Supplementary Information

TGF-β Stimulation of EMT Programs Elicits Non-genomic ERα Activity and Anti-estrogen Resistance in Breast Cancer Cells

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Running Title: EMT Drives Anti-estrogen Resistance in Breast Cancer

Supplementary Table 1: Pharmacological agonists and inhibitors

Name	Target	Concentration	Supplier (USA)
Human TGF-β1	TGF-β Rec.	5 ng/ml	R&D Systems
TβR-I Inh II	TβR-I	100 ng/ml	Calbiochem
U0126	MEK1/2	10 μM	Promega
PP2	Src	5-10 μM	Calbiochem
17-β-estradiol	ER-α	0.1-1 nM	Sigma
Tamoxifen	ER-α	0.1 μM	Sigma
4-OH-tamoxifen	ER-α	0.1 μM	Sigma
Fulvestrant	ER-α	0.1 μM	Sigma
AG1024	IGFR	1 μM	Calbiochem
AG1478	EGFR	1 μM	Cayman Chemicals

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

Supplementary Table 2: Real-time PCR primer pairs

Gene	Genbank Access #	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
ZO-1	NM_003257	5'-TCTAAGGGAGCACATGGTGA	5'-ATCTTCTCGGTTTGGTGGTC
Twist	NM_000474	5'-CCTTCTCGGTCTGGAGGAT	5-TTCTCCTTCTCTGGAAACAATG
Snail1	NM_005985	5'-CTAGGCCCTGGCTGCTAC	5'-GACATCTGAGTGGGTCTGGA
Zeb1	NM_030751	5'-TTTCCCATTCTGGCTCCTAT	5'-GATGCTGAAAGAGACGGTGA
Zeb2	NM_014795	5'-GCCCTTTAGGAGTTCATCCA	5'-GGCTTCCATCCCTACACCTA
E-cad	NM_004360	5'-CATCTTTGTGCCTCCTGAAA	5'-TGGGCAGTGTAGGATGTGAT
N-cad	NM_001792	5'-CCTGCTTATCCTTGTGCTGA	5'-CCTGGTCTTCTTCTCCTCCA
Vimentin	NM_003380	5'-CAAAGCAGGAGTCCACTGAG	TAAGGGCATCCACTTCACAG
CK19	NM_002276	5'-GATAGTGAGCGGCAGAATCA	5'-GACCTTGGAGGCAGACAAAT
EGFR	NM_005228	5'-TTCTTGCAGCGATACAGCTC	5'-GGGAACGGACTGGTTTATGT
IGF1R	NM_000875	5'-CAATGCTTCAGTTCCTTCCA	5'-GAGGGTTCCACTTCACGATT
MMP9	NM_004994	5'-CCACCACAACATCACCTATTG	5'-CTGTACACGCGAGTGAAGGT
ER-α	NM_000125	5'-TCCAAAGAGAAGACCCTATCAATGTA	5'-AGTAAGTCCCTTATTTGTTCAGC
ER-β	NM_001437	5'-TCGCTAGAACACACCTTACC	5'-TTCACACGACCAGACTCCATA
Cox2	NM_000963	5'-CCTTCCTCCTGTGCCTGATG	5'-ACAATCTCATTTGAATCAGGAAGCT
β-Catenin	NM_001904	5'-TGCTAAATGACGAGGACCAG	5'-TGAGGAGAACGCATGATAGC
GAPDH	NM_002046	5'-TCCATGACAACTTTGGTATTCGT	5'-AGTAGAGGCAGGGATGATGTT

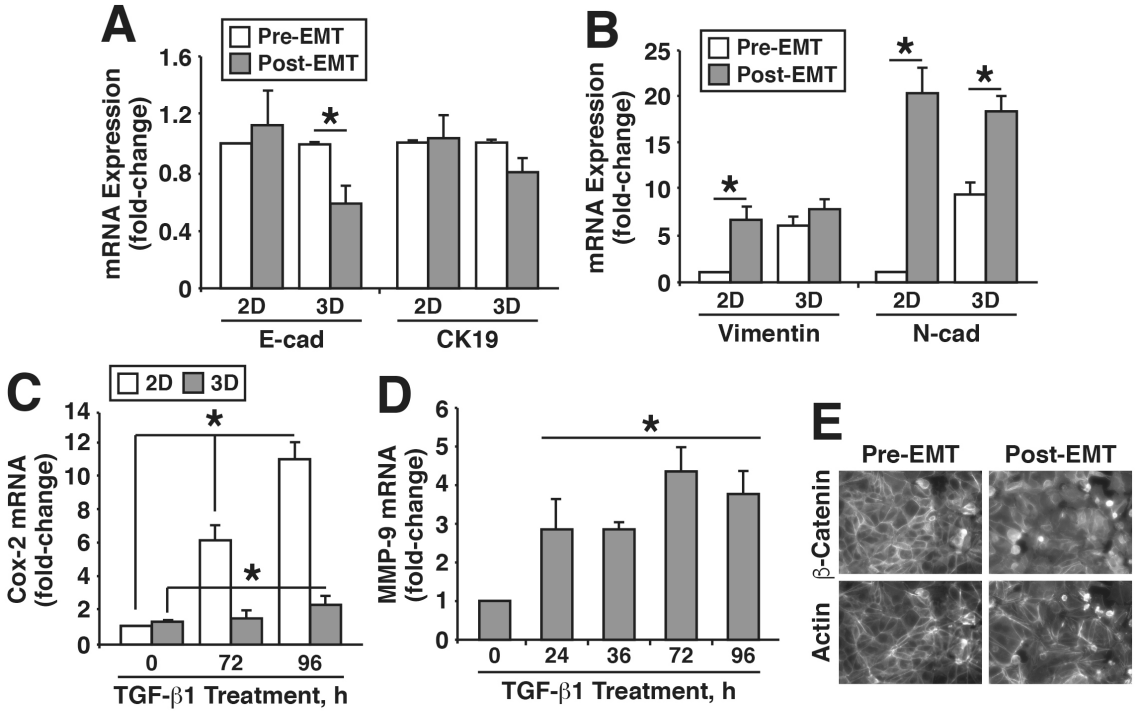
Shown are the sense and antisense primers used to amplify the indicated target gene.

Supplemental Table 5: Immunoblotting antibodies

Antibody	Dilution	Supplier (catalog #)
Phospho-Smad2	1:1000	Cell Signaling (#3101)
Total Smad2/3	1:1000	Cell Signaling (#3102)
β-catenin	1:1000	BD Biosciences (#610154)
Phospho-IGF1R	1:1000	Cell Signaling (#3024)
Total IGF1R	1:1000	Santa Cruz (#sc-713)
Total EGFR	1:1000	Cell Signaling (#2646)
Phospho-Erk1/2	1:1000	Cell Signaling (#9101)
Total Erk1/2	1:1000	Cell Signaling (#4695)
Phospho-p38 MAPK	1:500	Cell Signaling (#4060)
Total p38 MAPK	1:1000	Santa Cruz (#sc-728)
Phospho-Src	1:500	Cell Signaling (#2113)
Total Src	1:1000	Cell Signaling (#2108)
E-Cadherin	1:5000	BD Biosciences (#610182)
N-Cadherin	1:1000	Cell Signaling (#4061)
HDAC1	1:1000	Santa Cruz (#sc-8410)
β-Actin	1:1000	Santa Cruz (#sc-1616)

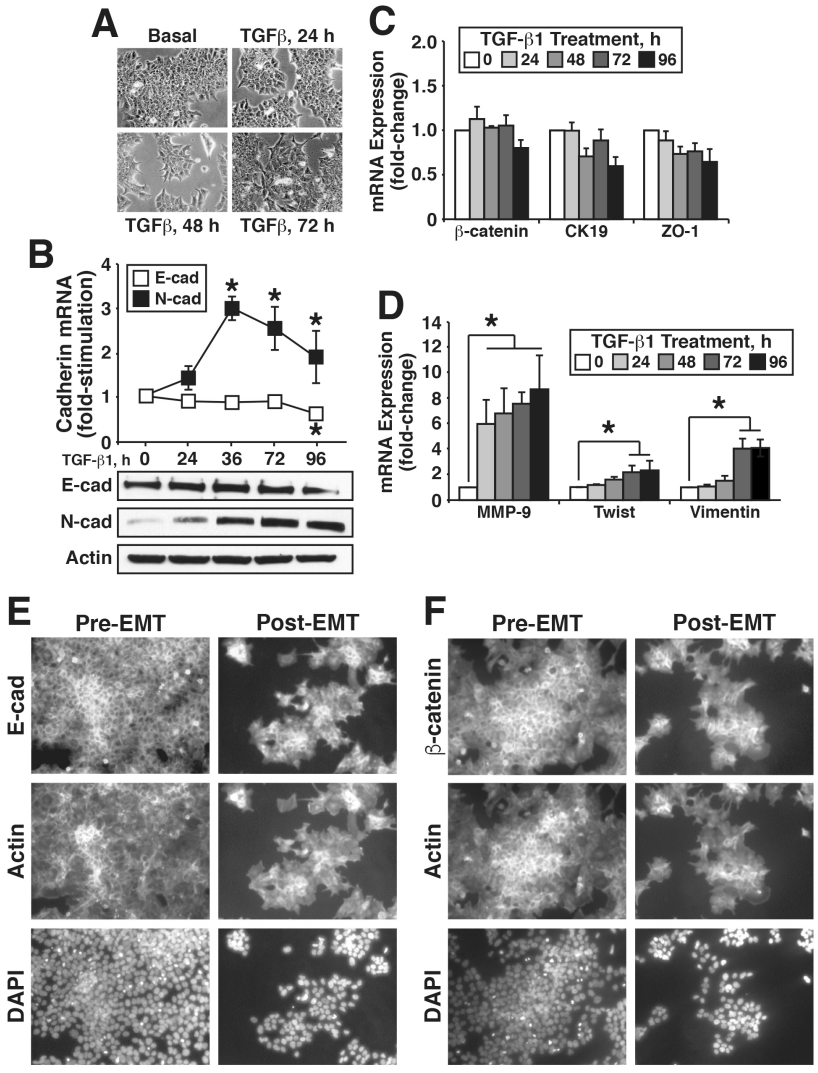
Shown are the antibodies and dilutions used to visualize the indicated proteins. Also provided are the vendors where these reagents were obtained.

Supplementary Figure S1: ***Tian & Schiemann***



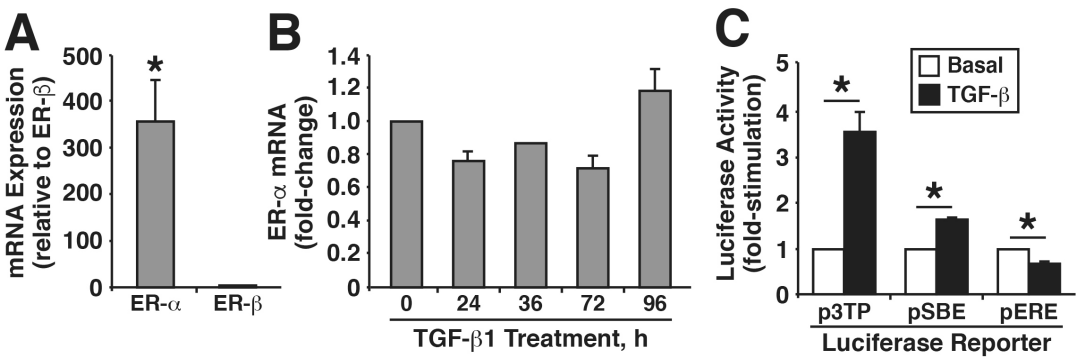
Supplementary Figure S1: TGF-β induces EMT phenotypes in MCF-7 cells in both 2D- and 3D-cultures. **(A-C)** MCF-7 cells were propagated in either2D- or 3D-culture systems and treated with TGF-β1 (5 ng/ml) for either 72 h **(A** and **B)** or 96 h **(C)** in 2D-cultures, or for either 8 days **(A** and **B)** or 96 h (**C**) as indicated. Afterward, total RNA has harvested and subjected to real-time PCR analyses to monitor the expression of the epithelial markers E-cad and CK19 **(A)**, of the mesenchymal markers vimentin and N-cad **(B)**, or of Cox-2 **(C)**. **(D)** MCF-7 cells were stimulated with TGF-β1 for 0-96 h as indicated, at which point the levels of MMP-9 were monitored by real-time PCR. Data are the mean fold-changes (±S.E.; n=3; **P*<0.05; Student's *t*-test). **(E)** MCF-7 cells were stimulated with TGF-β1 (5 ng/ml) for 96 h, and subsequently were fixed in paraformaldehyde and processed for indirect immunofluorescence of β-catenin, as well as for direct immunofluorescence of actin. Images are representative of 2-independent experiments (X 400).

Supplementary Figure S2: ***Tian & Schiemann***



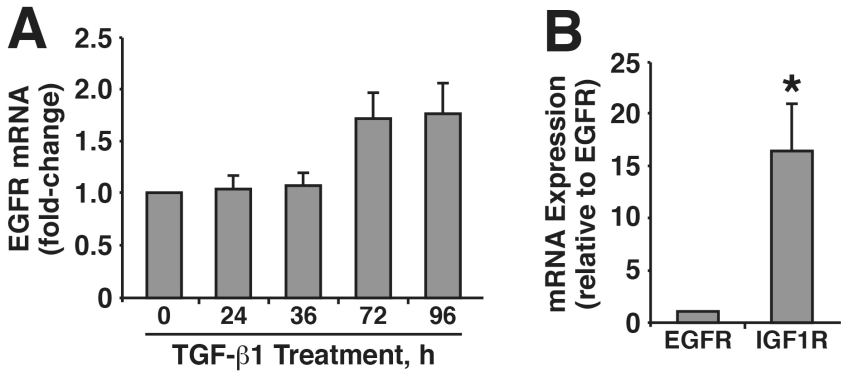
Supplementary Figure S2: TGF-β induces EMT phenotypes in BT474 cells. **(A)** BT474 cells were treated with TGF-β1 (5 ng/ml) for 0-72 h to induce an EMT program. Photomicrographs depict accompanying alterations in cell morphology (X 400). **(B)** BT474 cells were stimulated with TGF-β1 (5 ng/ml) for 0-96 h as indicated, at which point differences in the expression of E-cad and N-cad mRNA (*top*) and protein (*bottom*) were determined. mRNA data are the mean fold-changes (±S.E.; n=3; **P*<0.05; Student's *t*-test), while protein data are representative images from 3-independent experiments. **(C** and **D)** BT474 cells were treated with TGF-β1 (5 ng/ml) for 0-96 h, at which point the expression of β-catenin, CK19, and ZO-1 **(C)** or of MMP-9, Twist-1, and vimentin **(D)** were monitored by real-time PCR. Data are the mean fold-changes (±S.E.; n=3;**P*<0.05; Student's *t*-test). **(E** and **F)** BT474 cells were stimulated with TGF-β1 (5 ng/ml) for 72 h, and subsequently were fixed in paraformaldehyde and processed for indirect immunofluorescence of either E-cad **(E)** or β-catenin **(F)**, as well as for direct immunofluorescence of actin **(E** and **F)**. Nuclei were visualized by DAPI staining. Images are representative from 3-independent experiments (X 400).

Supplementary Figure S3: ***Tian & Schiemann***



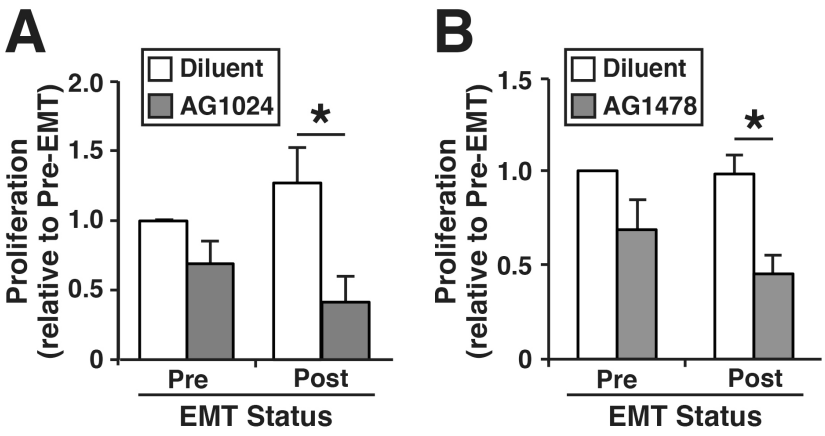
Supplementary Figure S3: TGF-β regulates estrogen signaling and EGFR expression in BT474 cells. **(A)** Differential mRNA expression of ER-α and ER-β in quiescent BT474 cells. **(B)** BT474 cells were stimulated with TGF-β1 (5 ng/ml) for 0-96 h as indicated. Afterward, total RNA was harvested and subjected to real-time PCR to monitor differences in ER-α expression. **(C)** BT474 cells were transiently transfected overnight with p3TP-, pSBE-, or pERE-luciferase and pCMV-β-gal cDNAs prior to their stimulation for 24 h with TGF-β1 (5 ng/ml). Afterward, luciferase and β-gal activities contained in detergent-solubilized cell extracts were measured. Data are the mean fold-changes (±S.E.; n=3;*P<0.05; Student's t-test).

Supplementary Figure S4: ***Tian & Schiemann***



Supplementary Figure S4: TGF-β regulates EGFR and IGF-1R expression in BT474 cells. **(A)** BT474 cells were stimulated with TGF-β1 (5 ng/ml) for 0-96 h as indicated, at which point differences in EGFR expression were monitored by real-time PCR. **(B)** Differential mRNA expression of EGFR and IGF-1R in quiescent BT474 cells. Data are the mean fold-changes (±S.E.; n=3; **P*<0.05; Student's *t*-test).

Supplementary Figure S5: ***Tian & Schiemann***



Supplementary Figure S5: Post-EMT cells are sensitized to inhibitors against IGF-1R and EGFR. Pre- and post-EMT MCF-7 cells were cultured in the absence (diluent) or presence of AG1024 (10 μM; **A**) or AG1478 (10 μM; **B**) for 5 days prior to analyzing differences in cell growth and survival by MTS assays. Data are the mean growth (±S.E.; n=3; **P*<0.05; Student's *t*-test).