Supplementary Materials: NGF-Enhanced Vasculogenic Properties of Epithelial Ovarian Cancer cells is Reduced by Inhibition of the COX-2/PGE_2 Signaling Axis

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Table S1. Primer sequences used for traditional and real time PCR.

Gene	Primers	Application	Amplicon	Seq
COX-2	Sense: 5′-TTC AAA GAG ATT GTG GGA AAA TTG CT-3′ Antisense: 5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′	Traditional PCR	303 pb	NM_000963.3
Survivin	Sense: 5′-CTG GCA GCC CTT TCT CAA GGA-3′ Antisense: 5′-GCA ACC GGA CGA ATG CTT TT-3	Real-time PCR	225 pb	NM_001168.2
GAPDH	Sense: 5′-GAG TCA ACG GAT TTG GTC GT-3′ Antisense: 5′-ATC CAC AGT CTT CTG GGT G-3′	Traditional PCR	547 pb	NM_002046.6
beta-actin	Sense: 5′- TGGCAC CCA GCA CAA TGA AGA -3′ Antisense: 5′GAA GCA TTT GCG GTG GAC GAT 3′	Real-time PCR	166 bp	NM_001101.4

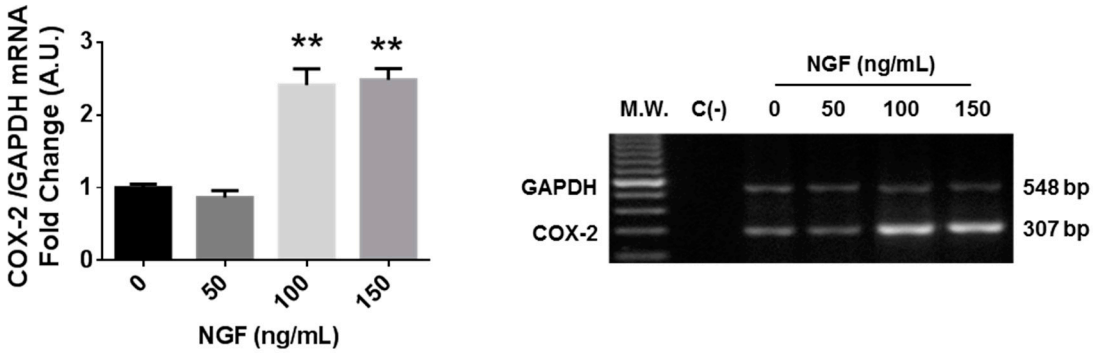

Figure S1. NGF increases COX-2 mRNA in EOC cells. A2780 cells were stimulated with NGF (50, 100 and 150 ng/mL) for 2 h and then analyzed by RT-PCR. Left: semi-quantification of COX-2 mRNA. Right: Representative image of COX-2 products in an agarose gel. $n = 4$. C: negative control. MW: molecular weight. ** = $p < 0.01$ with respect to basal condition. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).

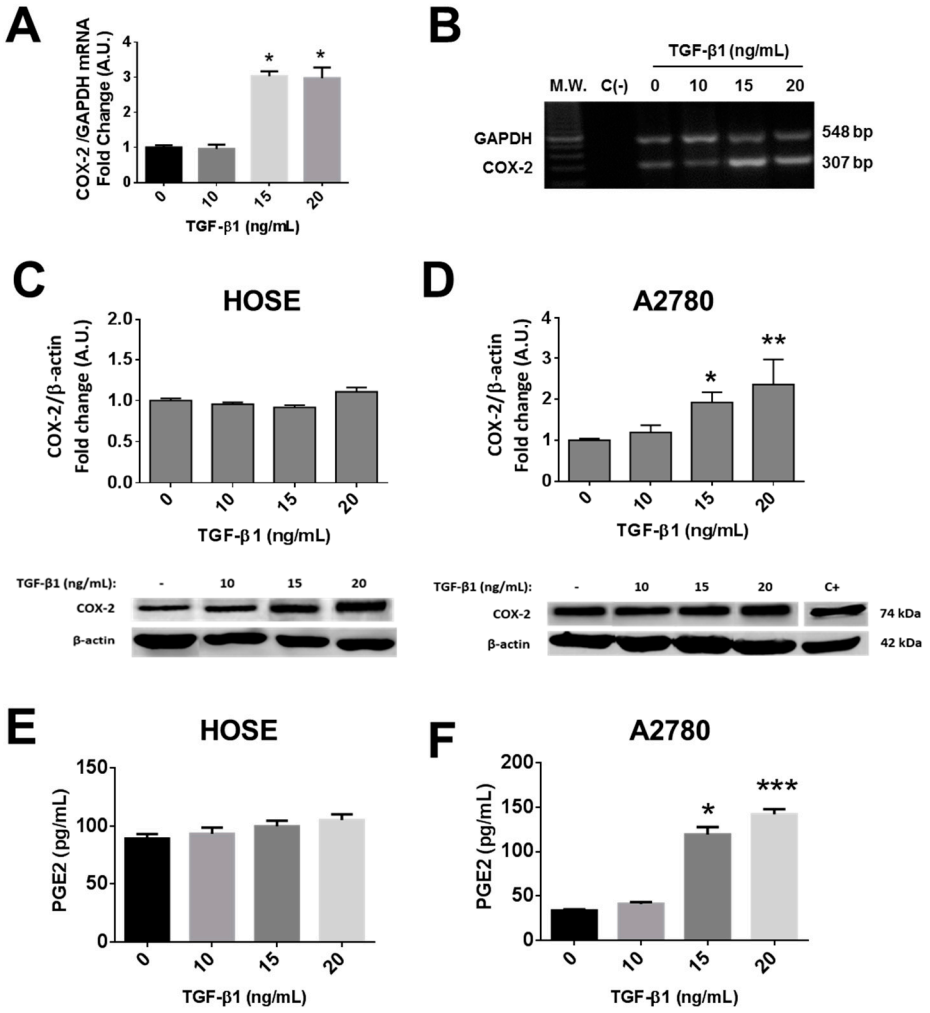

Figure S2. EOC cells respond to TGF-β stimulation increasing COX-2 and PGE_2. The ovarian cell lines HOSE and A2780 were stimulated with TGF-β (10, 15 and 20 ng/mL) for 2 h. (**A**): Semi-quantitative analysis of COX-2 mRNA levels induced by TGF-β stimulation. (**B**): A representative image of COX-2 products in an agarose gel. MW: molecular weight standard. C: negative control. (**C–D**): COX-2 protein levels induced by TGF-β stimulation in HOSE and A2780 cells evaluated by Western blotting. C+: extract from HEK cells that overexpress COX-2 was employed as a positive control. (**E–F**): PGE_2 levels in the culture supernatants of HOSE and A2780 cells, respectively, after TGF-β stimulation. n = 4. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$ as indicated or with respect to basal condition. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).

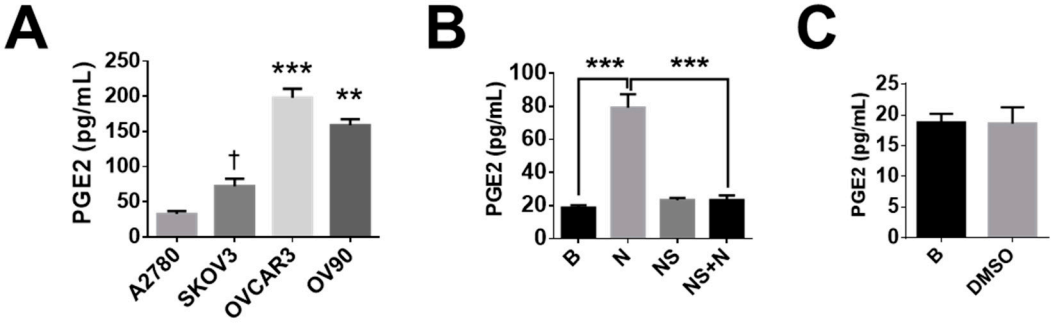

Figure S3. PGE_2 levels in media from different EOC cells before and after COX-2 inhibition. (**A**): Basal PGE_2 in the EOC cell lines A2780, SKOV3, OVCAR3 and OV90. ** = $p < 0.01$ and *** = $p < 0.001$ respect

to A2780 (Kruskal Wallis test and Dunn´s post-test). † = $p < 0.05$ respect to A2780 (Mann Whitney test). (**B**): A2780 cells were treated with the COX-2 inhibitor (NS, 20 μM) for 24 h and stimulated with NGF (N, 150 ng/mL) during the last 2 h. PGE_2 was measured in culture supernatants. (**C**): PGE_2 in culture supernatants of A2780 cells under basal conditions (B, without stimuli) or treated with DMSO (NS vehicle). $n = 4$ or more. *** = $p < 0.001$ as indicated. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results were expressed as mean ± standard error of the mean (SEM).

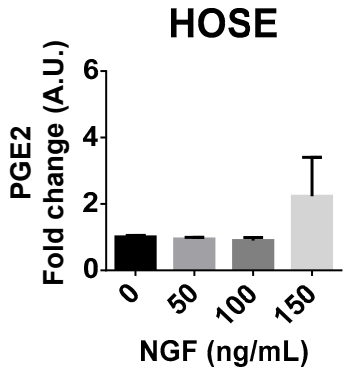

Figure S4. PGE_2 levels after NGF stimulation of HOSE cells. HOSE cells were stimulated with NGF (50, 100 and 150 ng/mL) for 2 h and PGE_2 was measured in the culture supernatants by ELISA.

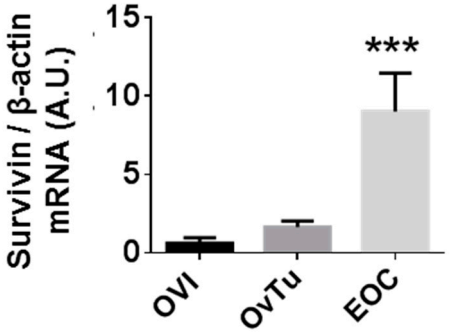

Figure S5. mRNA levels of survivin during EOC progression. Survivin mRNA was obtained from ovarian epithelium of inactive ovaries (OVI, $n = 4$), benign tumor (BeT, $n = 5$), borderline tumor (BorT, $n = 6$) and either well (EOC I, $n = 2$), moderately (EOC II, $n = 5$) or poorly differentiated (EOC III, $n = 8$) ovarian cancers. Then, samples were analyzed by real time PCR. *** = $p < 0.01$ vs. OVI. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).

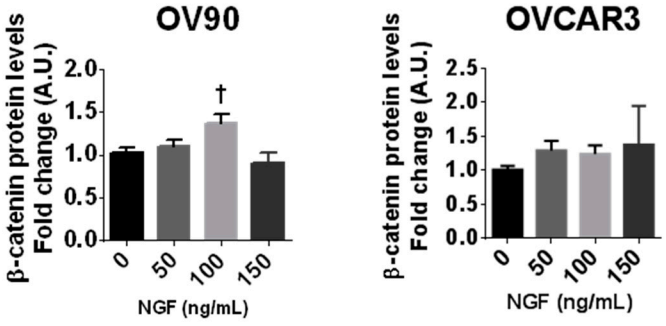

Figure S6. Effect of NGF on β-catenin protein levels in EOC cells. OV90 and OVCAR3 cells were serum-deprived for 24 h and then stimulated with NGF (0, 50, 100 and 150 ng/mL) for 8 h. $n = 4$ or more. † = $p < 0.05$ with respect to the basal condition (Mann Whitney test).

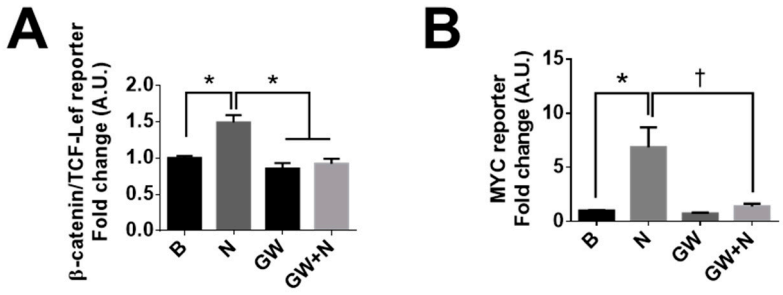

Figure S7. β-catenin/TCF-Lef and MYC transcriptional activity in A2780 cells. β-catenin/TCF-Lef (**A**) and MYC transcriptional activity (**B**) were measured in gene-reporter assays as described. B = basal condition (without stimuli), N = NGF (100 ng/mL), GW: GW441756 (specific TRKA inhibitor). $n = 4$ or more. * = $p < 0.05$ (Kruskal Wallis test and Dunn´s post-test) and † = $p < 0.05$ (Mann Whitney test) as indicated. Results are expressed as the mean ± standard error of the mean (SEM).

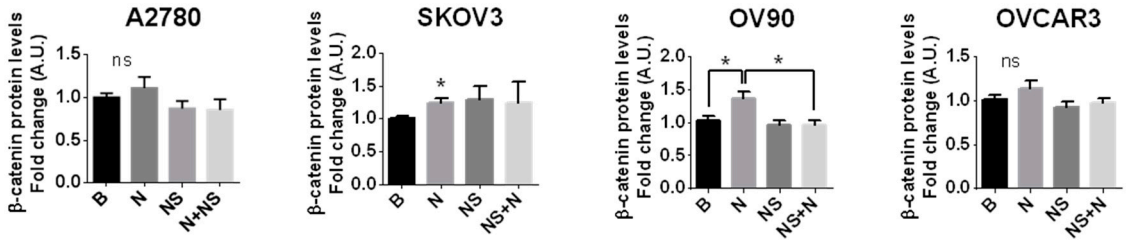

Figure S8. β-catenin protein levels after NGF stimulation and NS398 treatment of EOC cells. EOC cells were treated with the specific COX-2 inhibitor (NS, 20 μM, 24 h) and/or NGF (N; 150 ng/mL for 2h in SKOV3 cells; 100 ng/mL for 8 h in OV90 cells and 150 ng/mL for 8 h in OVCAR3 cells) and β-catenin levels were then assessed by western blotting and scanning densitometry. $n = 4$ or more. * = $p < 0.05$ with respect to basal condition (B, without stimuli). Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).

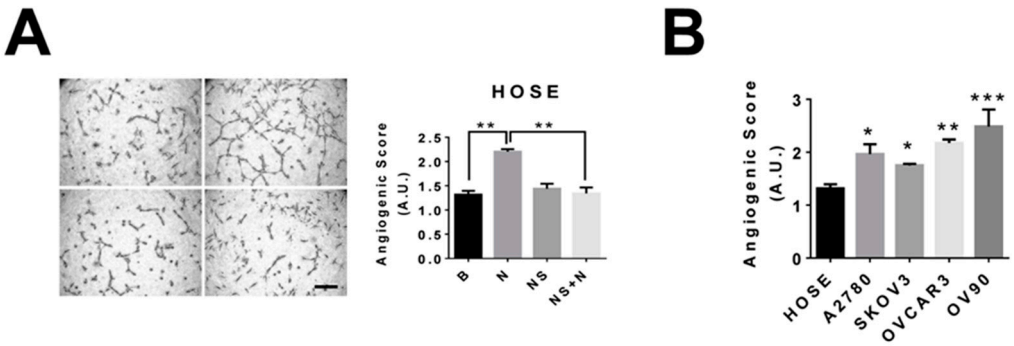

Figure S9. Angiogenic score of HOSE cells after NGF stimulation and NS treatment. (**A**): HOSE cells were stimulated with NGF (N, 100 ng/mL, 2h) and/or treated with the specific COX-2 inhibitor (NS, 20 μM, 24h). Then, culture supernatants were employed in tubule formation assays in matrigel using endothelial cells. Bar = 100 μm (**B**): basal angiogenic score of the different cell lines used. * = $p < 0.05$ and $p < 0.01$ with respect to the basal condition (without stimuli) or as indicated. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).

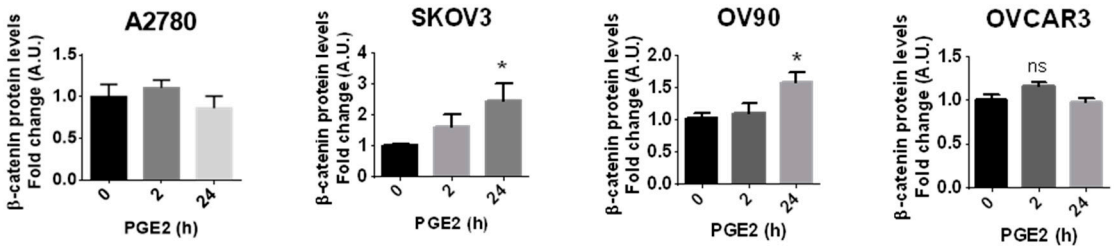

Figure S10. β-catenin protein levels after PGE_2 stimulation of EOC cells. EOC cells were serum-deprived for 24 h and then stimulated with PGE_2 (20 μM, 2 and 24 h). β-catenin levels were assessed by western-blotting and scanning densitometry. $n = 4$ or more. * = $p < 0.05$ with respect to the basal condition (without stimuli). ns: statistically not significant. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).

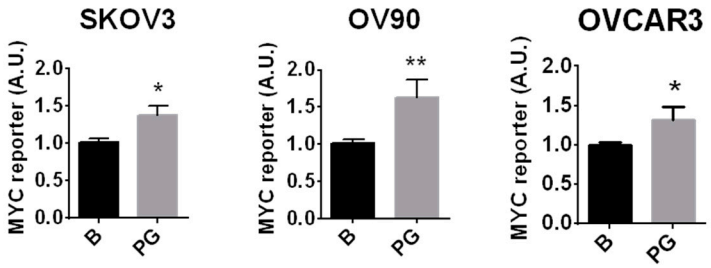

Figure S11. PGE_2 increases MYC transcriptional activity in EOC cells. EOC cells were incubated with PGE_2 (PG, 20 μM) for 24 h and reporter activity was measured. $n = 4$, * = $p < 0.05$ and $p < 0.01$ (Mann Whitney test). Results are expressed as the mean ± standard error of the mean (SEM).

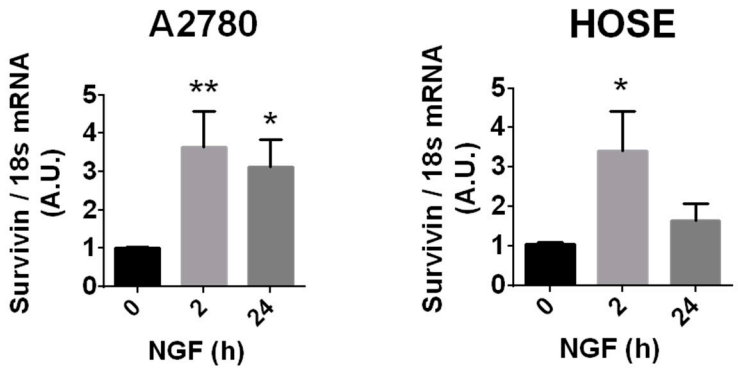

Figure S12. Survivin mRNA post- NGF stimulation in ovarian cells. A2780 and HOSE cells were stimulated with NGF (100 ng/mL, 2 or 24 h) and survivin mRNA was detected by qPCR. $n = 4$ independent experiments in duplicate. ** = $p < 0.01$ and *** = $p < 0.001$. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).

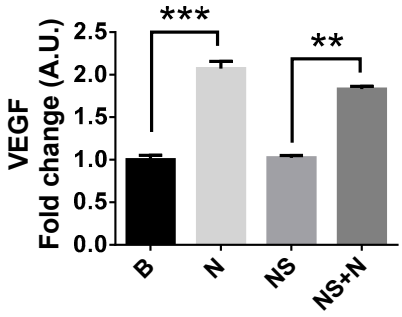

Figure S13. Effect of COX-2 inhibition for short periods of time on VEGF liberation by EOC cells. A2780 cells were incubated with the COX-2 specific inhibitor (NS, 20 μM) and stimulated with NGF (N, 150 ng/mL) for 2 h. Then, VEGF protein levels in the culture supernatants were measured. $n = 4$. ** = $p < 0.01$ and *** = $p < 0.001$. Statistical analysis: Kruskal Wallis test and Dunn´s post-test. Results are expressed as the mean ± standard error of the mean (SEM).