**Microenvironment and Immunology**

**Endoglin Regulates Cancer-Stromal Cell Interactions in Prostate Tumors**

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**Abstract**

Endoglin is an accessory receptor for TGF-β that has been implicated in prostate cancer cell detachment, migration, and invasiveness. However, the pathophysiologic significance of endoglin with respect to prostate tumorigenesis has yet to be fully established. In this study, we addressed this question by investigation of endoglin-dependent prostate cancer progression in a TRAMP (transgenic adenocarcinoma mouse prostate) mouse model where endoglin was genetically deleted. In this model, endoglin was haploinsufficient such that its allelic deletion slightly increased the frequency of tumorigenesis, yet produced smaller, less vascularized, and less metastatic tumors than TRAMP control tumors. Most strikingly, TRAMP:eng⁺⁻/⁻-derived tumors lacked the pronounced infiltration of carcinoma-associated fibroblasts (CAF) that characterize TRAMP prostate tumors. Studies in human primary prostate-derived stromal cells (PrSC) confirmed that suppressing endoglin expression decreased cell proliferation, the ability to recruit endothelial cells, and the ability to migrate in response to tumor cell–conditioned medium. We found increased levels of secreted insulin-like growth factor–binding proteins (IGFBP) in the conditioned medium from endoglin-deficient PrSCs and that endoglin-dependent regulation of IGFBP– secretion was crucial for stromal cell–conditioned media to stimulate prostate tumor cell growth. Together, our results firmly establish the pathophysiologic involvement of endoglin in prostate cancer progression; furthermore, they show how endoglin acts to support the viability of tumor-infiltrating CAFs in the tumor microenvironment to promote neovascularization and growth. Cancer Res; 71(10): 3482–93. ©2011 AACR.

**Introduction**

Prostate cancer is the second leading cause of male cancer death in the United States, mainly because of metastatic disease (1). Endoglin expression is altered in prostate cancer (2) and high endoglin levels are associated with decreased survival in patients with tumor Gleason scores between 6 and 7 (3). We have shown that endoglin, a TGF-β coreceptor, is involved in prostate cancer cell migration and invasion. Importantly, endoglin expression is lost in human metastatic prostate cancer cells (4). When restored, endoglin inhibits cell migration in vitro via modulation of both Smad-dependent and Smad-independent signaling mechanisms (5, 6). Furthermore, endoglin expression in human prostate cancer cells represses their tumorigenicity in SCID (severe combined immunodeficient) immunosuppressed mice (6) and metastasis in an orthotopic mouse model of prostate cancer (7). These studies, however, did not address the mechanisms underlying endoglin function with regard to stromal cell support of tumor vascularization and growth.

Solid tumors are a heterogeneous population of malignant and nonmalignant cell types. The latter include inflammatory cells, stem cells, fibroblasts, and endothelial cells (8). These cell populations constitute the tumor stroma, which provides key regulatory determinants for tumor progression and metastasis (9). We have previously described the effects of endoglin expression in prostate tumor cells in vitro (4–6) and in vivo (6, 7). However, the in vivo role of endoglin expression in other tumor cell types is unknown. To address this question, we developed a genetic model of prostate cancer that combined endoglin haploinsufficiency (eng⁺⁻/⁻; ref. 10) with the TRAMP (transgenic adenocarcinoma mouse prostate) mouse, a well-characterized transgenic model for the study of prostate cancer (11). TRAMP mice express the SV40 large T antigen, under the control of the prostate epithelium-specific probasin promoter, and develop prostate cancer from hyperplasia through more aggressive and metastatic stages (11, 12). In this model, the resulting level of endoglin in all eng⁺⁻/⁻ mouse-derived tissues is deficient compared with eng⁺⁺/⁺ tissues (10). Our results show that endoglin is essential for the...
presence of carcinoma-associated fibroblasts (CAF) in prostate tumors. Furthermore, data suggest that the prostate tumor CAFs impaired by endoglin deficiency in the TRAMP model are myogenic in origin and that endoglin suppression impairs CAF-mediated endothelial cell recruitment and CAF migratory response to tumor-derived factors. Finally, data support the hypothesis that endoglin downregulation in cancer affects CAF insulin-like growth factor–binding protein-4 (IGFBP-4) expression, supporting a novel mechanism of cancer–stromal cell cross-talk mediated through endoglin.

Materials and Methods

Mouse strains

Endoglin-targeted mice were screened for the presence of a neocassette in the truncated, engineered, endoglin allele, as previously described (10). TRAMP mice (The Jackson Laboratory) were screened for the presence of the SV40 large T antigen, as described on The Jackson Laboratory Web site (research.jax.org). Both TRAMP and endogin heterozygous mice were maintained in the C57BL/6 mouse strain background. Mice were bred, maintained, and experimentation was conducted according to the NIH standards established in the Guidelines for the Care and Use of Experimental Animals.

Necropsy and analysis of mouse tissues

Mice were weighed and euthanized at 21 or 25 weeks of age. All mice were genotyped twice: after birth and following sacrifice. The internal organs were examined and dissected in accordance with established guidelines (13), and metastases were determined as previously described (14). Harvested tumors and prostates were fixed in 4% paraformaldehyde for 48 hours and embedded in paraffin. Hematoxylin and eosin (H&E), Masson’s trichrome, and platelet/endothelial cell adhesion molecule (PECAM-1) staining were done as described (15). Antibodies used for immunohistochemistry included anti-endoglin antibody M7/18 (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA); anti-stromal-derived factor 1 (SDF-1), anti-smooth muscle actin (αSMA), anti-IGF-I, and anti-IGF-IR (Abcam); anti-Ki67 (DAKO); and anti-IGFBP-4 (R&D Systems). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was done with the In Situ Cell Death Detection Kit (Roche) according to manufacturer’s instructions.

For immunofluorescence analysis, anti-FSP-1 (S100A4 Ab-8 from NeoMarkers; 1:50 dilution), anti-SM22α (Abcam; 1:200 dilution), and anti-IGFBP-4 (R&D Systems; 1:50 dilution) were used as previously described (16, 17).

The slides were examined with a Carl Zeiss Axioskop microscope. Imaging was carried out using the Scion Image software program, and processed with Adobe Photoshop software as previously described (18).

Protein analysis

The tumors were ground and homogenized in lysis buffer (150 mmol/L NaCl, 300 mmol/L sucrose, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mmol/L Tris–HCl, pH 7.5) containing a cocktail of protease (Roche) and phosphatase (Calbiochem-EMD) inhibitors. Immunoprecipitation and Western blot analysis were carried out with anti-endoglin (Becton Dickinson Transduction Laboratories), and anti-β-actin (Sigma) as previously described (16, 19).

Cell culture, gene silencing, and growth factor treatment

Human primary prostate stromal cells (PrSC; Clonetics) were grown in stromal cell growth medium (SCGM; Clonetics). PrSCs were used between passages 5 to 10. PC3-M-C and PC3-M-FL cells were grown by the procedure described by Romero and coworkers (6). Human primary umbilical vein endothelial cells (HUVEC, passages 3–6) were cultured as previously described (19). TRAMP-C2 cells were obtained from the American Type Culture Collection (Manassas, VA), and maintained as described in the Supplementary Data and by Carter and coworkers (20).

siRNA for human endoglin interference was cloned in pSilencer 5.1 (Ambion). A pSilencer control vector (nonspecific) was purchased from the same company. The cells were transfected using Effectene (QiAGEN). RNA isolation and reverse transcriptase (RT)-PCR for endoglin and GAPDH were done as previously described (6). Alternatively, constructs expressing 21-nucleotide endoglin-specific short hairpin RNAs (shRNA) targeting human endoglin [shENG(1–3)] or nontargeting control (shSC; Sigma; SHC002) were obtained from Sigma–Aldrich. Constructs were packaged into lentivirus pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G). Transduction was conducted by incubating PrSCs with lentivirus, and stably transduced cells were subsequently used for studies without drug-marker selection (see Supplementary Data and Supplementary Table S1). All cell lines were verified by morphology, and human endoglin-specific PCR, certified mycoplasma-negative by PCR (Lonza), and primary cell cultures used within the indicated passage numbers. Human recombinant IGF-I, IGFBP-4, and IGFBP-6 proteins, and the neutralizing anti-IGFBP-4 were obtained from R&D Systems.

Cell migration

Migration assays were conducted as described (21). Briefly, 5 × 10^5 cells (HUVECs or PrSCs) were suspended in migration buffer [stromal cell basal medium (SCBM) containing 1 mmol/L MgCl₂, 0.2 mmol/L MnCl₂, and 0.5% BSA], plated in the upper chamber of Transwell migration chambers (8.0 μm; Costar), and allowed to invade through a polycarbonate membrane toward conditioned medium for 4 to 8 hours at 37°C. Cells remaining on the upper surface of the membrane were removed and cells that had migrated to the underside were stained with crystal violet. Cell migration was quantified in at least 3 independent experiments using triplicates, either by counting or by extraction of crystal violet and quantifying absorbance at 600 nm.

Analysis of conditioned media

A total of 1.2 × 10^6 PrSCs were plated onto culture plates 10 centimeters in diameter. Forty-eight hours later, they were rinsed 3 times in SCBM (Clonetics), and 5 mL per plate of fresh...
SCBM was added. Forty-eight hours later, the conditioned media were filtered (0.2-μm-pore-size mesh), concentrated, and stored at –20 °C until further analysis.

For isotope-coded affinity tag (ICAT) tandem mass spectrometry (MS/MS), the conditioned media were concentrated by ultracentrifugation, labeled, and purified using the Cleavable ICAT Reagent Kit for Protein Labeling (Applied Biosystems), and analyzed with a tandem quadrupole time-of-flight mass spectrometer (QSTAR; MDS-SCIEX) as described by Koleva and coworkers (19). Analysis of mass spectrometric data was conducted using ProteinPilot software program (Life Technologies). Detailed description of methods is provided in Supplementary Data.

Results

TRAMP:eng+/– mice have a greater number of tumors, which are smaller and less metastatic than those in TRAMP:eng+/+ mice

To generate TRAMP:eng+/+ and TRAMP:eng+/– transgenic mice, we crossed endoglin heterozygous (eng+/–) males (10) with TRAMP females (12). We analyzed tumor formation in TRAMP:eng+/+ and TRAMP:eng+/– 21-week-old (n = 12), and 25-week-old males (n = 10), obtaining similar results.

Western blot analysis indicated that TRAMP:eng+/– tumors showed lower levels of endoglin than TRAMP:eng+/+ tumors, although heterogeneity was observed as expected (ref. 10; Fig. 1A). Quantitative analysis indicated that endoglin protein expression in TRAMP:eng+/– tumors was approximately one-third of the levels detected in TRAMP:eng+/+ tumors (Fig. 1B).

The frequency of prostate tumorigenesis was slightly higher in TRAMP:eng+/– mice than TRAMP:eng+/+ mice (Fig. 2A). Two-thirds of the TRAMP:eng+/– derived tumors were nonmetastatic, whereas all of the TRAMP:eng+/+ derived tumors were metastatic (Fig. 2A). Metastases were observed in lung and lymph nodes with similar frequencies in TRAMP:eng+/+ and TRAMP:eng+/– mice: 50% of the metastases occurred in local lymph nodes and 50% in lungs.

Tumors in TRAMP:eng+/– mice were smaller than those in TRAMP:eng+/+ mice (Fig. 2B). Quantification of the percentage of cells positive for the proliferation marker Ki67 and TUNEL staining indicated that proliferation and apoptotic rates were similar in the tumor cells of TRAMP:eng+/+ and TRAMP:eng+/– mice (data not shown), suggesting that the tumor microenvironment promoted more sustained growth of TRAMP:eng+/– derived tumors over time.
Endoglin is a marker of tumor neoangiogenesis [reviewed in (22)]. To investigate differences in tumor vascularization, the endothelial cell marker PECAM-1 and endoglin (Fig. 2C) were used to quantify the microvascular density (Fig. 2D). The number of PECAM-1-positive vessels was 5-fold higher in TRAMP:eng+/+ -derived tumors versus TRAMP:eng+/-/C0-derived tumors, whereas endoglin-positive vessels were 25% to 30% higher in TRAMP:eng+/+ -derived tumors versus TRAMP:eng+/-/C0-derived tumors, suggesting that TRAMP:eng+/+ -derived tumors benefit from higher amounts of metabolites and oxygen.

Endoglin is associated with CAF investment of TRAMP:eng+/+ -derived tumors

H&E and Masson’s trichrome staining revealed that TRAMP:eng+/+ and TRAMP:eng+/-/C0-derived tumors were poorly differentiated adenocarcinomas, with a predominant solid mass of epithelial-derived cells and very rare gland formation, as defined in (13). Furthermore, we observed that TRAMP:eng+/+ -derived tumors contained areas enriched in fibroblast-like cells. In contrast, all the TRAMP:eng+/-/C0-derived tumors analyzed were nonfibrotic, indicating the absence of stromal fibroblasts (Fig. 3A and B). Image analysis confirmed that the average area occupied by epithelial-like cells was approximately 75% in TRAMP:eng+/+ -derived tumors versus 99% in TRAMP:eng+/-/C0-derived tumors.

CAFs are a major and heterogeneous constituent of the tumor stroma (23). CAFs are characterized by the expression of αSMA and SDF-1 (8), which were both detected in the TRAMP:eng+/+ CAFs but not in TRAMP:eng+/-/C0 CAFs (Fig. 3C).

One of the cellular components of CAFs is the SM22α-positive myofibroblast (24), which plays an important role in

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**Figure 2.** Prostate tumorigenesis and tumor angiogenesis are altered in TRAMP:eng+/+ versus TRAMP:eng+/-/C0 mice. A, frequency of prostate tumorigenesis and metastasis in TRAMP:eng+/+ and TRAMP:eng+/-/C0-21-week-old mice (n = 12). B, tumor size in TRAMP:eng+/+ (n = 4) and TRAMP:eng+/-/C0 (n = 5) 21-week-old mice (average weight ± SD). C, immunohistochemistry for PECAM-1 and endoglin (arrows) in TRAMP:eng+/+ and TRAMP:eng+/-/C0 tumors from 21-week-old mice, counterstained with hematoxylin. Bars, 300 μm. D, the number of microvessels stained for PECAM-1 and endoglin, determined in at least 8 fields per sample (n = 4). *, P < 0.05 (Student’s t test).
tumor behavior (25). SM22α was restricted to the TRAMP:eng+/+ stromal fibroblast; however, it was largely absent from TRAMP:eng+/−-derived tumors (Fig. 3D). Immunofluorescence staining for fibroblast-specific protein 1 (FSP-1) was more pronounced in TRAMP:eng+/+ and TRAMP:eng+/−-derived tumors confirming the identity of prostate-associated fibroblasts (17). However, double immunofluorescence analysis using anti-endoglin and anti-SM22α or anti-FSP-1 antibodies revealed that endoglin expression was associated with SM22α-positive cells but not FSP-1-positive cells (Fig. 4A and B). These results indicate that TRAMP:eng+/−-derived tumors are largely comprised of endoglin-expressing myofibroblast-derived CAFs.

**Endoglin expression is necessary for the viability of cultured prostate stromal cells**

We attempted to establish primary cultures of CAFs derived from TRAMP:eng+/+ and TRAMP:eng+/−-derived tumors. However, although we were able to propagate TRAMP:eng+/+ and TRAMP:eng+/−-derived CAFs in culture, the TRAMP:eng+/−-derived CAFs were not viable under a variety of culture conditions (data not shown). To overcome this limitation, we used human PrSCs. Consistent with TRAMP immunohistochemistry, human PrSCs robustly expressed endoglin, as detected by RT-PCR (Fig. 5A, left panel). Endoglin expression was transiently knocked down in PrSCs with a specific interfering RNA construct, siENG. The efficiency of endoglin RNA silencing was approximately 60%, as detected by both RT-PCR and immunoprecipitation (Fig. 5A, right panel). This reduction of endoglin protein level approximated the difference seen in tumors (Fig. 1A) and was sufficient to significantly impair PrSC growth in vitro (Fig. 5B, left panel), suggesting that endoglin expression promotes CAF proliferation in prostate tumor.

Because growth factor secretion is a recognized CAF function (23), we analyzed the effect of the conditioned medium from PrSCs in their proliferation. PrSC growth was stimulated when they were cultivated in their own conditioned medium. Moreover, PrSC-conditioned medium partially rescued the inhibitory effect of decreased endoglin levels (Fig. 5B, middle panel). These results suggest that endoglin influences prostate stromal cell viability via secretion of soluble factors.
Stromal fibroblasts stimulate the proliferation of prostate cancer cells through an endoglin-dependent mechanism

CAFs contribute to tumor development, in part, because they stimulate tumor cell proliferation (8). To further investigate the link between endoglin expression in PrSCs and prostate cancer cell proliferation, we used PC3-M cells that did not express endoglin (4, 6; PC3-M-C, control) or that stably overexpressed endoglin (PC3-M-FL, full-length; ref. 6). PC3-M-C and PC3-M-FL cells were grown in the presence of basal medium, or in the presence of conditioned medium from PrSCs transfected with an interfering RNA against endoglin or nontargeting control. Control PrSC-conditioned medium strongly stimulated the proliferation of both PC3-M-C and

Figure 4. Endoglin is associated with tumor myofibroblasts. A, double immunofluorescence for endoglin, SM22α, and FSP-1 in TRAMP:eng+/tumors. The nuclei were stained with DAPI. Arrows, endoglin (ENG) and SM22α double-positive cells. Bars, 200 μm. B, the number of ENG:SM22α and ENG:FSP-1 double-positive cells were counted in at least 5 fields per sample (average ± SD; ref. 18).
PC3-M-FL cells. The conditioned medium from endoglin knockdown in PrSCs had a lower stimulatory effect in PC3-M-C cells, and no effect in PC3-M-FL cells (Fig. 5B, right panel). Considered together, these results are consistent with the view that endoglin expression in stromal cells is necessary to stimulate cancer cell proliferation via a mechanism that involves secreted factors.

### Figure 5

Endoglin knockdown reduces PrSC proliferation and affects PrSC-dependent modulation of PC3-M cell proliferation. PrSCs were transfected with siRNAs directed against endoglin (siENG) or a control scrambled sequence (siSC) for 48 hours. Endoglin expression was analyzed by RT-PCR and immunoprecipitation (A). PC3-M-C and endoglin-expressing PC3-M-FL cells (6): negative and positive controls, respectively. HC, immunoglobulin heavy chain. B, left, PrSC proliferation following siENG transfection: Two independent experiments using triplicates were done. r, ratio of siENG- versus siSC-treated cells. Middle, PrSC siENG- or siSC siRNA–derived conditioned SCBM was used to treat new cultures of PrSCs. r, number of cells divided by the number of siSC cells in basal media. Right, PC3-M-C or PC3-M-FL cells were prepared in SCBM or PrSC-conditioned medium. The number of cells per well was determined 48 hours later as described above. r, number of cells divided by the number of cells in basal media. C, PrSCs were transduced with shRNA constructs targeting human endoglin (left, inset). Endoglin Western blot of PrSCs. Left, HUVECs were tested for ability to migrate toward basal PrSC shSC or shENG(1–3) medium (25 μg protein). CM and BM, conditioned and basal media, respectively. D, TRAMP-C2 cells were used to prepare conditioned medium as described above. Following shRNA transduction, PrSCs were used for migration assays as above. * P < 0.05 and ** P < 0.005 (Student’s t test). See Supplementary Data for detailed methods.

**Endoglin deficiency in PrSCs impairs endothelial cell migration and tumor cell recruitment**

To further suppress endoglin expression in PrSCs, 3 separate shRNA constructs were delivered using a lentivirus (26). PrSC shENG(1–3) shRNAs resulted in either partial or complete suppression of endoglin protein levels, respectively (Fig. 5C, inset). Conditioned medium collected from shENG1,
shENG2, and shENG3 all reduced the ability of HUVEC migration, reflecting the degree of endoglin suppression. Furthermore, TRAMP-C2-conditioned medium was tested for its ability to recruit PrSCs. Endoglin-deficient PrSCs were significantly impaired in their capacity to migrate in response to tumor cell-conditioned medium (Fig. 5D). TRAMP-C2 endoglin knockdown did not affect cell recruitment (data not shown), suggesting that endoglin is required for CAF-dependent recruitment of endothelial cells and their response to tumor cell factors.

Endoglin-dependent modulation of IGFBP-4 secretion by PrSCs is involved in the regulation of tumor cell growth

To identify peptides secreted by PrSCs, we carried out ICAT-MS/MS (27) to compare the conditioned media from control and endoglin knockdown PrSCs. Among the proteins overexpressed by endoglin knockdown in PrSCs were (i) tissue inhibitors of metalloproteinases 1 and 2 (TIMP1, TIMP2); (ii) sulphydryl oxidase 1; (iii) SPARC, and (iv) 2 members from the IGFBP family—IGFBP-4 and IGFBP-6 (Table 1). These proteins are implicated in the induction of cell growth arrest and in cell invasiveness (15, 28–31).

Mass spectrometric sequencing of the putative IGFBP-4 and IGFBP-6 peptides confirmed their identities and corroborated the quantitative data indicating their upregulation in endoglin-deficient PrSCs (Supplementary Figs. S1–S7). IGFBPs play important roles in neoplastic processes and prostate cancer (32, 33), and TGF-β signaling regulates tumor–stromal interactions via IGF-I (34). Therefore, we quantified the cell growth of PC3-M-C cells in response to recombinant IGF-I, IGFBP-4, and IGFBP-6 treatment. IGF-I and IGFBP-6 stimulated PC3-M-C proliferation (Fig. 6A). IGFBP-4 alone did not affect cell proliferation; however, in combination with IGF-I, it inhibited IGF-I–dependent stimulation of cell proliferation (Fig. 6A). When these treatments were carried out in PrSC-conditioned medium, the growth-stimulation effect of IGF-I and IGFBP-6 was enhanced, and, surprisingly, IGFBP-4 alone inhibited cell proliferation. These effects were likely due to the presence of PrSC-derived IGF-I in the medium (35). It is reasonable to postulate that IGFBP-4 inhibits PC3-M-C proliferation when the treatment was carried out in control PrSC-conditioned medium (Fig. 6B). When added in the presence of endoglin knockdown PrSC-conditioned medium, the neutralizing antibody had the same partial blocking effect on IGFBP-4–dependent inhibition of

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Abbreviation: ECM, extracellular matrix.

*H* indicates heavy isotope (siENG)-tagged peptide versus light isotope (siSC)-tagged peptide, average of confirmed samples.

*Number of peptides identified and quantified by more than 95% CI.

See Supplementary Data.
PC3-M-C cell growth (Fig. 6B). This experimental approach confirmed the presence of functional IGFBP-4 in endoglin knockdown PrSC-derived medium, which is consistent with the reduced size of TRAMP:eng+/C0-derived tumors.

TRAMP:eng-derived tumor sections were stained for these IGF signaling components. In TRAMP:eng+/+-derived tumors, IGFBP-4 was detected in both fibroblast-like and epithelial-derived cancer cells. The epithelial staining appeared to be peripheral, suggesting that most of the IGFBP-4 detected was associated with the stromal compartment (Fig. 6C, arrow). TRAMP:eng+/- derived tumors showed minimal staining for IGFBP-4 (Fig. 6C) due to the lack of CAFs, IGF-I and IGF-IR were detected mainly in the nonstromal compartment (Fig. 6C).

Immunofluorescence analysis of TRAMP:eng+/+-derived tumor showed more myofibroblast incursion (SM22α-positive cell proliferation (Fig. 6B)).
cells), and less IGFBP-4 staining, which predominantly colocalized with SM22α staining. In contrast, TRAMP: \textit{eng}^{+/−}-derived tumor showed less SM22α-positive areas but more prominent IGFBP-4 staining that was localized in the extracellular space adjacent to SM22α-positive cells (Fig. 6D). Thus, the expression pattern of IGFBP-4 in these tumors is consistent with endoglin-dependent modulation of IGFBP-4 availability and it affects stromal investment in prostate tumors.

**Discussion**

The role of endoglin in tumorigenesis \textit{in vivo} has been principally studied using tumor cell xenografts. Such studies indicate that endoglin expression represses migration and invasiveness of prostate cancer cells (4, 5), and that it attenuates their tumorigenicity (6). However, more accurate animal models are needed to elucidate the behavior of particular tumor types in their microenvironment. The present work is the first to study the effect of endoglin haploinsufficiency in an autologous model of cancer. This bigenic model is based on the TRAMP mouse, which develops \textit{in situ} and invasive carcinoma of the prostate (11) and, ultimately, late-stage metastatic cancer (37).

Endoglin expression inhibits prostate cancer cell migration \textit{in vitro} (4, 5), but, surprisingly, the frequency of metastasis in our \textit{in vivo} model was higher in TRAMP: \textit{eng}^{+/−} mice than in TRAMP: \textit{eng}^{+/+} mice. The increased vascularization of TRAMP: \textit{eng}^{+/+} tumors is likely the reason for this difference, as the intravasation of tumor cells into the blood stream is the first step in the establishment of distant-site metastatic lesions (9).

Histologic and immunohistochemical examination of TRAMP: \textit{eng}^{+/+} versus TRAMP: \textit{eng}^{+/−} tumors showed that endoglin was required for the presence of CAFs in the tumor. This phenotype is much more profound than expected from endothelial cell haploinsufficiency (50% reduction in endoglin level) or the asymptomatic reduction of endoglin systemically. Interestingly, studies of the effect of endoglin haploinsufficiency on xenografted Lewis lung carcinoma 3LL cell–derived tumors showed no such CAF phenotype (38). Moreover, endoglin expression in endothelial cells of \textit{eng}^{+/+} versus \textit{eng}^{+/−} mice cause relatively small effects (compared with the tumor CAF phenotype) in the context of skin carcinogenesis (39). These observations suggest that the endoglin-dependent CAF phenotype is specific to the prostate tumor stroma.

The origin of CAFs is unclear. Candidate CAF precursors include activated quiescent local fibroblasts (8) and circulating bone marrow mesenchymal stem cells (40). Moreover, recent work suggests the intriguing possibility that CAFs occur as a consequence of endothelial cells undergoing endothelial–mesenchymal transition (41). Our studies suggest that endoglin is required for continuous tumor CAF investment. Furthermore, CAFs are compared with myofibroblasts, defined as activated fibroblasts involved in processes such as wound healing (23). Endoglin is a marker of myofibroblasts (42) and its expression is increased in these cell type during atherosclerosis-related and vascular TGF-β-dependent myogenic differentiation (43) and cell migration (44). The current data suggest that endoglin is primarily associated with myofibroblast-related SM22α-positive fibroblasts. Based on our previous studies (45), we propose that endoglin expression is required for the viability or the lineage specification of the myofibroblast-related CAF precursors.

To study the role of endoglin in CAF function, we isolated CAFs from TRAMP: \textit{eng}^{+/−} and TRAMP: \textit{eng}^{+/+} tumors. However, we were not able to establish cell cultures of TRAMP: \textit{eng}^{+/−}-derived CAFs. Human PrSCs were utilized as an alternative. Two studies showed that co-injection of PrSCs with prostate cancer cells in mice enhances tumor incidence and growth (35, 46). We showed that endoglin is expressed in PrSCs and found that PrSC growth is impaired in conditions of reduced endoglin expression. In addition, reduction of endoglin expression in human prostate stromal cells reduced their ability to recruit endothelial cells and their capacity to migrate in response to tumor-secreted factors. These results suggest that endoglin is required for multiple aspects of CAF function including viability, endothelial cell recruitment, and tumor-induced migration.

CAFs recruit several cell types to the tumor area via growth factor secretion (8, 23). Therefore, decreased tumor angiogenesis in TRAMP: \textit{eng}^{+/−} mice may be directly related to the absence of CAFs needed to recruit endothelial cell precursors. However, the signals that CAFs use to communicate with adjacent tissue are poorly understood.

Quantitative isotope peptide tagging methods suggested that endoglin regulated PrSC secretion of several potentially important secreted proteins involved in cell recruitment. For example, endoglin knockdown resulted in increased TIMP1 and TIMP2 detected in PrSC-conditioned medium (Table 1).

Previous studies implicate tumor–stromal interactions in the regulation of TIMP expression and its role in prostate cancer progression (30), which is consistent with the view that reduced endoglin expression raised TIMP levels, impairing CAF invasion of the tumor.

Mass spectrometric data suggested that the IGF signaling system is an important mediator of endoglin-dependent cancer cell–stromal cell interactions. This hypothesis is supported by studies showing that IGF-I stimulates cancer cell proliferation (33) and promotes cell growth in several cancer cell lines including PC3, the precursors of PC-3-M cells (47). PrSCs secrete IGF-I, promoting the proliferation of human prostate cancer cells (35). IGFBP-4 and IGFBP-6 are modifiers of IGF pathway signaling. IGFBP-4 antagonizes the growth-stimulatory effect of IGF-I (31) and inhibits the proliferation and tumorigenicity of human prostate cancer cells (48). Additionally, inhibition of IGFBP-6 expression promotes colon cancer cell proliferation (49). In this article, we provide evidence suggesting that PrSCs secrete IGFBP-4 and -6 in response to decreased endoglin expression, which may repress tumor growth. In our experimental model, IGFBP-4 inhibits IGF-I–dependent stimulation of prostate cancer cell growth. Our interpretation is that PrSCs secrete IGF-I and several modulators of its activity. Under WT conditions of endoglin expression (\textit{eng}^{+/+}), the balance is switched toward the stimulation of prostate cancer cell proliferation. Therefore, we suggest endoglin expression is necessary for PrSC/IGF-dependent...
modulation of tumor growth, potentially by regulation of TGF-β signaling in CAFs (34). ICAT studies did not reveal endoglin-dependent contributions from other secreted factors including Wnt family members. Further studies are needed to elucidate the mechanisms underlying endoglin-dependent modulation of IGFBP secretion.

The present study supports the view that endoglin plays a critical role in prostate cancer stromal cell function in the microenvironment. Experiments in the TRAMP:eng mouse model, combined with conditional transgenic approaches (16) will help elucidate the effect of systemic endoglin levels on stromal investment at several stages of tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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2. Kassouf W, Jovanovic B, Pins M, Lee C, Bergan RC. Overexpression of endoglin plays a critical role in prostate cancer stromal cell function in the microenvironment. Experiments in the TRAMP:eng mouse model, combined with conditional transgenic approaches (16) will help elucidate the effect of systemic endoglin levels on stromal investment at several stages of tumorigenesis.

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Endoglin Regulates Tumor–Stromal Cell Cross-Talk


Correction: Endoglin Regulates Cancer–Stromal Cell Interactions in Prostate Tumors

In this article (Cancer Res 2011;71:3482–93), which was published in the May 15, 2011, issue of Cancer Research (1), a funding source was left out of the original statement. The additional funding source is provided below. The authors regret this error.

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