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Tumor Endothelin-1 contributes to macrophage infiltration and metastatic colonization of murine lungs by human cancer cells

Neveen Said [1], Steven Smith [1], Marta Sanchez-Carbayo [2] and Dan Theodorescu [1*]

[1] Department of Molecular Physiology, University of Virginia, Charlottesville, USA
[2] Spanish National Cancer Research Center (CNIO), Madrid, Spain

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[*]**Corresponding Author:** Dan Theodorescu: Departments of Surgery and Pharmacology and University of Colorado Comprehensive Cancer Center, Aurora, CO 80045, Tel: 303-724-7135, Fax: 303-724-3162
E-Mail: dan.theodorescu@ucdenver.edu.

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SUPPLEMENTARY FIGURE LEGENDS

SUPPLEMENTARY FIGURE 1: The association between the expression ET family genes, muscle invasion and disease specific survival patient survival in human bladder cancer. Datasets were used from Sanchez-Carbayo *et al.* (*S-C et al*. N= 26 and 65, for NMI and MI disease, respectively), *GSE13507* (N= 103 and 62 for NMI and MI disease, respectively) and *UVA85* (N=36 and 24, for NMI and MI, respectively). Data for the expression of *ECE-2* **(A)**, *ET-2* **(B)**, *ET-3* **(C)**, and ET_BR **(D)** were plotted using probsets identified in material and methods. Dotplots represent standardized (z-scored) logged (base 2) expression of probes comparing NMI and MI tumors and differences in distributions were tested by the Mann-Whitney U-test. Kaplan Meier curves show the association between expression of *ECE-2* **(E)**, *ET-2* **(F)**, *ET-3* **(G)**, and ET_BR **(H)** expression and disease specific survival (*DSS*) of patients.

SUPPLEMENTARY FIGURE 2: Correlation between ET-1 expression and key inflammatory cytokines in human cancer. Scatter plots of showing the correlation of ET expression (*abscissa*) versus **(A)** *MMP9* **(B)** *MMP2*, **(C)** *CCL2/MCP-1*, **(D)** *IL-6* , and **(E)** *PTGS2 /Cox2* (*ordinate,* respectively) in two of the gene profiling studies (described in the material and methods). ET-1 expression is significantly correlated with the expression of each of these targets at the Spearman correlation coefficients and P-values indicated in both studies. Datapoints for the Sanchez-Carbayo *et al.* (red) and UVA-Den (blue) were logged and standardized (z-scored) study to enable facile visualization of their similar correlation patterns on the same scales, while correlation coefficients and *P*-values for each study are indicated in red or blue as appropriate.

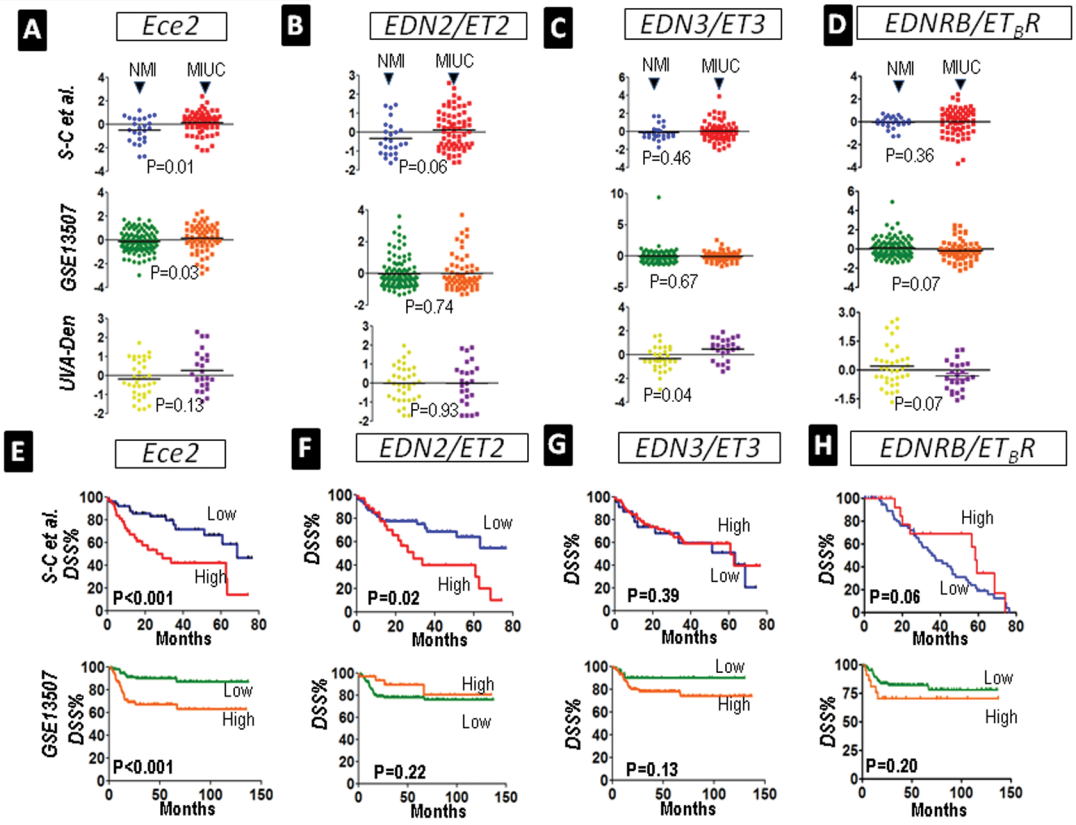
SUPPLEMENTARY FIGURE 3: Effect of co-culture of UMUC3 and U937 on cell number. UMUC3 cells (5000cells/ 1ml medium containing 2%FBS) were plated in wells of 24-well plates, and U937 (5000cells/100µl) were added on the upper well of 0.4µm inserts, either in single cell or co-culture. Each cell line was collected at 72 hours and cell number was determined by CyQuant assay.

SUPPLEMENTARY FIGURE 4: (A) Effect of ET_BR blockade on spontaneous MB49 metastasis to the lungs. Scatter plots showing the incidence and volume of lung metastases that developed 3W after subcutaneous inoculation of MB49 cells ($1x10^5$/cells) in C57Bl6 mice treated or not with ET_BR blocker BQ788 before (BQ-pre) or after (BQ-post) MB49 injection. **(B)** Mac2 immunostaining of lungs harboring spontaneous MB49 metastases (100x magnification) revealed no significant difference in macrophages infiltrating the metastatic foci (in-tumor) or the surrounding lung tissue (parenchyma). **(C)** Effect of ET axis on in vitro MB49 cell proliferation. **(D)** ET-1, cytokines and Cox-2 levels in lungs 1 week after subcutaneous inoculation of MB49 cells ($1x10^5$/cells) in C57Bl6 mice.**P*<0.05 compared to corresponding normal lung, two-tailed Student's *t*-test.

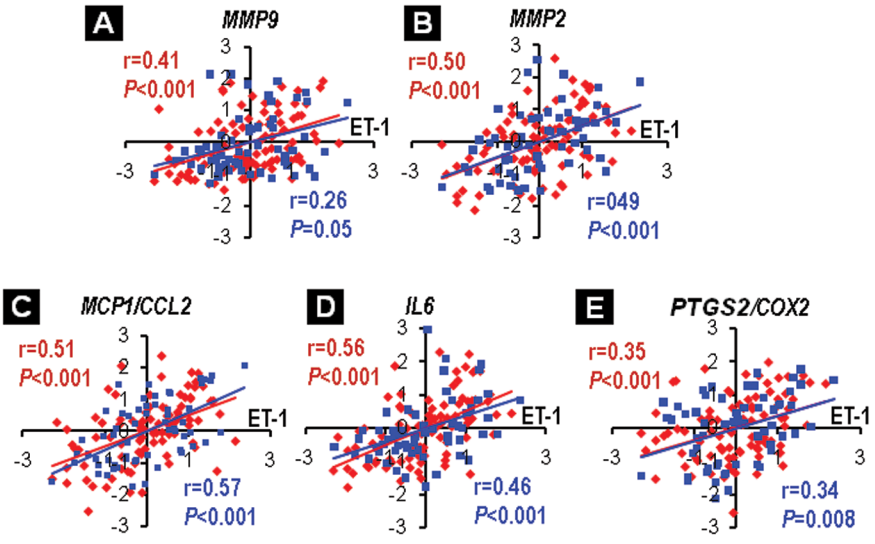
SUPPLEMENTARY FIGURE 5: (A) Expression of ET-1 axis proteins in tissue lysates. Western blots showing the expression of ECE-1, ET_AR and ET_BR proteins in lung lysates 6W after tail vein injection of control (NTsh) or UMUC3 cells depleted of ET-1 (shET-1). **(B)** Female nude mice (N=5) were injected via tail vein with 10^6 T24 (non-metastatic) and T24T (metastatic) cells and lungs harvested at 6 weeks. Experiment was repeated with T24 stably transfected with pcDNA or pcDNA expressing ET-1 as described in methods (*inset*, western blot for ET1 and tubulin)

SUPPLEMENTARY FIGURE 6: **Effect of endothelin axis manipulations on subcutaneous tumor growth. (A)** Depletion of ET1 in UMUC3 cells by shRNA, and pharmacologic blockade of either ET_AR or ET_BR had no significant effect on the in vivo growth of UMUC3 cells after subcutaneous implantation of 1x106 cells/100µl phenol red-free medium and follow up for 6 weeks. **(B)** Mac2 immunostaining of SC tumors (100x magnification) revealed no difference in macrophage infiltration. ET-1 levels **(C)**, Cox-2 activity **(D)** as well as human and murine IL-6 and MCP-1 **(E)** showed no significant difference between UMUC3-shET1 tumors and their control UMUC3-NTsh as well as between tumors from mice treated with either ZD4054 or BQ788 and their vehicle controls.

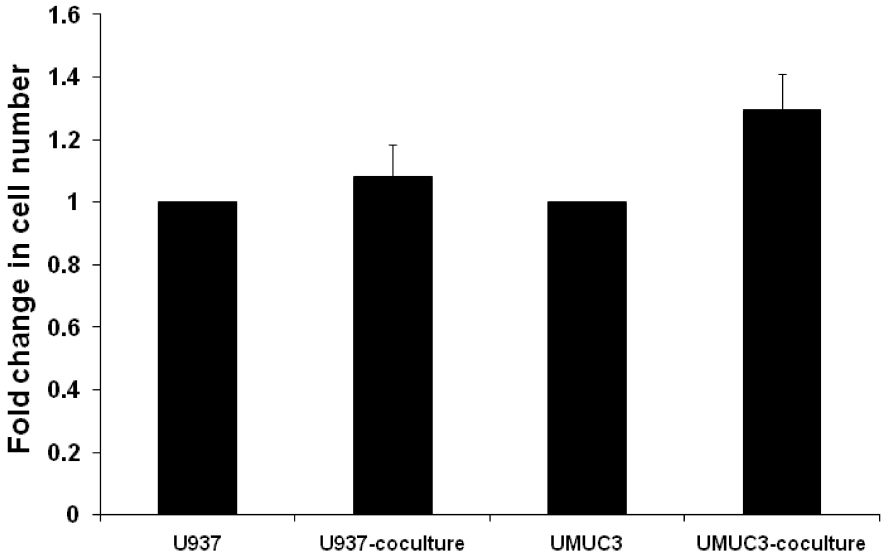
Supplementary Figure 1



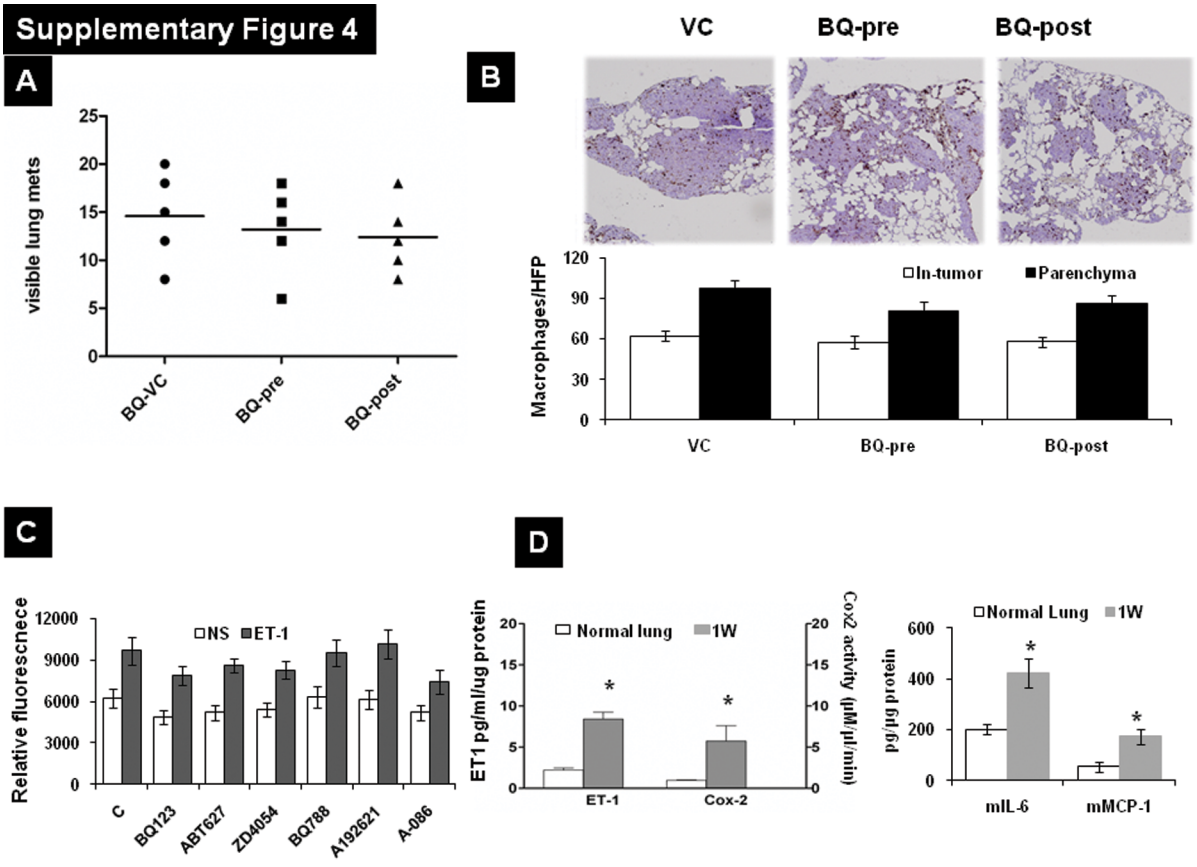
Supplementary Figure 2



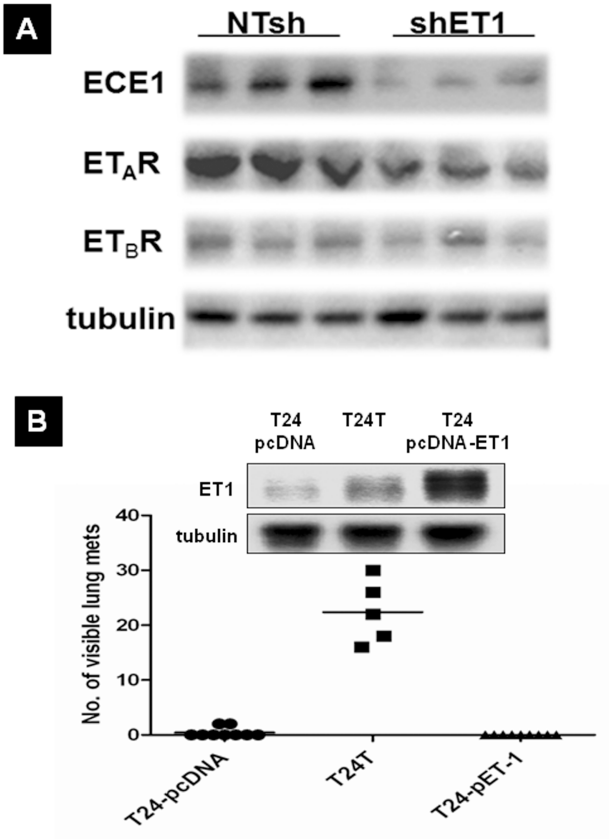
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6