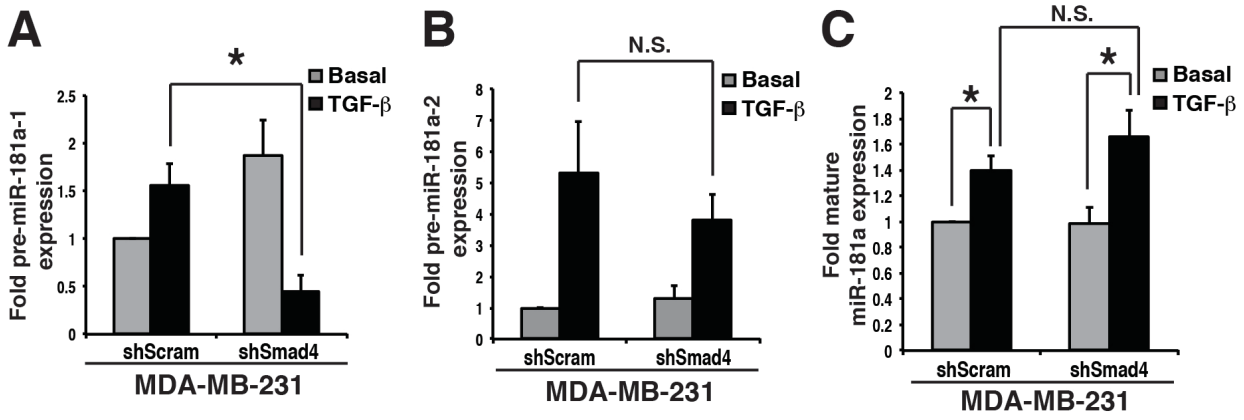
Supplementary Figure 2: Taylor *et al*



Supplemental Figure 2

Time-course of miR-181a expression induced by TGF-β in nonmetastatic and metastatic human and murine breast cancer cells. Murine 67NR, 4T07, and 4T1 cells **(A)** or human MCF-7 or MDA-MB-231 cells **(B)** were stimulated with TGF-β1 (5 ng/ml) for varying times over a span of 48 h as indicated, at which point the expression of miR181a was determined by semi-quantitative real-time PCR.

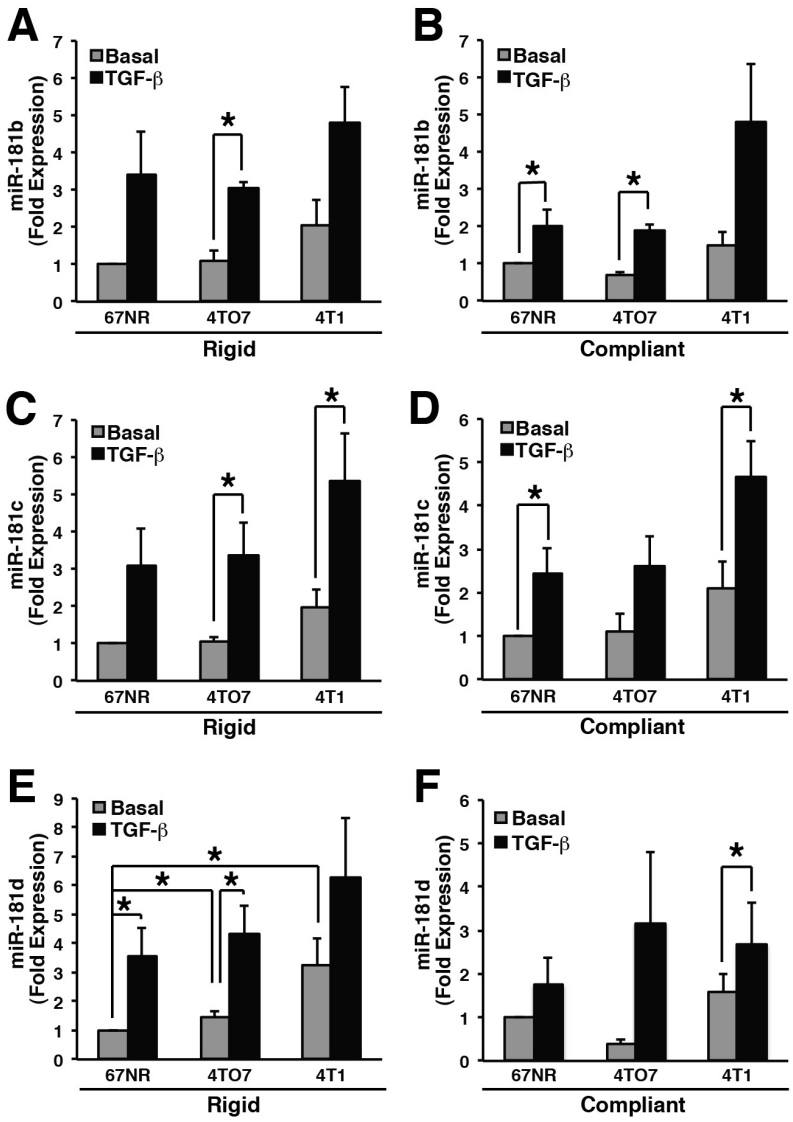
Supplementary Figure 3: Taylor *et al*



Supplemental Figure 3

Regulation of pre-miR-181a expression and processing by canonical Smad4 signaling. Control (shScram) and Smad4-deficient (shSmad4) MDA-MB-231 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h, at which point the expression of pre-miR-181a-1, pre-miR-181a-2, and miR-181a was determined by semi-quantitative real-time PCR. Individual signals were normalized to those of U6. Data are the mean (±SE; n=3) fold expression of pre-miR-181a-1 **(A)**, pre-miR-181a-2 **(B)**, or miR-181a **(C)** relative to basal expression levels. (**P*<0.05; Student's *t*-Test).

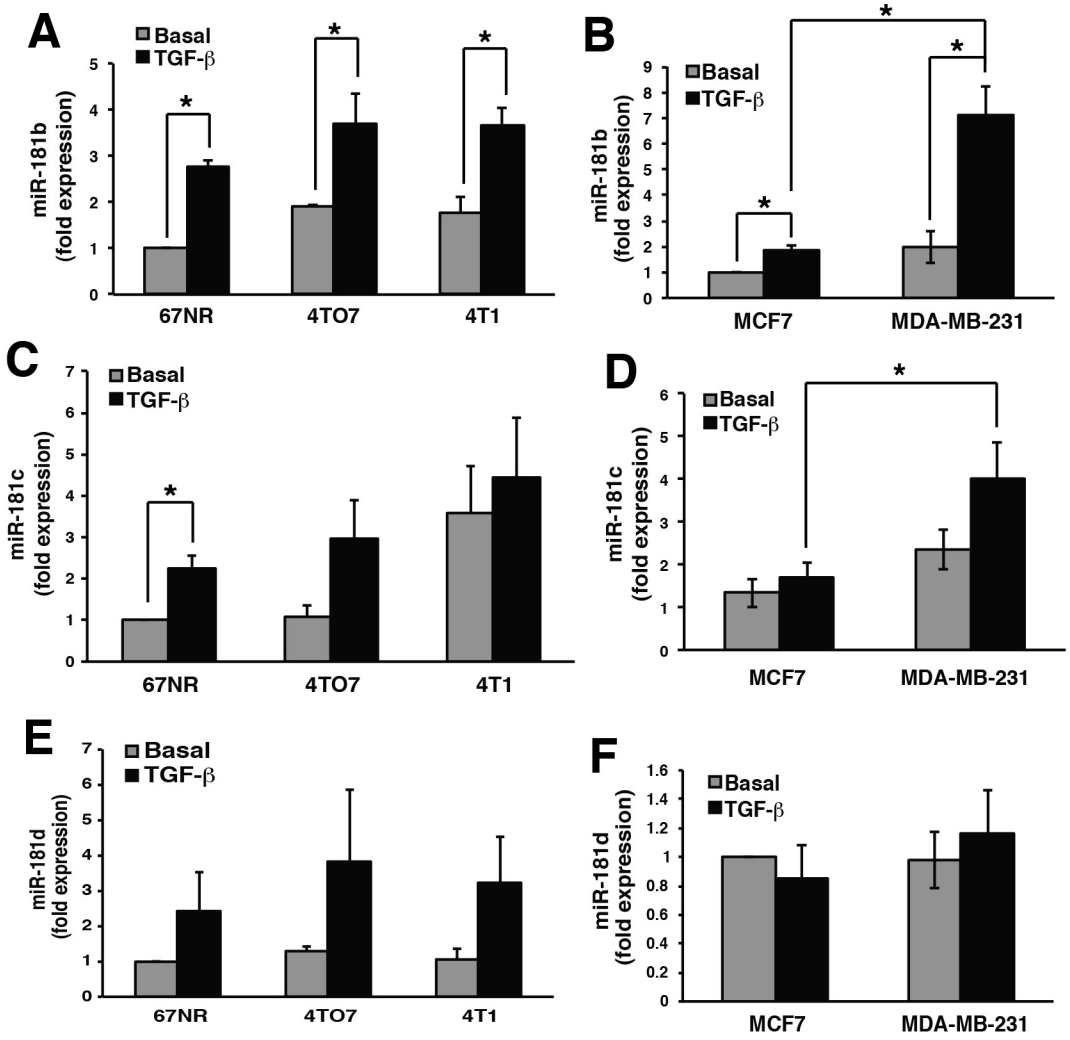
Supplemental Figure 4: Taylor *et al*



Supplemental Figure 4

TGF-β stimulates the expression of miR-181 family members in 3D-organotypic breast cancer cultures. TGF-β1 (5 ng/ml) treatment of 67NR, 4T07, and 4T1 cells for 6 days in rigid (**A, C, E**) or compliant (**B, D, F**) 3D-organotypic cultures universally induced the expression of miR-181b (**A&B**) ,miR-181c (**C&D**), and miR-181d (**E&F**) as determined by semi-quantitative real-time PCR. Individual miR signals were normalized to those of U6. Data are the mean (±SE; n=3) fold expression of miR-181 family members relative to those detected in basal 67NR cells (**P*<0.05; Student's *t*-Test).

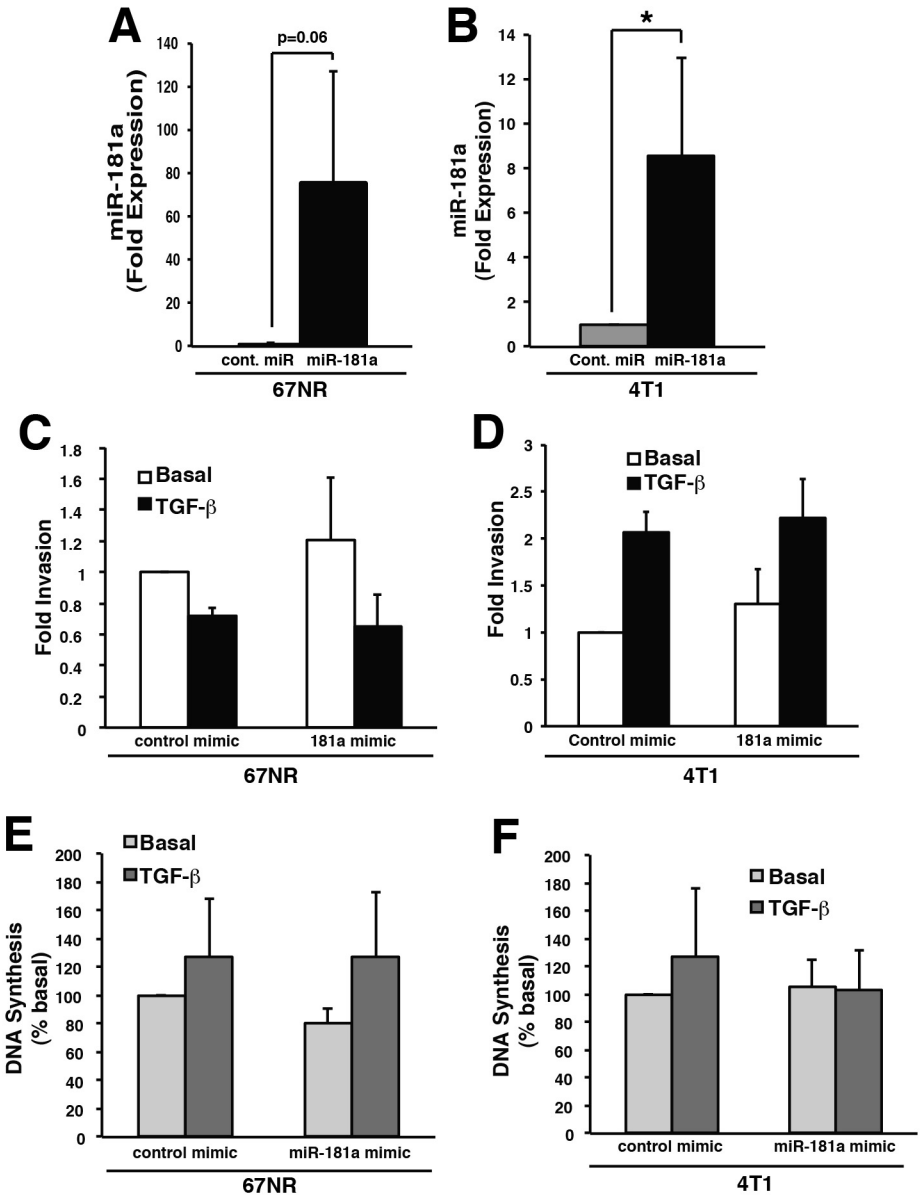
Supplementary Figure 5: Taylor *et al*



Supplemental Figure 5

TGF-β stimulates the expression of miR-181 family members in murine and human breast cancer cells. Murine 67NR, 4T07, and 4T1 cells (**A, C, E**) or human MCF-7 or MDA-MB-231 (**B, D, F**) were stimulated with TGF-β1 (5 ng/ml) 48 h, at which point the expression of miR-181b (**A&B**), miR-181c (**C&D**), and miR-181d (**E&F**) was determined by semi-quantitative real-time PCR. Individual miR-181 signals were normalized to those of U6. Data are the mean (±SE; n=3) fold expression of miR-181 family members relative to those detected in basal 67NR **(A, C, E)** or MCF-7 (**B, D, F**) cells (**P*<0.05; Student's *t*-Test).

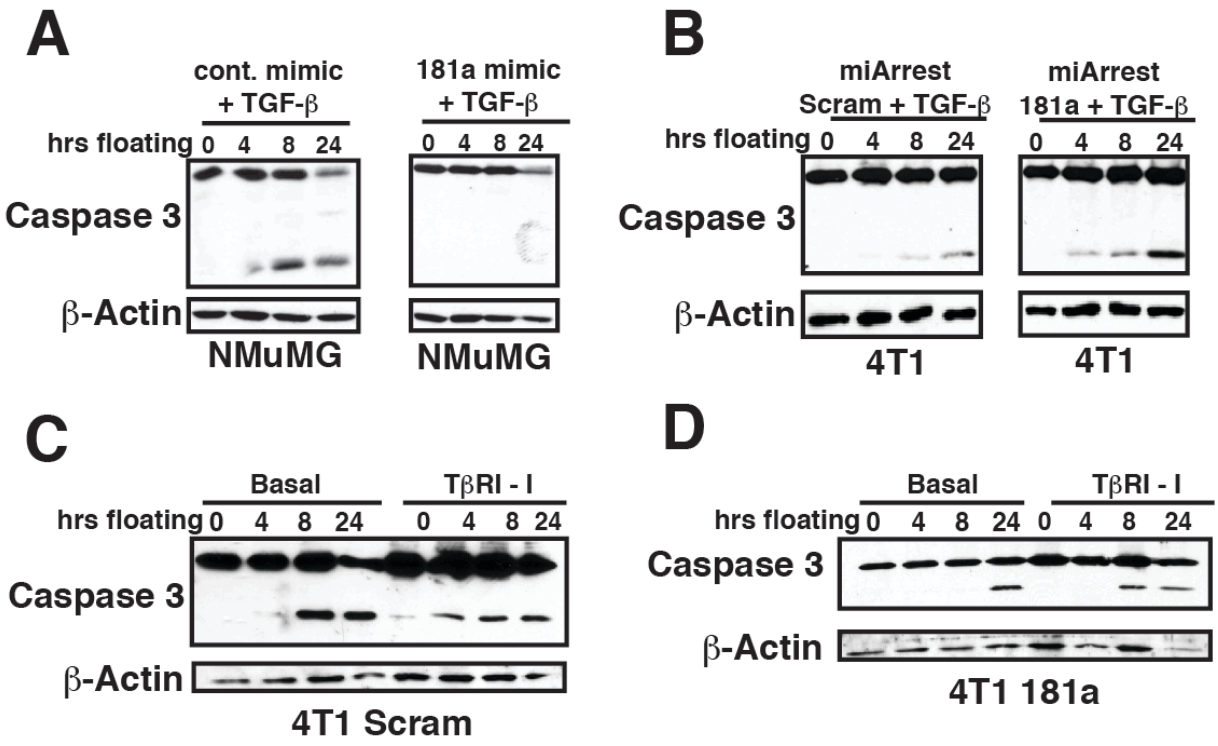
Supplementary Figure 6: Taylor *et al*



Supplementary Figure 6

Overexpression of miR-181a mimics fail to enhance breast cancer cell invasion and proliferation. (**A&B**) Transient transfection of miR-181a mimics elevated miR-181a expression in 67NR (**A**) and 4T1 (**B**) cells as measured by semi-quantitative real-time PCR. Individual miR-181a signals were normalized against those measured for U6. (**C-F**) The aforementioned 67NR and 4T1 variants were incubated in the absence (*i.e.,* basal) or presence of TGF-β1 (5 ng/ml) to monitor changes in cell invasion (**C&D**) or DNA synthesis (**E&F**). All data are the mean (±SE; n=3) relative to corresponding basal activity (**P*<0.05; Student's *t*-Test).

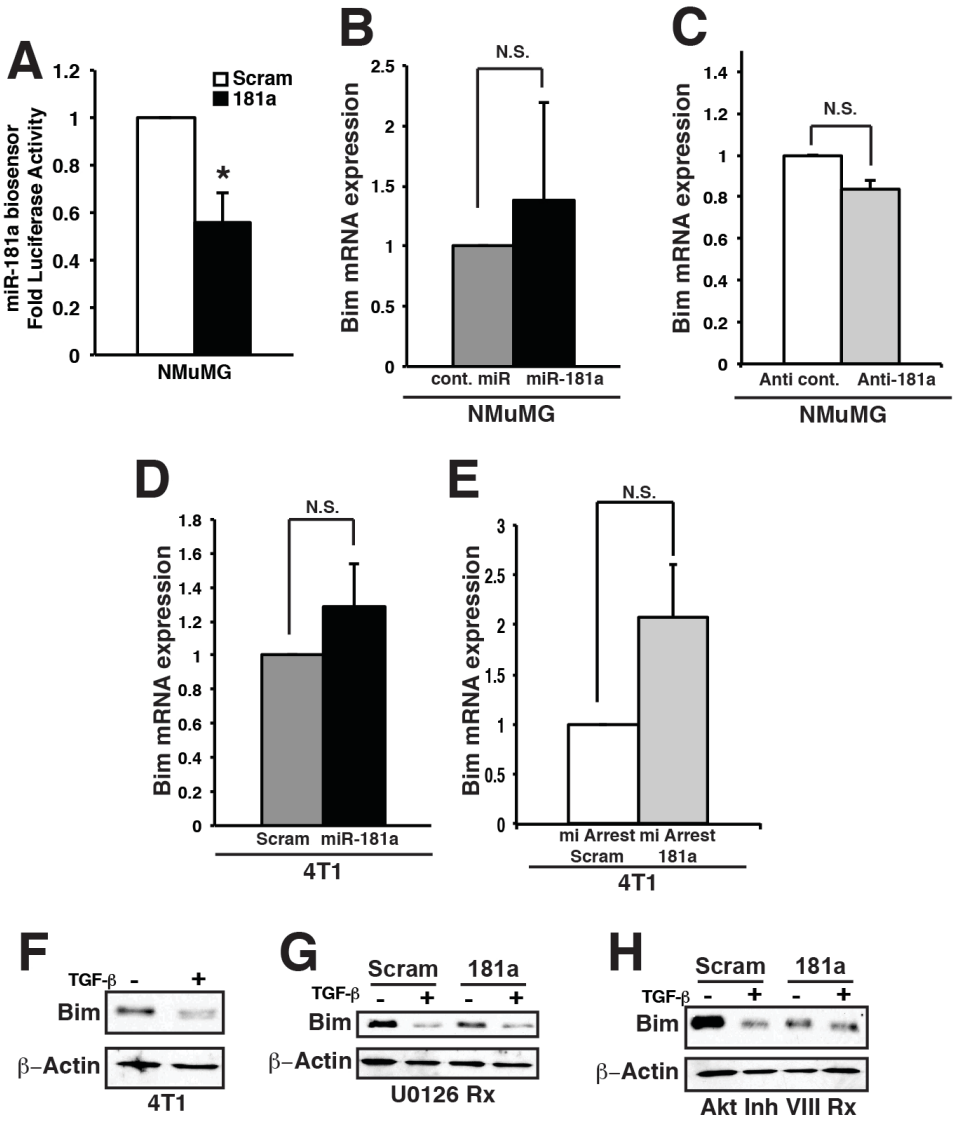
Supplementary Figure 7: Taylor *et al*



Supplementary Figure 7

Abrogation of TGF-β signaling sensitizes normal and malignant MECs to undergo anoikis. **(A&B)** NMuMG **(A)** or 4T1 **(B)** cell derivatives were suspended over poly-HEMA-coated culture dishes and treated with TGF-β1 (5 ng/ml) for 0-24 h as indicated. The extent of anoikis was monitored by immunoblotting for cleavage of caspase-3. **(C&D)** 4T1 derivatives indicated were pre-treated for 48h with the TβR-I inhibitor (100 ng/ml) as indicated prior to their being suspended for 24 h over poly-HEMA-coated culture dishes to induce anoikis. Afterward, caspase-3 cleavage was monitored by immunoblotting detergent-solubilized whole-cell extracts with anti-caspase-3 antibodies. Differences in protein loading were assessed with anti-β-actin antibodies. Shown are representative images from 3 **(A&B)** or 2 **(C&D)** independent experiments.

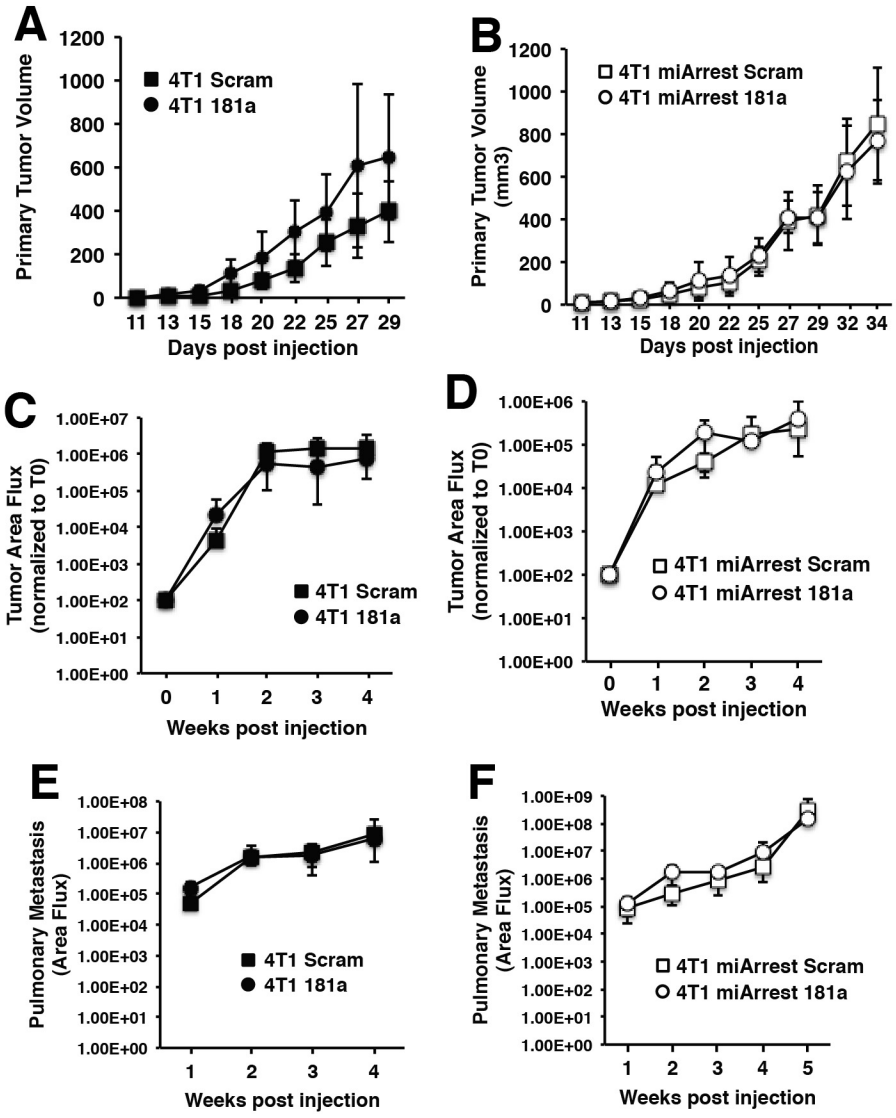
Supplementary Figure 8: Taylor *et al*



Supplementary Figure 8

miR-181a suppresses Bim expression by repressing the translation of its mRNA. (**A**) Stable overexpression of miR-181a in NMuMG cells decreased miR-181a biosensor activity indicative of elevated miR-181a activity. (**B&C**) Neither stimulation of miR-181a expression (**B**), nor inhibition of its activity (**C**) in NMuMG cells affected Bim mRNA expression levels as measured by semi-quantitative real-time PCR. Bim transcript levels were normalized to those for GAPDH. (**D&E**) Neither stimulation of miR-181a expression (**D**), nor inhibition of its activity (**E**) in 4T1 cells affected Bim mRNA expression levels as measured by semi-quantitative real-time PCR as above. Data in **Panels A-E** the mean (±SE; n=3; **P*<0.05; Student's *t*-Test). **(F-H)** Immunoblotting 4T1 cell extracts demonstrated that TGF-β decreased Bim protein levels **(F)**, while neither MEK inhibition **(**U0126, 10 μM; **G)** or Akt inhibition **(**Akt Inh VIII, 1 μM; **H)** abrogated the ability of TGF-β or miR-181a to decrease Bim protein levels. Differences in protein loading were assessed by β-actin immunoblotting. Shown are representative images from 2 independent experiments.

Supplementary Figure 9: Taylor *et al*



Supplementary Figure 9

miR-181a expression fails to affect primary tumor growth and metastatic dissemination. (**A&B**) 4T1 cells engineered to overexpress miR-181a (**A**) or possess diminished miR-181a activity (**B**) were engrafted onto the mammary fat pads of 6-week-old Balb/c mice. Data are the mean (± SEM; n=5) tumor volumes quantified at the indicated times post engraftment. (**C&D**) Data are the mean (± SEM; n=5) bioluminescent tumor area flux units detected in the aforementioned tumor-bearing mice. (**E&F**) Data are the mean (±SE) bioluminescent pulmonary metastasis area flux at the indicated time points.