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Endoglin suppresses human prostate cancer metastasis

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Abstract

Endoglin is a transmembrane receptor that suppresses human prostate cancer (PCa) cell invasion. Small molecule therapeutics now being tested in humans can activate endoglin signaling. It is not known whether endoglin can regulate metastatic behavior, PCa tumor growth, nor what signaling pathways are linked to these processes. This study sought to investigate the effect of endoglin on these parameters. We used a murine orthotopic model of human PCa metastasis, designed by us to measure effects at early steps in the metastatic cascade, and implanted PCa cells stably engineered

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to express differing levels of endoglin. We now extend this model to measure cancer cells circulating in the blood. Progressive endoglin loss led to progressive increases in the number of circulating PCa cells as well as to the formation of soft tissue metastases. Endoglin was known to suppress invasion by activating the Smad1 transcription factor. We now show that it selectively activates specific Smad1-responsive genes, including JUNB, STAT1, and SOX4. Increased tumor growth and increased Ki67 expression in tissue was seen only with complete endoglin loss. By showing that endoglin increased TGF β -mediated suppression of cell growth in vitro and TGF β -mediated signaling in tumor tissue, loss of this growth-suppressive pathway appears to be implicated at least in part for the increased size of endoglin-deficient tumors. Endoglin is shown for the first time to suppress cell movement out of primary tumor as well as the formation of distant metastasis. It is also shown to co-regulate tumor growth and metastatic behavior in human PCa.

Keywords

Endoglin; Prostate cancer; Metastasis; Transforming growth factor β ; Circulating tumor cells

Introduction

Prostate cancer (PCa) is the second most common cause of cancer related death for men in the United States [1]. The development of metastatic disease is responsible for essentially all deaths, and for the severe morbidity seen in individuals with advanced disease [2]. In order to metastasize, cancer cells must progress through a series of steps, which together are termed the metastatic cascade [3]. Cell invasion represents an initial step in this cascade, and the invasive capacity of cells represents a major determinant of their metastatic potential [3,4]. Therefore, proteins that regulate cell invasion represent determinants of critical biological behavior.

We have previously demonstrated that endoglin inhibits human PCa cell invasion [5,6]. Further, endoglin expression was shown to be lower in PCa cells as compared to normal prostate epithelial cells from the same patient, as well as lower in metastatic variant cells as compared to the parental cell line [5]. Endoglin is a transmembrane protein that belongs to the transforming growth factor (TGF) β receptor superfamily [7]. Signaling through TGF β superfamily receptors is complex, and is best understood for TGF β itself, which is considered the canonical pathway (reviewed in [8]). Soluble TGF β ligand interacts with a type II receptor subtype (RII; TGF β RII in the case of TGF β), which in turn phosphorylates a type I receptor subtype (RI; ALK5 in the case of TGF β), and this in turn phosphorylates and activates Smad proteins (Smad3 in the case of TGF β), which act as transcription factors. Endoglin is considered an accessory, or type III, TGF β superfamily receptor subtype.

Findings from our initial investigations provided evidence that endoglin represented a primary regulator of human prostate cancer cell motility [9]. In those studies we had used a gene expression array to screen for genes that were differentially regulated during changes in human prostate cancer cell motility. Of thousands of genes evaluated, only endoglin was affected. Since then, accumulating evidence further supports the notion that endoglin has a primary regulatory role. This is because endoglin has been shown to regulate signaling by facilitating the activation of specific RI subtypes, thereby acting as a signaling pathway gatekeeper. Endoglin's role in this regard has been shown by Bertolino et al. in endothelial cells [10], as well as by us in human prostate cells [6]. In particular, we demonstrated that endoglin selectively enhanced signaling through the RI subtype, ALK2 [6]. ALK2 is considered a bone morphogenetic protein (BMP) receptor. We went on to demonstrate that endoglin and ALK2 activated the BMP responsive Smad, Smad1 [6]. Smad1 suppressed

human prostate cell invasion, and was necessary for endoglin-mediated suppression of invasion. In contrast to Smad1, Smad3 increased invasion. Endoglin-mediated activation of Smad1 was not dependent upon TGF β , nor upon signaling through the ALK5/Smad3 axis [6]. Importantly, we demonstrated that it was the balance between anti-invasive Smad1 and pro-invasive Smad3 that served as the determinant of PCa cell invasion. Endoglin increased the ratio of activated Smad1 to activated Smad3. Endoglin did not affect Smad3 activation, but increased this ratio by increasing activated Smad1.

In a related series of studies we demonstrated that the endoglin signaling axis was an important target of small molecule therapeutics [11]. Specifically, 4',5,7-trihydroxyisoflavone (genistein) has been shown to activate Smad1 and to suppress PCa cell invasion in a manner that is dependent upon the kinase activity of ALK2. In mice, we demonstrated that genistein inhibits human PCa cell metastasis [12]. In a series of studies in man, we demonstrated that genistein was well tolerated and that it inhibits the expression of matrix metalloproteinase 2 (MMP-2) in prostate tissue [13,14].

It is not known whether endoglin regulates metastatic behavior, what genes are regulated by endoglin in human prostate, and nor what role, if any, endoglin plays in regulating tumor growth. We hypothesize that endoglin suppresses human PCa metastasis. In the current study we demonstrate for the first time that endoglin suppresses the entry of cancer cells into the circulation, suppresses metastasis, selectively increases the expression of the Smad1-responsive genes, JUNB, STAT1, and SOX4 in tumor tissue, and that it also suppresses tumor growth at least in part by suppressing cell proliferation through a TGF β -dependent mechanism. We propose a model wherein endoglin's effects upon multiple signaling pathways are integrated at the cellular level thereby defining the cell's ultimate phenotype.

Materials and methods

Cell culture

The engineering and phenotypic characterization of endoglin variant cell lines, from parental PC3-M cells, has been described by us [5]. HI-ENG1 and HI-ENG2 cells express high levels of L-endoglin, VC cells express low but detectable levels of endoglin, and NO-ENG1 and NO-ENG2 cells do not express detectable levels of endoglin protein. In NO-ENG cells, endoglin is suppressed by an antisense vector. We have previously shown that the corresponding sense vector control cells behave in an identical fashion to both VC and non-transfected cells (i.e., wild type PC3-M cells) with respect to cell adhesion, migration and invasion in vitro [5]. Therefore, only VC cells were used in the current study. Cell lines were authenticated according to methods described in the American Type Culture Collection Technical Bulletin No. 8, Cell Line Verification Test Recommendations [15]. Specifically, cells from low passage (i.e., <15 passages) frozen stocks were used and were replenished after 20 passages, cells underwent routine microscopic examination to confirm uniform and standard cellular architecture and no microbial infection, and cells were tested (within 3 months) and found negative for mycoplasma infection.

In some experiments, PC3-M cells stably transfected with green fluorescent protein (GFP) were used. The engineering and characterization of PC3-M-GFP cells has previously been described by us [12]. When implanted into the prostates of mice, cells were suspended in 35 μ l of RPMI 1640 (Gibco, Grand Island, NY), as described by us [12].

Cell invasion

Cell invasion assays were performed as previously described by us [11]. Briefly, 10⁴ cells in 52 μ l serum free media containing 0.1% bovine serum albumin were placed into the upper chamber of a 48-well Boyden chamber apparatus (Neuro Probe, Gaithersburg, MD). Cells

invaded toward NIH-3T3 conditioned media in the lower chamber for 15 h. Chambers were separated by a Nuclepore Track-Etch Membrane that contained 8 μ m pores (Whatman, Clifton, NJ) and that was coated with denatured collagen (product 214340; Difco-Becton Dickinson, Sparks, MD). Cells were then fixed and stained with the Diff-Quick cell-staining kit (Dade Behring AG; Duding, Switzerland) according to manufacture's instructions, and the membranes were mounted onto slides. The number of invading and non-invading cells was then counted under light microscopy, using predetermined field coordinates, and the percentage of invading cells was determined. Two separate experiments were performed at separate times, with four replicate samples run within each experiment, for each experimental condition.

Animal housing and orthotopic implantation

Inbred 4 week old male athymic BALB/C mice (Charles River Laboratories) were treated under a Northwestern University ACUC approved protocol. Mice were housed in a barrier facility with 12 h light/dark cycles, and given food (Harlan Teklad 2016S® chow) and water ad libitum. Orthotopic implantation of cells into the dorsal lobe of the prostate was performed as previously described by us [12]. Briefly, 10^6 cells in 35 μ l serum free media were injected under prostatic capsule under direct visualization, thus allowing confirmation of the formation of a bleb. Mice not recovering from surgery or that became moribund, and were therefore euthanized, within the 48 h postoperative period, were considered perioperative mortalities.

Examination of organs and quantification of lung metastasis

Necropsy was performed at 4 or 5 weeks after implantation, as indicated, and metastasis quantified, as previously described by us [12]. Briefly, the prostate tumor volume was calculated as $0.52 \times (\text{width})^2 \times (\text{length})$ from measures taken in perpendicular dimensions. Lungs and prostate tumor were fixed in 10% formalin. A portion of prostate tumor samples were snap frozen. All major organs were examined for microscopic evidence of metastasis on 5 μ m hematoxylin and eosin (H&E) stained sections. Lungs were completely step sectioned at 30 μ m increments in the sagittal plane to expose all lobes in one plane, and 5 μ m H&E stained slides were prepared at each step section. All slides from each mouse were then examined by a single person (ML) in a blinded fashion under light microscopy. Metastatic human prostate cancer cells could be readily distinguished from other cells in the lung on H&E slides (see Fig. 2a). The number of metastatic cells was counted on each H&E stained slide, on all H&E stained slides from a given mouse. In this manner the total number of metastatic cells present in the lungs of each mouse was determined. For prostate tumors, tissue was step sectioned at 1 mm increments and processed for H&E and for immunohistochemical staining as described below.

Measurement of viable prostate cancer cells in the blood

A terminal blood draw was performed via cardiac puncture, and the resultant number of viable PCa cells was measured as previously described [16,17]. Briefly, blood was collected into a preservative free lithium heparin coated tube. After centrifugation, the resultant buffy coat and serum layers were plated into α -MEM (Gibco) with 5% fetal bovine serum. The following day, plates were rinsed twice with phosphate buffered saline (PBS), and cultured in RPMI 1640 (Gibco), 10% FBS, in the presence of G418. Ten days after plating, groups of ≥ 50 cells were scored as colonies, and counted. A terminal blood draw was performed on all animals. However, only animals from which 100 μ l or more blood was harvested did we then go on to measure cancer cells in the blood. The tumor blood burden per ml blood was taken as the number of colonies divided by the volume of blood taken.

Immunohistochemistry

Immunohistochemical staining was performed as previously described by us, with modifications [18,19]. Primary tumor tissues were rehydrated, treated with hydrogen peroxide to quench endogenous peroxidase activity, and blocked with goat serum. Tissues were then stained with either anti-Ki67 (rabbit polyclonal; DAKO, Carpinteria, CA), diluted 1:200 in 5% goat serum, or with anti-GFP antibody (clone A11122, Molecular Probes), diluted 1:50. Signal was detected by using the EnVision™ + System (DAKO), which employed a streptavidin–biotin polymer conjugated secondary antibody, along with 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA), both per manufacturer's instructions. Tissue was counterstained with hematoxylin (Zymed). Adjacent sections of primary tumor were stained for TUNEL using the Apoptag detection kit (Chemicon, Temecula, CA), per manufacturer's instructions.

Adjacent sections of immunostained tissue were read by a single GU pathologist (VA) in a blinded and batch fashion. Immunohistochemical scoring employed a semi-automated digital scanning system, designed to minimize reader bias. Slides were scanned at 20× on a ScanScope CS® (Aperio Technologies, CA). On resultant digitized H&E slides, 10 regions of interest (ROIs) were prospectively identified. Areas of infarction and necrosis were avoided. ROIs were imported onto adjacent slide sections, stained for Ki67 or TUNEL, as digital overlays. Ki67 was scored using the inbuilt 'positive pixel count algorithm', which computes the number of weak, moderate and strong staining pixels within the ROI, as well as the mean intensity within each of these categories. Settings were adjusted to exclude non-specific background staining. TUNEL was scored using the 'CoLocalization' algorithm, which identifies co-localized brown and blue pixels—and thus 'nuclear' staining—and computes their staining index as above. For Ki67 and TUNEL, H-scores were calculated by determining the product of percent staining and average intensity, thus providing a measure of overall staining within the ROI.

Western blots

Protein isolation from frozen tissue was performed as described by us, with modifications [18,20]. Briefly, snap frozen tumor tissue was extracted with RIPA buffer containing protease (aprotinin, leupeptin, pepstatin, and 1 mM EDTA) and phosphatase inhibitors (NaVO₄, NaF, and Phosphatase Inhibitor Cocktails #1 and #2; Sigma–Aldrich). Immunoblotting was performed as described by us [6], and used the following antibodies: anti-cleaved caspase 3 and anti-Smad3 (Cell Signaling Technology); anti-Smad1 (Upstate Biotechnology, Lake Placid, NY, USA); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA). All Western blots were repeated once, at a separate time.

RNA isolation and quantitative reverse transcription/polymerase chain reaction (qRT/PCR)

RNA was isolated from snap frozen prostate tumor tissue using RNeasy RNA isolation kit from Qiagen (Valencia, CA), per manufacturer's instructions. RNA was treated with RNase free DNase, its quality and quantity assessed by optical density, and qRT/PCR performed on a dedicated ABI 7500 qPCR workstation, all as previously described by us [21,22]. Validated gene specific primer/probe sets for ID1, ID2, STAT1, JUNB, SOX4, MMP-2, MMP-9 and GAPDH were from Applied Biosystems. All primers were exon spanning, except for SOX4, which only contains 1 exon. RT minus control reactions were run as a negative control. Negative control reactions, in particular those for SOX4, were always negative under all reaction conditions. Assays were run in replicates of 2 and the resultant mean threshold cycles were used for further analysis. Assays were repeated at a separate time, also in replicates of 2. The threshold cycle (Ct) for individual reactions was identified through Applied Biosystems 7500 Real Time PCR System software. Gene expression was

normalized to that of GAPDH, and relative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method [23].

Thymidine uptake

Thymidine uptake was measured as previously described by us [24]. Briefly, 24 h after plating into 96 well plates, cells were changed to serum free media, and treated for 24 h with TGF β , as indicated. For the final 6 h, cells were treated with tritium-labeled thymidine, and incorporation into cellular DNA measured by scintillation counting. Cells were cultured under exponential growth sub-confluent conditions. Assays were run in replicates of 3, and repeated at a separate time, in replicates of 3.

Statistical analysis

Two or more treatment groups were compared by means of Chi-squared or Fisher's Exact test as appropriate for categorical outcomes, and by two-sided Student's *t* test or one-way ANOVA as appropriate for continuous variables, as denoted. Statistical significance was considered present for *P*-values of ≤ 0.05 . To evaluate the association between tumor weight and metastatic burden, the Spearman correlation coefficient was used. Statistical tests were performed with the statistical software package "R" version 2.8 (r-project.org) and SAS, V9.1 (Cary, NC).

Results

Characterization of endoglin variant cell lines

In this study we used human PCa cells that were previously engineered and characterized by us [5] to stably express high levels of L-endoglin (HI-ENG1 and HI-ENG2 cells), native levels of endoglin (VC cells; for vector control), and cells expressing no detectable levels of endoglin (NO-ENG1 and NO-ENG2 cells). Because endoglin is a cell surface protein, we sought to detect it on the surface of cells by immunohistochemical staining without cell permeabilization. HI-ENG1 and HI-ENG2 cells were thereby shown to express high levels of endoglin, VC cells low but detectable levels, while NO-ENG1 and NO-ENG2 cells expressed no detectable cell surface endoglin, and looked identical to isotype antibody stained negative control cells (Fig. 1a).

The ability of individual cell lines to invade was next assessed in a Boyden Chamber cell invasion assay. Equal numbers of cells were placed into the upper chamber of an invasion apparatus, and the number of cells invading towards conditioned media was then measured 15 h later, and expressed as the percent of VC cells. Compared to VC cells, HI-ENG1 and HI-ENG2 cells both invaded significantly less, while NO-ENG1 and NO-ENG2 cells both invaded significantly more (two-sided Student's *t* test $P < 0.05$) (Fig. 1b). HI-ENG1, HI-ENG2, NO-ENG1 and NO-ENG2 cells each constitute clonal cell lines derived from a single cell. However, multiple clones are seen in metastatic PCa lesions in man [25]. We therefore combined HI-ENG1 and HI-ENG2 cells in a 1:1 ratio, thus giving a mixed clonal population of HI-ENG cells. An identical approach was taken for NO-ENG1 and NO-ENG2 cells, giving a mixed clonal population of NO-ENG cells. In each instance, individual clonal cells were combined just before conducting invasion assays, or before implantation into animals, as indicated. VC cells already consisted of a mixed clonal population and were therefore not altered. HI-ENG and NO-ENG cells exhibited significantly lower and higher invasion, respectively, compared to VC cells (two-sided Student's *t* test $P < 0.05$) (Fig. 1c).

Alternative splicing of the endoglin transcript produces two isoforms: long (L)-endoglin, which has 47 amino acids in the cytoplasmic domain, and short (S)-endoglin, which has 14 amino acids [26]. We focused the current studies on L-endoglin. We have previously shown

that L- and S-endoglin both equally suppress human PCa cell invasion [5]. We have also shown that transient up or down regulation of L-endoglin (i.e., after transient transfection) affects cell invasion in an identical fashion to stably transfected cells, and does so across several different human prostate cell lines [6].

Endoglin suppresses human prostate cancer metastasis

To investigate the hypothesis that endoglin suppresses human PCa metastasis, mice were orthotopically implanted with HI-ENG, VC or with NO-ENG cells. Four weeks later, the number of metastatic cells in the lung of each mouse, the tumor volume, and the animal weight were measured. Representative photomicrographs of H&E stained primary tumor and metastatic lung deposits are depicted in Fig. 2a. The characteristically large PC3-M cells and nuclei (black arrow) allowed for easy detection by histomorphologic examination, under conventional light microscopy, of H&E stained lung tissue. By counting the number of metastatic cells present on all H&E stained slides from a given mouse, the number of metastatic cells for that mouse was determined. Even though metastatic human PCa cells were readily distinguishable from other cell types present in mouse lung tissue by morphologic appearance, we went on to confirm their origin. In separate studies mice were implanted with PC3-M cells transfected with green fluorescent protein, i.e., with PC3-M-GFP cells (Fig. 2b). PC3-M cells are the cell line from which HI-ENG, VC, and NO-ENG cells were derived. Immuno-staining of primary prostate tumor and of lung tissue for GFP confirms the identity of these cells (Fig. 2a).

In experiment #1, 8 mice per cohort were implanted with a given endoglin variant cell type. One VC implanted mouse died in the perioperative period. The number of mice developing lung metastasis and the number per mouse is depicted in Fig. 2c. Considering data from all three cohorts demonstrates that endoglin significantly suppressed the formation of metastases (chi-squared P value <0.001). For NO-ENG, VC, and HI-ENG cohorts, the percentage of mice with metastasis was 88, 29, and 0%, respectively. Further, for mice with metastases, the mean number of metastases per mouse was 94 (range 3–431), 9 (1–17), and 0 (N/A), respectively. Tumor volume did not differ significantly between VC and HI-ENG cohorts, but mean tumor volume was 6.5-fold higher in the NO-ENG as compared to the VC cohort (two-sided Student's t test $P = 0.05$) (Fig. 2d). Body weight was 9% lower in the NO-ENG cohort, and 9% higher in the HI-ENG cohort, compared to the VC cohort (two-sided Student's t test $P \leq 0.05$ for both) (Fig. 2d).

These findings demonstrate for the first time that endoglin can suppress metastasis. They also show for the first time that tumor growth is enhanced when endoglin is at very low levels. These findings were substantiated by conducting an expanded repeat experiment. In experiment #2, a fourth cohort was included in which mice were implanted with HI-ENG cells, but were maintained for an additional week (i.e., for 5 weeks). We had previously demonstrated that increased incubation time correlated with increased metastases in this model [12]. Thus, the HI-ENG $\times$ 5 week cohort provided a measure of endoglin's ability to suppress metastasis under conditions that fostered increased metastasis. Because of the negative impact of systemic cancer upon animal health beyond 4 weeks, it was not possible to maintain mice in other cohorts beyond 4 weeks. In experiment #2, 7 mice were implanted per cohort. Two mice each in the HI-ENG and HI-ENG $\times$ 5 week cohorts died in the perioperative period.

The findings of experiment #2 were similar to those of experiment #1 (Fig. 2c, d). While more metastases were observed across all cohorts in experiment #2 compared to experiment #1, importantly, within each experiment the same differences between cohorts was observed. Specifically, a consideration of data from the NO-ENG, VC, and HI-ENG 4 week cohorts demonstrates that endoglin significantly suppressed the formation of metastasis (chi-

squared P value = 0.02). For NO-ENG, VC, and HI-ENG cohorts, the percentage of mice with lung metastasis was 100, 71, and 40%, and for those with metastases, the mean number per mouse was 347 (range 6–1387), 402 (2–1421), and 193 (23–363), respectively. Tumor volume did not differ significantly between VC and HI-ENG cohorts, but was ninefold higher in the NO-ENG as compared the VC cohort (two-sided Student's t test P value = 0.002) (Fig. 2d). Body weight was significantly lower in the NO-ENG cohort by 8% (two-sided Student's t test P value <0.05), and non-significantly higher in the HI-ENG as compared to the VC cohort (Fig. 2d).

Additional findings related to the HI-ENG $\times$ 5 week cohort. Tumor growth continued during the additional week of incubation, giving a mean tumor volume 1.5-fold greater than the HI-ENG $\times$ 4 week cohort (not statistically significant). Despite the extended incubation time and increased tumor growth, only 60% of mice had metastasis, and for those with metastasis the mean number per mouse was only 11 (range 3–24). These values did not differ significantly from the respective values of 40% and 193 observed in the HI-ENG $\times$ 4 week cohort (two-sided Student's t test). Finally, the weight of the HI-ENG $\times$ 5 week cohort did not differ from that of either VC or HI-ENG $\times$ 4 week cohorts (two-sided Student's t test).

Endoglin suppresses viable prostate cancer cells in the blood

Endoglin inhibits PCa cell detachment and invasion, which represent initial steps in the metastatic cascade [5]. We hypothesized that if endoglin was inhibiting metastasis at least in part by inhibiting early steps in the metastatic cascade, then it should suppress the movement of PCa cells out of the primary tumor and into the blood. The presence of cancer cells in the blood provides a measure of their ability to complete the early steps in the metastatic cascade, and in particular, the steps required for cell movement out of the primary organ and into the circulation. Circulating cancer cells in the blood are increasingly being evaluated clinically as potential markers of the future development of metastasis [27–30]. The number of viable PCa cells in the blood of mice at 4 weeks after orthotopic implantation was determined in both experiments. Considering data from all mice, endoglin significantly suppressed circulating viable PCa cells (Fisher's exact P -value = 0.015) (Fig. 3a). Circulating viable PCa cells were present in 38, 18, and 0% of NO-ENG, VC, and HI-ENG mice, respectively. The mean number of colonies/mouse/ml blood, for those with circulating cells, was 643 (range 25–1926), 19 (15–23), and N/A, respectively.

Colonies arising from the blood were of human cell origin. In separate experiments wherein mice were implanted with PC3-M-GFP cells, all emergent colonies contained GFP as assessed by fluorescent microscopy. Figure 3b depicts a typical PC3-M-GFP cell colony. Cells lacking GFP do not fluoresce (Fig. 3b).

Loss of endoglin increases cell proliferation

Having shown for the first time that complete loss of endoglin increased tumor size, we conducted a series of studies to evaluate the underlying mechanism. We hypothesized that increased tumor size would be due to increased cell proliferation and/or decreased apoptosis. Ki67 is known to be elevated in proliferating human PCa cells, and increased Ki67 is a poor prognostic marker in men with PCa [31–33]. Tumor tissue was stained for Ki67; a representative photomicrograph is depicted in Fig. 4a. Photomicrographs of H&E, Ki67, and TUNEL stained tissue from all mice examined are depicted in supplemental Fig. 1. On H&E stained slides, regions of interest (ROIs) were prospectively identified in a blinded fashion. ROIs are denoted by the squares superimposed on the tissue in Fig. 4a. ROIs were imported as digital overlays onto adjacent Ki67-stained slide sections, and Ki67 was scored using an inbuilt algorithm of the Aperio ScanScope CS[®] digital slide scanner workstation. The resultant quantification of data from all mice is depicted in Fig. 4b. Ki67 was 2.3-fold higher

in NO-ENG compared to VC mice (two-sided Student's *t* test *P*-value = 0.008), but did not differ between VC and HI-ENG mice. These findings indicate that complete loss of endoglin increases cell proliferation. Further, they suggest that increased cell proliferation may be responsible for the increased tumor size observed in NO-ENG mice.

To evaluate the possibility that changes in cell death contributed to the increased tumor size observed in NO-ENG mice, we performed a series of studies. Importantly, TUNEL staining of tumor tissue revealed no increase in TUNEL activity in NO-ENG compared to VC tissue (Fig. 5a, b). Interestingly, TUNEL staining was 2.3-fold higher in HI-ENG, compared to VC tissue. However, this increase was not significant (two-sided Student's *t* test *P* value >0.05). Further, the tumor size did not differ between these two cohorts (see Fig. 2d). Despite this, we conducted additional investigations to investigate this finding. Specifically, we measured cleaved caspase 3 protein levels in tumor tissue by Western blot, demonstrating that there were no differences amongst the three cohorts of mice (Fig. 5c, d). Of importance, there was no increase in cleaved caspase 3 in NO-ENG tumors compared to VC. Together, these findings provide no evidence that the increased tumor size observed in NO-ENG mice is due to a decrease in cell death. Taken with the Ki67 findings, they support the notion that endoglin loss increases cell proliferation.

Taken together the above findings support the notion that endoglin affects two cellular processes: metastasis and cell proliferation, the latter in turn affects tumor size. If metastasis was simply driven by increased tumor size, then tumor size should correlate with the number of metastases in a given mouse. We went on to demonstrate that this was not the situation by comparing tumor volume and number of metastases for all mice at the 4 week time point. The resultant Spearman correlation coefficient, *R*, for NO-ENG, VC, and HI-ENG cohorts was -0.22, -0.24, and -0.35, respectively. For all mice in all cohorts considered together, *R* was -0.11.

Loss of endoglin inhibits TGF β -mediated regulation of cell proliferation and expression of TGF β -responsive genes

While endoglin is known to bind TGF β [34], the biological relevance of this is not clear, particularly for human PCa. Specifically, we have shown that endoglin suppresses human PCa cell invasion [5]. In the same study we demonstrated that TGF β induces the opposite effect (i.e., increases PCa cell invasion). Further we went on to show that although TGF β and endoglin exert opposite effects upon invasion, the relative action of each is not affected by the presence or absence of the other. For example, endoglin will suppress invasion in TGF β treated cells, as well as in control cells, and does so by a similar proportion. Examination of cell signaling at the molecular level corroborated these cellular-based findings. Specifically, it was shown that endoglin did not affect activation of the TGF β -responsive Smad, Smad3. These findings demonstrate that endoglin does not play a role in affecting TGF β -mediated regulation of cell invasion. However, TGF β is known to inhibit PCa cell proliferation [35], and our current findings indicate that endoglin loss leads to increased cell proliferation. These findings support the hypothesis that endoglin loss decreases TGF β signaling. To test this, we first treated HI-ENG, VC, and NO-ENG cells with increasing concentrations of TGF β , and measured thymidine uptake. It can be seen that with progressive endoglin loss there was a progressive loss of TGF β sensitivity (Fig. 6a).

However, cell proliferation in mice was only increased in NO-ENG cells, while in vitro there was a progressive increase in cell proliferation with progressive endoglin loss. This finding supports the notion that factors in addition to TGF β signaling act to co-regulate cell proliferation in vivo. If this were the situation, then we hypothesized that endoglin status in mice should directly affect TGF β signaling, as it did in vitro. To investigate this we assessed whether loss of endoglin expression would decrease the expression of TGF β -responsive

genes. The expression of both matrix metalloproteinase 2 (MMP-2) and MMP-9 genes is regulated in human PCa cells by TGF β , which increases their expression [14,36]. As each of these MMPs act to increase cell invasion and to impart a metastatic phenotype when expressed at high levels, they provide a very rigorous test of our hypothesis. This is because with endoglin loss cell invasion and metastasis increase. It would therefore be expected that MMPs would increase with endoglin loss. As can be seen in Fig. 6b, the mean expression of both MMP-2 and MMP-9 transcript levels decreases with progressive endoglin loss, consistent with a loss of TGF β signaling, and consistent with our in vitro findings. Considering all three cohorts of mice, the decrease in MMP-2 and MMP-9 expression with progressive endoglin loss were both significant (one-way ANOVA $P < 0.01$).

Endoglin increases expression of Smad1-responsive genes

Endoglin is known to activate bone morphogenetic protein (BMP) Smad1-responsive genes in several cell types, and we have shown that Smad1 activation is necessary for endoglin-mediated suppression of human PCa cell invasion [6,10,37]. Smad1 regulates gene transcription. For human PCa studies the role of Smad1 was investigated through a siRNA-mediated knockdown approach. Therefore, it is not known whether endoglin affects the expression of BMP Smad1 responsive genes, or if it does, are some genes selectively regulated. We hypothesize that endoglin increases the expression of BMP Smad1-responsive genes, but does so in a selective manner. We evaluated the effect of endoglin on the expression of the following panel of BMP Smad1-responsive genes in tumor tissue by qRT/PCR: SOX4, STAT1, JUNB, ID1 and ID2 [38,39]. With endoglin loss, JUNB, STAT1, and SOX4 expression significantly decreased (one-way ANOVA $P = 0.04$, <0.001 and 0.002 , respectively), while ID1 and ID2 were unaffected (Fig. 7a). If endoglin were altering gene expression by activating Smad1, then it should not affect Smad1 or Smad3 protein levels in tumor tissue. This was shown to be the case (Fig. 7b, c).

Discussion

We show for the first time that endoglin suppresses cancer metastasis, and did so in the particular case of human PCa. With progressive loss of endoglin, a progressive increase in metastasis was observed. We recognize that metastasis to the bone is a dominant clinical feature of PCa [2], and that metastasis to the bone was not a feature of the murine model we used. However, the current murine model does emulate key aspects of human PCa metastases. It is important to consider that in humans PCa moves from the prostate gland, passes through the circulation, and autopsy studies demonstrate that PCa metastases are in fact wide spread to organs throughout the body, including lung [25]. Therefore proteins that inhibit initial steps in the metastatic cascade, such as invasion out of the primary organ, are of particular importance because they preclude the development of later steps, no matter what the end organ is. Because endoglin suppressed PCa cell invasion [5] and was equally efficacious at doing so on metastatic cell lines as well as on early transformed phenotype prostate cell lines [6], we hypothesized that it would suppress metastasis, and that effects would be evident at initial steps in the metastatic cascade. To test this hypothesis we employed a model developed by us to specifically test action at initial steps in the metastatic cascade, including inhibition of invasion [12]. We elected to test this hypothesis using PC3-M cells because they are highly metastatic, and would thus pose a rigorous test of this hypothesis.

By demonstrating in the current study that endoglin decreased the number of circulating PCa cells in the blood, activity at early steps in the metastatic cascade was further supported. Passage of cells through the blood represents a middle step in the movement of cancer cells from their primary organ of origin to a distant organ [3]. The presence of cancer cells in the blood, including PCa, are increasingly being evaluated clinically as potential markers of the

future development of metastasis [27–30]. The proportion of individuals with circulating PCa cells in the blood is higher in subjects with more advanced stages of PCa, and with metastasis in particular, as compared to those with early stage PCa. Findings in our current model emulate the clinical scenario in humans in that with our model the proportion of mice with circulating tumor cells increases in cohorts of mice coincident with increases in the proportion of mice with metastasis. Our understanding of the biology of circulating tumor cells is still emerging and is not well understood. It should be noted that far fewer mice were found to have circulating tumor cells as compared to those found to have distant metastasis. A likely explanation is that circulating tumor cells are released in a cyclical fashion. This explanation is also supported by recent findings related to the mechanism of cell invasion [40,41]. For cells to move from a primary organ they must have the capability to invade through three dimensional protein structures, including the plasma membrane as well as the extracellular matrix. There is accumulating evidence that cell movement through such three dimensional protein structures involves grouped cell movement. This movement is characterized by leading cells that create a tunnel consisting of altered cell matrix, and this preconditioned matrix then facilitates the movement by following cells [40,41]. In contrast to the historical notion of single cells invading, this latter mechanism would result in the sporadic release of groups of cells into the circulation. This is consistent with our observations as well as those in human studies. It will therefore be important in future studies to specifically define the kinetics of release of circulating tumor cells, and to examine through dedicated in vivo imaging approaches the mechanism underlying those kinetics. Finally, the current findings warrant future investigations in humans aimed at understanding the relationship between endoglin expression, presence of circulating tumor cells, and development of metastasis.

We also show for the first time that endoglin suppresses human PCa tumor growth, and went on to demonstrate that this was due to changes in cell proliferation. Further, we identified a mechanism by which endoglin could regulate the growth of cells in tumor tissue by demonstrating that loss of endoglin led to a loss of TGF β -mediated inhibition of cell proliferation in vitro. This mechanism is also supported by additional facts. First, TGF β is ubiquitous in tissue and is an important suppressor of human PCa cell proliferation [35]. Second, we demonstrated in the current study that loss of endoglin led to a loss of TGF β signaling in tissue, just as it did in vitro. By examining the effect of endoglin upon MMP-2 and -9 gene expression in tissue, we pursued a rigorous examination of endoglin's effect upon TGF β signaling. This is because these MMPs are recognized mediators of cell invasion, and their expression has been shown to increase in invading human prostate cells [14,36]. Therefore, it would be expected that their expression would increase with progressive endoglin loss and the associated progressive increase in cell invasion. However, the opposite was observed. As the expression of both MMP genes is increased by TGF β , this finding directly supports the notion that loss of endoglin decreased TGF β signaling in tissue. Third, the increased tumor growth in NO-ENG mice was not due to decreased cell death, but was associated with increased Ki67, a measure of increased cell proliferation. This is consistent with in vitro findings that demonstrate that endoglin loss leads to a loss of TGF β -mediated inhibition of cell proliferation.

Our findings indicate that there are regulatory factors present in vivo that are not present under conditions of in vitro cell culture, and highlight the importance of examining endoglin biology in vivo. Specifically, in cell culture studies, progressive endoglin loss led to a progressive loss of TGF β -mediated suppression of cell proliferation. However, increased tumor growth was only observed in mice under conditions of complete endoglin loss. It will be important in future studies to identify the factors present in tissue that serve to co-regulate the pathways affected by endoglin.

Together, our findings suggest that endoglin's regulation of metastasis is separate from its regulation of tumor growth. This is highlighted by the fact that HI-ENG and VC mice have identical tumor size, while the later have increased metastases. Also, within individual cohorts, tumor size did not correlate with metastasis. The mechanism of this differential regulation of separate cellular functions likely relates to the fact that endoglin was found to regulate different TGF β superfamily signaling pathways. While our investigations focused upon Smad1 and TGF β /Smad3 signaling, given the complexity and interdependence of TGF β superfamily signaling, it is likely that additional regulatory components are involved. Finally, it was observed that body weight decreased with progressive loss of endoglin. This is likely due an increase in the total body burden of cancer (i.e., metastasis plus tumor) with progressive loss of endoglin.

By considering findings from the current study, as well as from prior studies related to endoglin function in human PCa, we propose the model outlined in Fig. 8 for endoglin function in human PCa [5,6]. TGF β is ubiquitous in tissue and acts to drive cell invasion. Invasion through the extracellular matrix increases cell entry into the blood, thereby forming circulating tumor cells, and distant metastases. Endoglin counteracts the effect of TGF β by suppressing cell invasion, circulating tumor cells, and the formation of distant metastases. When endoglin is lost during cancer progression, TGF β 's promotility effects are not counterbalanced, ultimately resulting in increased metastases. With respect to cell proliferation, TGF β acts to suppress it and to thereby limit tumor size. In this instance, endoglin acts to enhance TGF β signaling. Specifically, endoglin enhances TGF β -mediated suppression of cell proliferation, thus limiting tumor size. When endoglin is lost, there is diminished TGF β -mediated suppression of cell proliferation, which permits increased tumor growth.

Our findings would appear to contradict reports that link endoglin expression to cancer progression, but in fact they do not. It is important to realize that endoglin is expressed at high levels by endothelial cells [7]. With cancer-associated angiogenesis, endothelial cells increase, and therefore so does endoglin [10]. Specifically, blood levels of circulating endoglin have been identified as a marker of angiogenesis and tumor burden, and a poor prognostic indicator [42–45]. Further, it has recently been reported that anti-endoglin antibody inhibits angiogenesis, thereby blocking tumor growth and metastasis [46]. Together these studies suggest that in endothelial cells, endoglin enhances angiogenesis and cancer progression. In contrast, our studies have shown that in prostate epithelial cells, endoglin suppresses cancer progression [5,6,11]. Our findings are supported that of others (reviewed in [47]). Specifically, endoglin has been shown to suppress invasion and colony formation in esophageal epithelial cells [48] and to suppress cancer formation in skin epithelial cells [49]. Interestingly, in breast epithelial cells, endoglin has been reported to enhance invasion [50], suggesting that it may have altered function in different cell types. In fact, we had previously demonstrated this to be the situation at the molecular level by showing that in PCa cells endoglin cooperated with the ALK2 type I receptor subtype to activate Smad1 [6], whereas in endothelial cells endoglin is known to cooperate with ALK1 [51,52]. Finally, it is likely that this cell type-specificity is responsible for the fact that endoglin did not activate ID1 or ID2 in PCa epithelial cells (see Fig. 6a), while these two genes are considered endoglin-responsive in vascular endothelial cells [10].

In summary, we have shown for the first time that endoglin suppresses cancer metastasis, and that this was associated with decreased expression in several Smad1-responsive genes examined. Endoglin was shown to act at early steps in the metastatic cascade. This resulted in a decrease in circulating cancer cells in the blood. That a progressive loss of endoglin led to a progressive increase in metastasis, would support the notion that loss of endoglin expression in man imparts a continuum of risk across a spectrum of expression. This notion

is further supported by prior studies in which we demonstrated loss of endoglin expression during human PCa cell progression [5]. It will be important to confirm these studies in man. In the current study we also demonstrated for the first time that endoglin suppressed human PCa tumor growth. Growth suppression was not continuous, and was only lost when endoglin expression was undetectable. This finding supports the notion that there is a threshold level of endoglin expression in man below which tumor growth is enhanced. In future studies it will be important to examine endoglin expression in a comprehensive patient cohort to assess its relationship to primary tumor size and metastasis. The loss of TGF β 's ability to suppress PCa cell growth in vitro when endoglin was lost was identified as a possible and likely mechanism by which endoglin suppressed tumor growth in vivo. Related studies demonstrated that endoglin's affect upon TGF β signaling became more complex in the in vivo situation, reflecting the more complex environment of tumor as compared to cell culture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

H&E	Hematoxylin and eosin
MMP	Matrix metalloproteinase
PCa	Prostate cancer
qRT/PCR	Quantitative reverse transcription/polymerase chain reaction
TGFβ	Transforming growth factor β
TGFβRI	Type I transforming growth factor β receptor
TGFβRII	type II transforming growth factor β receptor

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Fig. 1.

Endoglin suppresses human prostate cancer cell invasion. **a** Endoglin expression varies across endoglin variant clonal cell lines. Representative photomicrographs are depicted of endoglin clonal variant cells stained by immunohistochemistry, using endoglin or isotype control antibody (100× magnification). **b, c** Endoglin suppresses cell invasion. Data are the mean $\pm$ SEM percent of vector control (VC) invading cells for a single experiment, run in replicates of $N = 4$. Similar results were seen in a separate experiment run at a separate time, also $N = 4$. * Student's t test $P \leq 0.05$ compared to VC. In (**b**), individual cell lines clones were evaluated. In (**c**), equal numbers of NO-ENG1 and NO-ENG2 cells were combined to form NO-ENG cells, and equal numbers of HI-ENG1 and HI-ENG2 cells were combined to form HI-ENG cells

Fig. 2.

Endoglin suppresses human prostate cancer cell metastasis and tumor growth. Mice were orthotopically implanted with the indicated endoglin variant cell line, and 4–5 weeks after implantation, tumor size and metastasis was assessed. **a** Representative photomicrographs of H&E stained and immunostained (for GFP) primary tumor (*white arrows*) and metastatic human PCa cells in lung tissue (*black arrows*). **b** Representative photomicrographs of PC3-M-GFP cells growing in cell culture, prior to implantation. **c** Endoglin suppresses metastasis. Data represent the number of lung metastasis per mouse 4–5 weeks after implantation, as indicated. Each graph depicts results from a separate experiment, conducted at a separate time. **d** Endoglin suppresses tumor growth. Data are the mean $\pm$ SEM tumor volume and mouse weight, as indicated. * $P \leq 0.05$ compared to VC

Fig. 3. Endoglin suppresses viable prostate cancer cells in the blood. **a** Four weeks after implantation of the indicated cell line, the number of viable PCa cells in the blood was measured. Data are the number of colonies per ml blood, for each mouse where at least 100 μ l blood could be harvested. **b** Colonies are of human origin. Representative photomicrographs of GFP positive and negative cell colonies are depicted

Fig. 4. Loss of endoglin increases Ki67 expression in tumor. **a** Representative photomicrographs of H&E and Ki67 stained tumor tissue under low magnification, with prospectively selected regions of interest (ROIs) denoted, and a high magnification photomicrograph of Ki67 stained cells in tissue. **b** Ki67 labeling index is increased in NO-ENG tumor cells. Data are the mean $\pm$ SEM Ki67 labeling index for all mice in the indicated cohorts at 4 weeks

Fig. 5.

Increased tumor growth is not associated with increased apoptosis. **a** Representative photomicrographs of TUNEL stained tissue at low and high magnification, with ROIs denoted. **b** TUNEL index is not increased in NO-ENG tumor bearing mice. Data are the mean $\pm$ SEM TUNEL index for all mice in the indicated cohorts. **c, d** Cleaved caspase 3 is not increased in NO-ENG tumor bearing mice. Western blot of tumor tissue protein is depicted in (**c**), and mean $\pm$ SEM of quantified bands, normalized to GAPDH, is graphically depicted (**d**)

Fig. 6.

Loss of endoglin inhibits transforming growth factor β -mediated effects on cell proliferation and gene expression. **a** Loss of TGF β -mediated growth inhibition is seen with loss of endoglin. NO-ENG, VC, or HI-ENG cells were treated with the indicated concentration of TGF β and thymidine uptake was measured. Data are the mean $\pm$ SEM thymidine uptake of a single experiment performed in replicates of $N = 3$, expressed as a percentage of non-TGF β treated cells; similar results were seen in a separate experiment performed at a separate time (also $N = 3$). **b** Loss of endoglin decreases TGF β -responsive gene expression. MMP-2 and MMP-9 transcript levels, normalized to that of GAPDH, were measured by qRT/PCR in tumor tissue. Data are the mean $\pm$ SEM level of gene expression, expressed as a percentage of the level measured in HI-ENG cells. Transcript levels were measured in tissue from five mice ($N = 5$) per cohort, for each of the three cohorts. Each qRT/PCR assay on each mouse was performed in replicates of two ($N = 2$), and the mean value was determined and taken as the level for that mouse. Similar results were seen in multiple replicate experiments performed at separate times (also $N = 5$ mice per cohort and $N = 2$ assay replicates per mouse). * $P \leq 0.05$ compared to HI-ENG tumor bearing mice

Fig. 7.

Endoglin increases the expression of specific Smad1 responsive genes. **a** Increases in endoglin are associated with increases in the expression of Smad1 responsive genes in mice. The expression of the indicated Smad1 responsive genes, normalized to GAPDH, was measured in tumor tissue from the indicated cohorts of mice by qRT/PCR. Data are the mean $\pm$ SEM of a single experiment, expressed as the percentage of the level measured in NO-ENG mice ($N = 5$ mice per cohort and $N = 2$ qRT/PCR assay replicates per mouse). Similar results were seen in a replicate experiment performed at a separate time (also $N = 5$ mice per cohort and $N = 2$ assay replicates per mouse). * $P \leq 0.05$ compared to NO-ENG tumor bearing mice. **b, c** Endoglin does not alter Smad1 or Smad3 protein expression in mice. Western blot of tumor tissue protein is depicted in (**b**), and mean $\pm$ SEM of quantified bands, normalized to GAPDH, is graphically depicted (**c**)

Fig. 8.

Model of endoglin-mediated regulation of metastatic potential and cell growth in human prostate cancer. **a** Endoglin present. Transforming growth factor β (TGF β) activates Smad3 and other TGF β -responsive elements. TGF β increases cell invasion and suppresses cell proliferation. Endoglin activates Smad1. Endoglin inhibits cell invasion, and thus counterbalances TGF β 's pro-invasion action. Overall, this gives cells a low invasion and metastatic potential. Further, endoglin enhances TGF β -mediated suppression of cell proliferation, thereby acting to limit tumor growth. **b** Endoglin absent. With cancer progression, endoglin is lost. Therefore, TGF β 's pro-invasion action is now unchecked, giving cells a high invasion and metastatic potential. Further, endoglin is not there to enhance TGF β -mediated suppression of cell proliferation. Therefore, cell proliferation increases, resulting in increased tumor growth. TGF β transforming growth factor β , TGF β RI and TGF β RII type I and II TGF β receptor, ALK activin-like kinase receptor, ENG endoglin