Endoglin and the immune system: immunomodulation and therapeutic opportunities for cancer

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Endoglin expression on cancer-associated fibroblasts regulates invasion and stimulates colorectal cancer metastasis

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ABSTRACT

Purpose

Cancer-associated fibroblasts (CAF) are a major component of the colorectal cancer tumor microenvironment. CAFs play an important role in tumor progression and metastasis, partly through TGF-β signaling pathway. We investigated whether the TGF-β family coreceptor endoglin is involved in CAF-mediated invasion and metastasis.

Experimental Design

CAF-specific endoglin expression was studied in colorectal cancer resection specimens using IHC and related to metastases-free survival. Endoglin-mediated invasion was assessed in vitro by transwell invasion, using primary colorectal cancer–derived CAFs. Effects of CAF-specific endoglin expression on tumor cell invasion were investigated in a colorectal cancer zebrafish model, whereas liver metastases were assessed in a mouse model.

Results

CAFs specifically at invasive borders of colorectal cancer express endoglin and increased expression intensity correlated with increased disease stage. Endoglin-expressing CAFs were also detected in lymph node and liver metastases, suggesting a role in colorectal cancer metastasis formation. In stage II colorectal cancer, CAF-specific endoglin expression at invasive borders correlated with poor metastasis-free survival. In vitro experiments revealed that endoglin is indispensable for bone morphogenetic protein (BMP)-9–induced signaling and CAF survival. Targeting endoglin using the neutralizing antibody TRC105 inhibited CAF invasion in vitro. In zebrafish, endoglin-expressing fibroblasts enhanced colorectal tumor cell infiltration into the liver and decreased survival. Finally, CAF-specific endoglin targeting with TRC105 decreased metastatic spread of colorectal cancer cells to the mouse liver.

Conclusions

Endoglin-expressing CAFs contribute to colorectal cancer progression and metastasis. TRC105 treatment inhibits CAF invasion and tumor metastasis, indicating an additional target beyond the angiogenic endothelium, possibly contributing to beneficial effects reported during clinical evaluations.

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide and accounts for 8.5% of all cancer-related deaths [1]. Ninety percent of all patients with cancer die from metastatic disease. Therefore, patients at risk to develop metastatic disease (stage III/IV) are eligible for (neo)adjuvant (chemo)-radiotherapy [2]. However, a significant proportion of patients with localized disease will still develop metastases, emphasizing that a better understanding of the mechanism underlying tumor metastasis is needed.

The tumor stroma can account for ≥50% of the tumor mass, and its extent is predictive for worse patient survival in patients with colorectal cancer [3]. The tumor stroma, or tumor microenvironment (TME), is composed of endothelial cells, pericytes, immune cells, and cancer-associated fibroblasts (CAF; ref. 4). CAFs interact with all other cells in the TME via direct cell–cell contact and secretion of cytokines [5, 6], thereby stimulating tumor progression and ultimately metastasis [7]. Therefore, CAFs are considered a potential novel target for cancer therapy.

TGF-β mediates the transdifferentiation of resident fibroblasts into CAFs [8, 9], as indicated by increased expression of α-smooth muscle actin (αSMA) and fibroblast activation protein (FAP; [ref. 10]). Endoglin is a coreceptor for TGF-β and bone morphogenetic protein (BMP)-9 that, upon ligand binding, can facilitate Smad1 phosphorylation [11]. Endoglin is highly expressed on the surface of activated endothelial cells and indispensable for developmental angiogenesis [12–14]. Furthermore, endoglin microvessel density is correlated with tumor progression and metastases in colorectal cancer [15, 16]. Different mutations in the endoglin gene have been reported in The Cancer Genome Atlas (TCGA) for colorectal cancer, although the affected cell types were not specified. The endoglin-neutralizing antibody TRC105 binds human endoglin with high affinity, competitively inhibits BMP-9 binding [17], and induces antibody-dependent cell-mediated cytotoxicity [18, 19]. Currently, TRC105 is in phase III clinical trials in patients with advanced cancer as antiangiogenic therapy.

Although endoglin expression on endothelial cells has been extensively studied and is the focus of targeted cancer therapy, endoglin is also expressed on other cells in the TME. Therefore, in this study, we investigated the role of endoglin expression on CAFs. We show that CAFs, located specifically at invasive borders of colorectal tumors and in metastatic lesions, express endoglin. We further demonstrate that targeting endoglin on CAFs with TRC105 modulates CAF function and that endoglin regulates tumor invasiveness in zebrafish and liver metastases in vivo. Taken together, our data suggest an additional working mechanism for endoglin-targeted therapy on CAFs, besides targeting the endothelium and highlights its therapeutic potential.

MATERIALS AND METHODS

Patient samples

Paraffin-embedded tissue samples were obtained from the Department of Pathology, Leiden University Medical Center (LUMC, Leiden, the Netherlands), used according to the guidelines of the Medical Ethical Committee of the LUMC, and conducted in accordance to the Declaration of Helsinki and the Code of Conduct for responsible use of Human Tissue and Medical Research as drawn up by the Federation of Dutch Medical Societies in 2011. This Code permits the further use of coded residual (historical) tissue and data from the diagnostic process for scientific purposes. Permission is granted by implementing an opt-out procedure for the patients; written informed consent in that case is not needed. The first cohort consisted of 25 adenomas, 140 stage II, and 94 stage III tumors from treatment-naïve patients with colorectal cancer and the same number of adjacent normal tissue samples. Patient characteristics have been included in Supplementary Table S1. The second cohort consisted of 31 patients, of which resection specimens of the primary tumor, lymph node, and liver metastases were available. Patient characteristics and >10-year follow-up were recorded. TCGA databases “COAD - TCGA Colonadenocarcinoma – June 2016" (350 patients) and “COADREAD – TCGA Colon and Rectum adenocarcinoma June 2016" (466 patients) were analyzed using SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp) to analyze potential correlations between patient survival, risk classification, and endoglin expression in colorectal tumors.

Tissue analysis

IHC and fluorescent staining were performed as described before (20), using antibodies as shown in Supplementary Table S2. CAF-specific endoglin expression was scored on a scale of 1 to 4 (≤10%; 10%–25%; 25%–50%, and ≥50% positive) in a blinded manner by two independent observers. Pictures were obtained using a Leitz Diaplan microscope (Leitz). Quantitative PCR analyses were performed as described before [6] using primer sequences shown in Supplementary Table S3.

Cell culture and signaling assays

Human CAFs, the human colorectal cancer cell lines HCT116 and HT29 (obtained from ATCC), and the mouse colorectal cancer cell line MC38 [21] (obtained from Kerafast) were cultured in DMEM/F12, supplemented with 10% FCS, 10 mmol/L HEPES, 50 μg/mL gentamycin, 100 IU/mL penicillin and 100 μg/mL streptomycin (all Thermo Fisher Scientific). The immortalized HUVEC cell line ECRF [22] was cultured as described before [23]. Human embryonic kidney (HEK293T) cells were obtained from ATCC and maintained in DMEM, supplemented with 10% FCS and penicillin/streptomycin (all

from Thermo Fisher Scientific). Primary human CAFs were isolated from nonnecrotic parts of the tumors and normal fibroblasts (NF) from adjacent healthy tissue as described before [6]. Tissues were cultured in DMEM/F12 as described above supplemented with 2.5 µg/mL Fungizone (Thermo Fisher Scientific). Mouse CAFs were isolated from colorectal cancer tissue by culturing 5 × 5 mm pieces of tumor in DMEM/F12 as described above. For both human and mouse isolations, fibroblast-like cell outgrowth was observed after 7 to 10 days. Murine embryonic fibroblasts (MEF) were obtained from E12.5 embryos as described before [24] from endoglin floxed mice [25]. MEFs were maintained in DMEM, supplemented with 10% FCS and penicillin/streptomycin (all Thermo Fisher Scientific). Cell lines were used for 20 passages, and all cell cultures were tested monthly for Mycoplasma contamination.

Constructs expressing human endoglin [26], Cre recombinase (pLV.mPGK.iCRE.IRES.PuroR, kindly provided by Dr. M. Gonçalves, LUMC), or endoglin short hairpin RNA (shRNA, Sigma Mission shRNA library, constructs SHC001, TRCN0000083138, TRCN0000083139, TRCN0000083140, TRCN0000083141 and TRCN0000083142) were delivered by lentiviral transduction to 80% confluent fibroblasts or endothelial cells. After 48 hours, transduced cells were selected using 1.5 µg/mL puromycin (Sigma). HEK293T cells were grown to 80% confluency and transfected with endoglin-expressing plasmids using 1 mg/mL polyethylenimine (PEI; Polysciences Inc.).

For signaling assays, fibroblasts were seeded in 6-well plates. Upon 90% confluency, cells were serum-starved overnight in medium containing 40 µg/mL TRC105 (TRACON Pharmaceuticals) or 40 µg/mL human IgG (Bio X Cell) for human cells. Mouse fibroblasts were incubated in the presence of 40 µg/mL M1043 (anti-mouse endoglin, Abzena) or 40 µg/mL Rat IgG (Bio X Cell). Next day, cells were stimulated with either 5 ng/mL TGF-β3 [27], 0.1 ng/mL BMP-9 (R&D Systems), or 100 ng/mL BMP-6 (PeproTech) for one hour. Cells were lysed in RIPA buffer, protein content was determined, and Western blot analysis was performed as described before [28]. Membranes were incubated overnight with primary antibodies against endoglin, phosphorylated (p)Smad1 or pSmad2 (Supplementary Table S2). Blots were stripped and reprobed with mouse anti-GAPDH or anti-actin antibodies as loading control. Blots were developed using the Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad).

TGF-β and BMP-9 ELISA

BMP-9 levels were measured by ELISA as described before [29]. TGF-β1 levels were analyzed using commercially available duo-set ELISA (R&D Systems) as described before [30, 31].

Invasion assays

A total of 1,000 HEK293T cells or 2,500 CAFs were seeded on top of 0.6% agarose (Sigma) coated 96-well plates and left to form spheroids for 48 hours. Spheroids

were collected and embedded in 1 mg/mL collagen-I matrix (Advanced BioMatrix) containing 10% FCS. At 0, 24, and 48 hours, pictures were taken using a Zeiss Axiovert 200M microscope (Carl Zeiss BV). Quantification of the invaded area was performed using Adobe Photoshop CC2014 software (Adobe Systems).

For transwell invasion assays, the upper surface of 8.0-μm pore size ThinCert (Greiner Bio-One) was coated with 200 μg/mL collagen-I in culture medium containing 0.5% FCS. The lower compartment of the transwell system contained medium with 0.5% FCS and 0.1 ng/mL BMP-9 or 5 ng/mL TGF-β3. When invasion toward colorectal cancer cells was assessed, 2 × 105 HT29, HCT116, or MC38 cells were seeded in the lower well. A total of 2.5 × 104 fibroblasts were seeded on the coated inserts in medium containing 0.5% FCS and left to invade for 24 hours, in the presence of ligands and inhibitors as described above. After 24 hours, invaded cells were fixed with 4% paraformaldehyde and stained with crystal violet. Using an Olympus BX51TF microscope (Olympus Life Science Solutions), five pictures per insert were obtained at ×20 magnification. Cell invasion was quantified in at least three independent experiments by counting the number of invaded cells or percentage of positive stained area using ImageJ software (NIH, Bethesda, MD).

Zebrafish

Zebrafish were maintained according to standard methods approved by the Leiden University animal welfare committee. Two-day-old Tg(fli1:GFP) [32] dechorionated zebrafish embryos were injected with 400 cells in the heart cavity: either 400 dTomato-labeled MC38 cells or 200 dTomato-labeled MC38 combined with 200 unlabeled MEFs. After injection, embryos were maintained at 33°C. Fluorescent imaging was performed using a Leica SP5 STED confocal microscope (Leica Microsystems) under sedation with 0.003% tricaine (Sigma). Confocal stacks were processed using Leica software. For survival analysis, embryos were injected and transferred to a 24-well culture plate, and viability was monitored daily for 6 to 12 days. For IHC, zebrafish were fixed, embedded in paraffin, and processed as described above.

Experimental metastasis model

Animal experiments were approved by the animal welfare committee of the LUMC. Twenty-week-old Crl:CD-1Fox1nu male mice (Charles River Laboratories) were injected intrasplenically with 5 × 105 HT29 cells expressing firefly luciferase under isoflurane anesthesia, either alone or combined with 105 human CAFs. CAFs were pretreated with 40 μg/mL TRC105 or 40 μg/mL human IgG. Mice were treated twice weekly, with 15 mg/kg TRC105 or 15 mg/kg human IgG, intraperitoneally. Metastatic spread was monitored twice weekly using bioluminescent imaging on the IVIS Lumina-II (Caliper Life Sciences). Twenty-five days after tumor cell injection, mice were sacrificed, and blood and tissue samples were collected.

Statistical analysis

Differences between two groups were calculated using Student t test; for multiple groups, one-way ANOVA analysis was used. Survival curves were generated using Kaplan–Meier analysis and log-rank test. Differences in bioluminescent signals over time were calculated using two-way ANOVA analysis. P values of ≤0.05 were considered statistically significant. Error bars represent either SEM or SD, depending on appropriateness, as described in figure legends.

RESULTS

Endoglin expression on CAFs correlates with metastasis-free survival in stage II colorectal cancer

To investigate endoglin expression in colorectal cancer, sequential sections of colorectal cancer tissues were stained for cytokeratin (epithelium), CD31 (endothelial cells), αSMA (CAFs), and endoglin. As previously described, endoglin is highly expressed on endothelial cells in the tumor, as shown by the overlap between CD31 and endoglin staining (Fig. 1A, white arrowheads). However, we also observed endoglin expression on CAFs at the invasive borders, as indicated by an overlap in endoglin and αSMA, a marker for CAF activation (Fig. 1A, black arrowheads; [ref. 33]). Endoglin expression by CAFs was further confirmed using immunofluorescent double staining (Fig. 1B; Supplementary Fig. S1A and S1B). Notably, fibroblasts in adjacent normal colonic tissue or CAFs in the tumor core did not express endoglin (Fig. 1C). This specific localization of endoglin-expressing CAFs at the invasive border and their absence in the tumor core suggest that endoglin on CAFs plays a role in colorectal cancer invasion and metastasis. Exploring this hypothesis, we stained primary colorectal cancer, lymph node, and liver metastases from the same patients for endoglin and αSMA. Endoglin expression was present on CAFs at the invasive border of primary tumors, while staining intensity was remarkably higher on CAFs in both lymph node and liver metastases (Fig. 2A). To assess CAF-specific endoglin expression at different colorectal cancer stages, we stained normal colonic tissue, polyps, and stage II and III primary colorectal cancer tissues for αSMA and endoglin. Average scores for endoglin-expressing CAFs increased significantly with tumor stage (Fig. 2B). To determine whether CAF-specific endoglin expression predicted the development of metastatic disease, we assessed the relation between endoglin expression and metastasis-free survival. In stage II colorectal cancer, high CAF-specific endoglin expression significantly correlated with poor metastasis-free patient survival (Fig. 2C). In stage III colorectal cancer, no relation between metastases-free survival and CAF-specific endoglin expression was observed (Supplementary Fig. S1C). Analyses of two colorectal cancer TCGA databases showed a correlation between

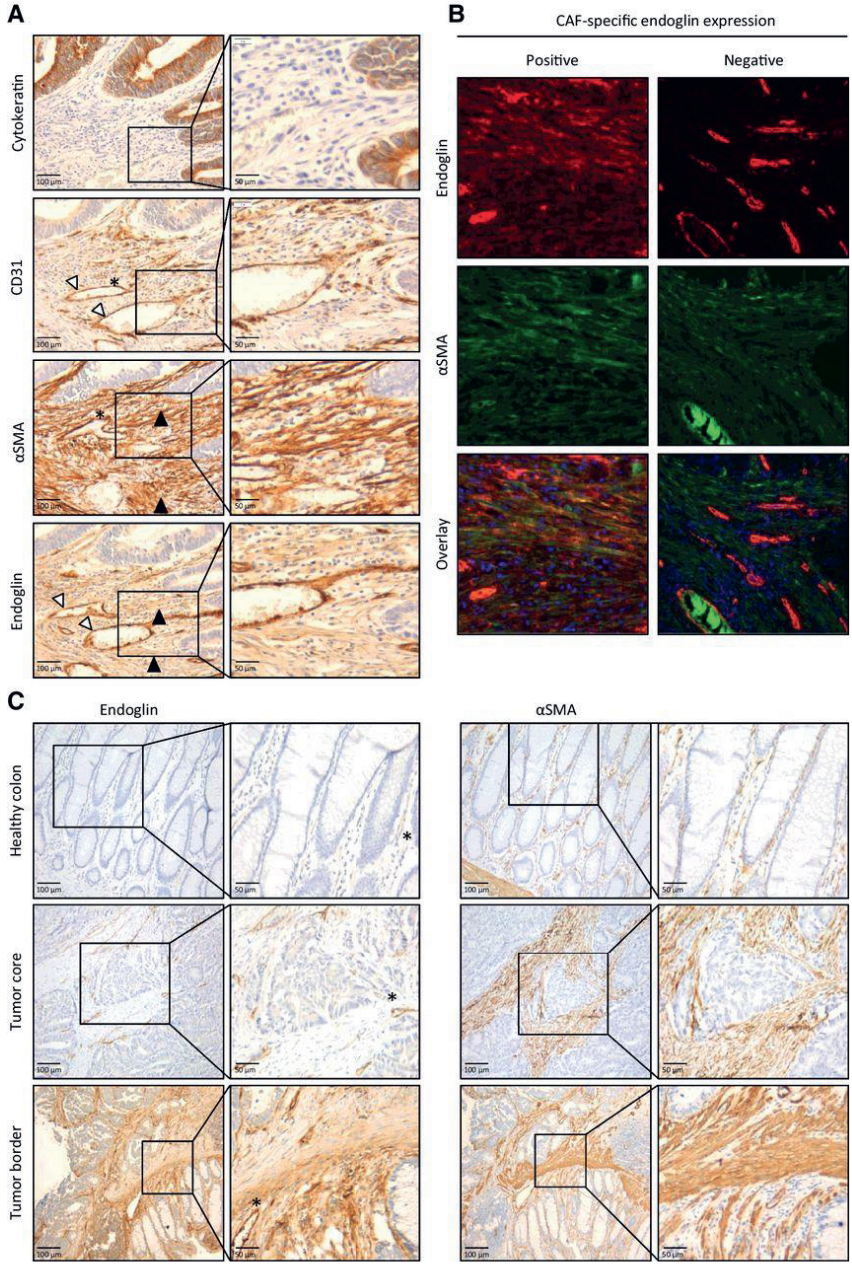

Figure 1. CAF-specific endoglin expression in colorectal cancer. A, IHC staining of colorectal cancer tissue for cytokeratin, CD31, αSMA, and endoglin. White arrowheads, high endoglin expression on the vasculature (CD31+/endoglin+); black arrowheads, endoglin-expressing CAFs (αSMA+/endoglin+); asterisks, αSMA+ smooth muscle cells surrounding vasculature. **B**, Immunofluorescent staining for endoglin (red) and αSMA (green) indicates high vascular endoglin expression and colocalization of αSMA and endoglin on CAFs (yellow, left). Colorectal cancer tissue containing endoglin-negative CAFs was used to show staining specificity (right). **C**, IHC staining for endoglin (left) and αSMA (right) in healthy colonic mucosa, tumor core, and invasive tumor border of the same patient with colorectal cancer. Asterisk indicates endoglin+ blood vessel.

endoglin expression and patient risk classification. Overall patient survival was not significantly different between the groups (Supplementary Fig. S1D), most probably due to the involvement of other factors besides endoglin expression, for example tumor stage, age, and sex. These data indicate that CAF-specific endoglin expression is predictive for metastasis-free survival in stage II colorectal cancer and could pose to be a relevant marker in selecting patients for adjuvant treatment.

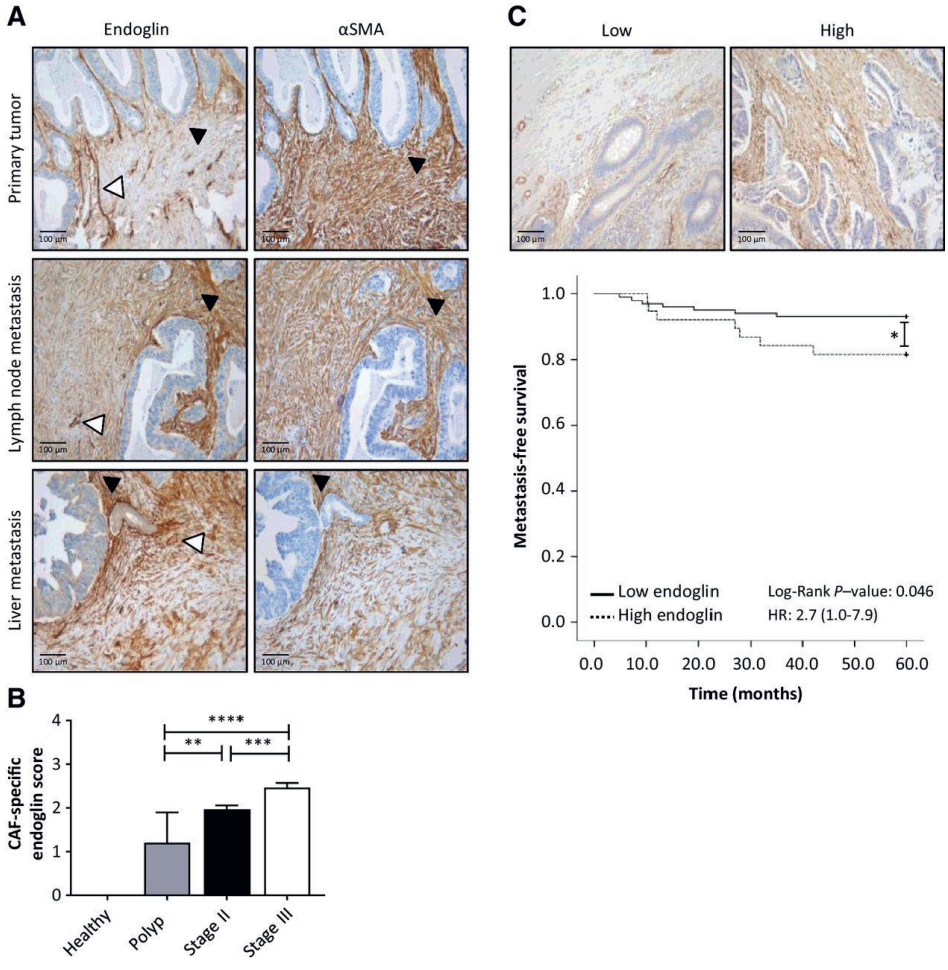

Figure 2. CAF-specific endoglin expression at the invasive border correlates to metastasis-free survival in stage II colorectal cancer. A, Primary tumor, lymph node, and liver metastases from the same patient with colorectal cancer show endoglin-expressing CAFs (black arrowheads). White arrowheads, endothelial endoglin expression. **B**, Average score of CAF-specific endoglin expression in healthy tissue, polyps, and stage II and III colorectal cancer (mean + SEM). **C**, Endoglin expression on CAFs at the invasive border of colorectal cancer correlates to metastasis-free survival in patients with stage II colorectal cancer (n = 140), analyzed as either high or low (exemplified in top boxes). Log-rank P value: 0.046; HR: 2.7 (1.0–7.9). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

NFs and CAFs display similar receptor expression profiles in vitro

To characterize TGF-β signaling in CAFs, we isolated NFs and CAFs from patients with colorectal cancer. CAFs were isolated from colorectal cancer tissues and NFs from adjacent normal mucosa (>10 cm from primary tumor) from four different patients and gene expression was assessed. Although NFs in tissue do not express endoglin (Supplementary Fig. S2A), endoglin expression is highly upregulated during in vitro culture. Therefore, endoglin mRNA expression did not differ between NFs and CAFs from the same patient, or between patients (Supplementary Table S4). Because endoglin can bind multiple TGF-β family members and mediate downstream signaling, expression of various TGF-β/BMP receptors was determined (Supplementary table S4). No differences in expression levels were observed between patients or between NFs and CAFs from the same patient after in vitro culturing, which might be due to the fact that in vitro culturing of NFs results in fibroblast activation, potentially influencing gene expression and therefore an accurate comparison between NFs and CAFs. To confirm CAF phenotype, protein expression of CAF markers αSMA and vimentin was confirmed by Western blot analysis and expression of the epithelial marker cytokeratin was excluded (Supplementary Fig. S2B). Platelet-derived growth factor receptor (PDGFR) expression can be used to distinguish certain CAF subpopulations [34]. However, no clear distinction in PDGFR expression was observed in NFs or CAFs from these patients in vitro (Supplementary table S4).

Fibroblast signaling in response to endoglin ligands

Canonical TGF-β signaling is regulated through different type-I receptors. Recruitment of ALK1, in the presence of endoglin, results in Smad1/5/8 phosphorylation [5, 36], whereas ALK5 directs phosphorylation of Smad2/3 [11]. Therefore, we determined endoglin signaling and downstream transcriptional regulation after TGF-β or BMP-9 stimulation. TGF-β stimulation of high endoglin-expressing human CAFs resulted in increased expression of the Smad2/3 target gene PAI-1, whereas this was unaffected by BMP-9 stimulation (Fig. 3A). BMP-9 induced expression of the Smad1 target gene inhibitor of differentiation-1 (ID-1). TGF-β also induced ID-1 expression, probably via ALK1 (Fig. 3A). Endoglin mRNA expression was not affected by TGF-β or BMP-9 stimulation (Fig. 3A). BMP-9 stimulation resulted in rapid and strong Smad1 phosphorylation, whereas TGF-β stimulation only slightly increased Smad1 phosphorylation (Fig. 3A, right). Next, the experiment was repeated in CAFs expressing very low levels of endoglin. In these CAFs, TGF-β–mediated effects were similar as observed for high endoglin-expressing CAFs (Fig. 3B). BMP-9 stimulation, however, did not induce ID-1 gene expression (Fig. 3B), nor Smad1 phosphorylation (Fig. 3B, right), confirming endoglin importance. Endoglin expression was unaffected by ligand stimulation (Fig. 3B). Mouse CAFs, which highly express endoglin, showed

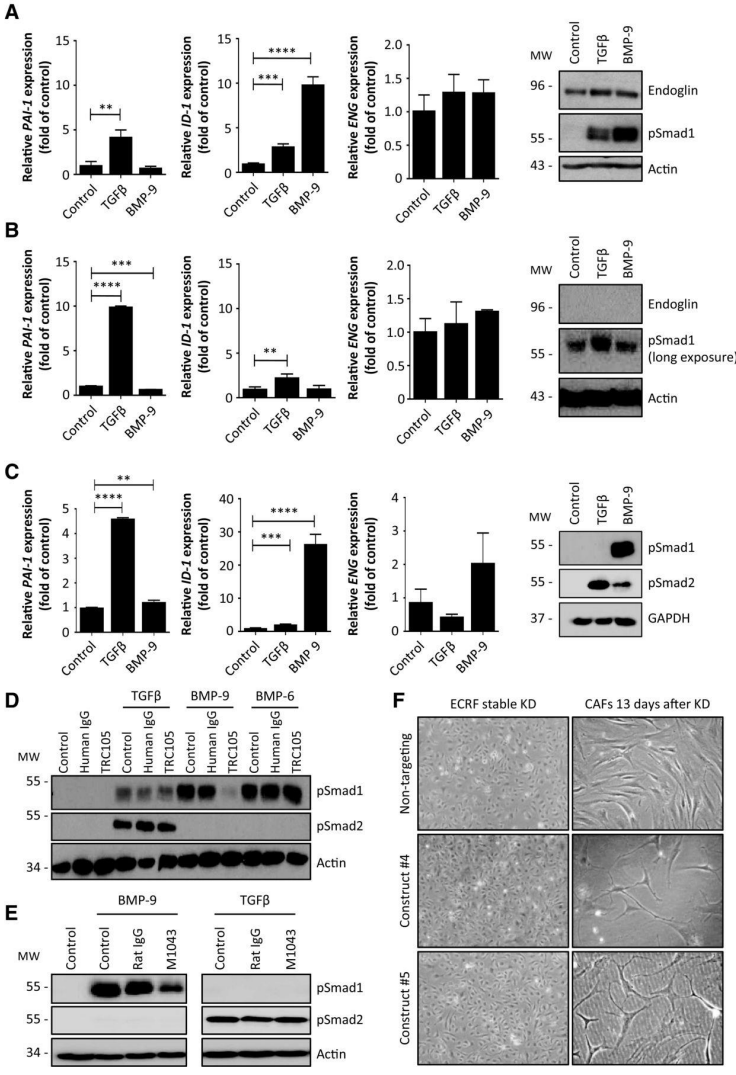

Figure 3. BMP-9–induced signaling in CAFs is endoglin dependent. A, BMP-9 stimulation of high endoglin–expressing human CAFs did not affect expression of PAI-1, but increased ID-1 expression. TGF-β slightly increased ID-1 expression and highly stimulated PAI-1. Both ligands did not affect endoglin expression. BMP-9 stimulation strongly induced pSmad1, whereas TGF-β showed slight pSmad1. Endoglin protein expression was unaffected upon stimulation. **B**, Low endoglin–expressing human CAFs showed a similar response to TGF-β and BMP-9 for PAI-1, whereas BMP-9 stimulation failed to induce ID-1 expression. Endoglin expression remained unaffected. No BMP-9–induced pSmad1 in low endoglin–expressing human CAFs. **C**, Mouse CAFs showed high induction of PAI-1 and ID-1 after stimulation with TGF-β or BMP-9, respectively. Endoglin expression was unaffected. BMP-9–induced pSmad1 in mouse CAFs; pSmad2 was strongly increased by TGF-β. **D**, TGF-β–induced pSmad2 and pSmad1 were unaffected by TRC105 in human CAFs. BMP-9–induced pSmad1 was abrogated by TRC105. Endoglin-independent pSmad1 induction by BMP-6 was unaffected by TRC105 treatment. **E**, In mouse CAFs, M1043 strongly decreased BMP-9–induced pSmad1. TGF-β increased pSmad2, which was unaffected by M1043. **F**, shRNA-mediated endoglin knockdown (KD) led to cell death in human CAFs, whereas endothelial ECRF cells remained viable. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. pSmad1, phosphorylated Smad1; pSmad2, phosphorylated Smad2. Graphs are representative of at least three independent experiments and show mean + SD.

similar gene expression results to those observed in high endoglin-expressing human CAFs (Fig. 3C). Furthermore, BMP-9 induced strong Smad1 phosphorylation, whereas TGF-β increased Smad2 phosphorylation in these cells (Fig. 3C, right). Ligand stimulation did not affect expression levels of total Smad2 in CAFs (Supplementary Fig. S2C). ELISA analysis was used to confirm endoglin protein expression on mouse CAFs (Supplementary Fig. S2D). To demonstrate that BMP-9 signals through endoglin to induce Smad1 phosphorylation in CAFs, we used the endoglin-neutralizing antibody TRC105, which competitively inhibits BMP-9 binding to endoglin. Human CAFs were stimulated with TGF-β, BMP-9, or BMP-6 in the presence or absence of TRC105. Stimulation with TGF-β increased Smad2 and, to a lesser extent, Smad1 phosphorylation, independent of TRC105 (Fig. 3D; Supplementary Fig. S3A). BMP-9 stimulation strongly increased Smad1 phosphorylation, which was abrogated by TRC105. BMP-6 induced endoglin-independent Smad1 phosphorylation and was unaffected by TRC105 (Fig. 3D; Supplementary Fig. S3A). In mouse CAFs, stimulation with BMP-9 strongly induced Smad1 phosphorylation (Fig. 3E; Supplementary Fig. S3B). This was efficiently blocked by the mouse endoglin-neutralizing antibody M1043, whereas TGF-β–induced Smad2 phosphorylation was unaffected (Fig. 3E; Supplementary Fig. S3B). TRC105 also inhibited Smad1 phosphorylation in mouse CAFs, although to a lesser extent (Supplementary Fig. S3B). Therefore, subsequent experiments using mouse cells were performed using M1043. Together, these results confirm that BMP-9–induced Smad1 phosphorylation is endoglin-dependent in CAFs, and this can be inhibited using endoglin-neutralizing antibodies.

Endoglin is required for CAF survival in vitro

After characterizing endoglin-mediated signaling in CAFs, its functional role was further evaluated. Short hairpin RNA (shRNA) constructs targeting endoglin were introduced using lentiviral transduction, and knockdown efficiency of the constructs at RNA and protein level was confirmed in endothelial cells (Supplementary Fig. S3C). Endoglin expression was reduced by 40% to 90% compared with non-targeting control. This degree of endoglin knockdown did not affect endothelial cell morphology or survival (Fig. 3F). However, CAFs transduced with endoglin shRNA constructs ceased to proliferate and cells adopted a senescence-resembling phenotype (Fig. 3F), progressing to cell detachment and death. The phenotype was confirmed in different CAFs and NFs, indicating that endoglin is indispensable for CAF survival in vitro.

Endoglin regulates CAF invasion in vitro

Different roles for endoglin in cell migration have been reported for endothelial and nonendothelial cells. Ectopic expression of endoglin in HEK293T cells enhanced cell invasion into a collagen-I matrix (Supplementary Fig. S4A and S4B). Spheroid diameters remained similar, suggesting that invasion rather than proliferation is the main

determinant of this effect. Next, we examined the role of endoglin in CAF invasion. High endoglin-expressing CAFs (F2) were compared with CAFs expressing 200-fold lower levels of endoglin (F1, Fig. 4A). After 48 hours, F2 CAFs invaded a collagen-I matrix to a higher extent than F1 CAFs (Fig. 4B), suggesting a role for endoglin in CAF invasion. Because 3-dimensional invasion assays with CAFs are difficult to accurately quantify, we overexpressed endoglin in F1 CAFs (Fig. 4C), and invasive capacity was determined using transwell invasion assays through collagen-I–coated inserts. Quantification of the number of invaded cells after 24 hours showed that endoglin overexpression significantly increased basal CAF invasion (Fig. 4D). Because endoglin knockdown is not possible in CAFs, we used MEFs isolated from endoglin fl/fl mouse embryos (25). To induce endoglin deletion (KO), MEFs were transduced with Cre recombinase or an empty vector. Endoglin KO did not affect MEF proliferation and cells remained viable for up to three passages after transduction, possibly due to their embryonic nature. Endoglin mRNA levels were reduced by 90% (Fig. 4E), and endoglin KO MEFs showed significantly reduced invasion in transwell invasion assays (Fig. 4F). These data demonstrate the importance of endoglin in CAF invasion.

Endoglin targeting reduces invasive capacity of fibroblasts

To confirm that CAF invasion is dependent on ligand binding to endoglin, and not merely on its presence, the invasive capacity of mouse CAF was assessed in the presence of M1043. M1043 treatment reduced basal mouse CAF invasion, without affecting cell morphology (Fig. 5A). Because both colorectal cancer cells and CAFs express the endoglin ligands TGF-β [6, 37] and BMP-9 (Supplementary Fig. S4C and S4D), these factors were used to determine their individual contributions to CAF invasion. TGF-β stimulation did not increase BMP-9 expression in CAFs (Supplementary Fig. S4E), excluding indirect effects on CAF invasion by TGF-β, through BMP-9 signaling. BMP-9– and TGF-β–induced invasion were inhibited by M1043 to a similar level, albeit not statistically significant (Supplementary Fig. S4F). Because interactions in the TME can be mediated by paracrine signaling, we assessed CAF invasion toward murine MC38 colorectal cancer cells. MC38 cells stimulated CAF invasion to a similar extent as BMP-9, which could not be further enhanced by combining MC38 cells with BMP-9 (Fig. 5B). M1043 treatment significantly decreased MC38-induced CAF invasion, compared with IgG control (Fig. 5C). In human CAFs, TRC105 decreased basal human fibroblast invasion (Fig. 5D), while cell morphology remained similar and proliferation was unaffected (Supplementary Fig. S4G). BMP-9 and TGF-β only marginally affected CAF invasion in this case, resulting in limited inhibitory effects of TRC105 on invasion (Supplementary Fig. S4H). In coculture experiments, treatment with TRC105 inhibited HCT116- and HT29-induced CAF invasion (Fig. 5E and F, respectively). Taken together, these experiments imply a substantial role for endoglin/BMP-9 signaling in CAF invasion in vitro.

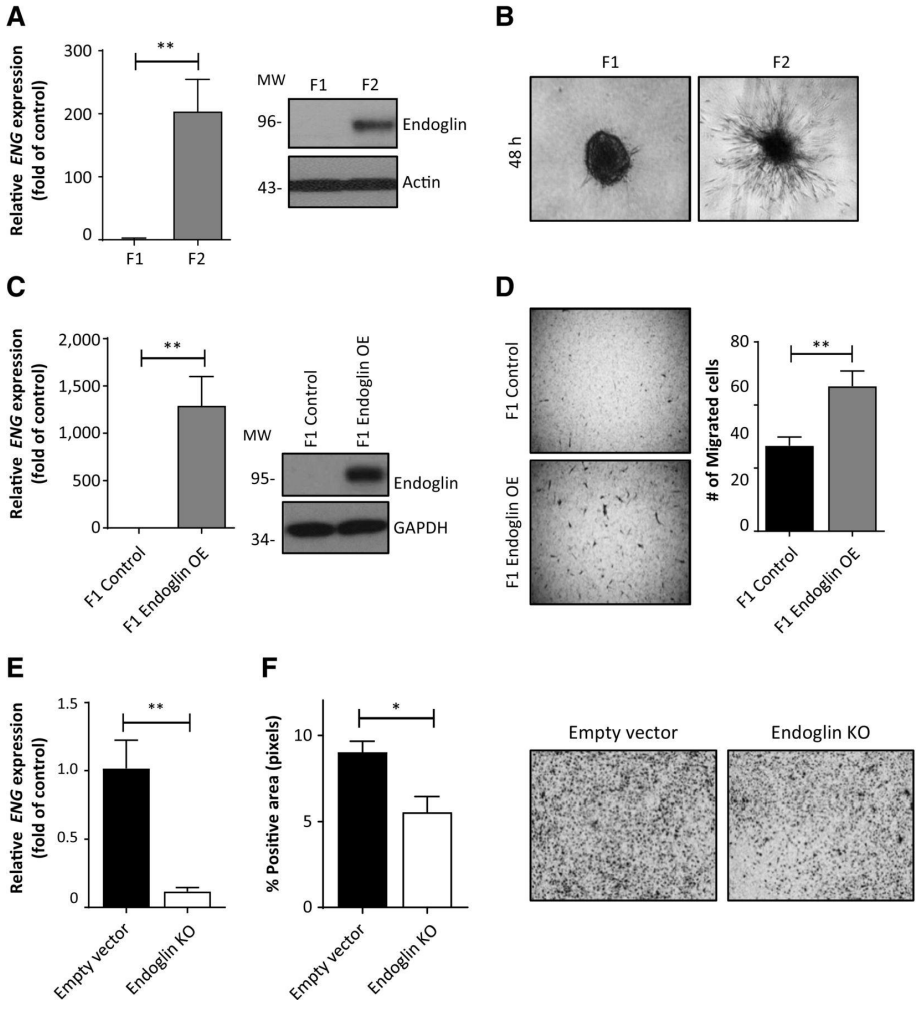

Figure 4. Endoglin regulates CAF invasive capacity in vitro. Low (F1) and high (F2) endoglin–expressing CAFs (**A**) were assessed for invasive properties. **B**, High endoglin–expressing F2 CAFs invaded collagen-I matrix more extensively than low endoglin–expressing F1 CAFs. Representative pictures of two experiments performed in triplicate. h, hours. **C**, Confirmation of endoglin overexpression (OE) in F1 CAFs. Representative graph from three independent experiments. **D,** Endoglin OE significantly enhanced F1 CAF invasion after 24 hours in a transwell invasion assay. Data represent mean of three independent experiments performed in triplicate. **E**, Reduced endoglin expression in endoglin knockout MEFs (endoglin KO) upon Cre expression. **F**, Endoglin KO significantly reduced MEF invasion in transwell invasion assays. Data represent mean of three independent experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq 0.01$. Quantification of expression data shown as representative mean + SD, invasion as mean + SEM of at least three independent experiments.

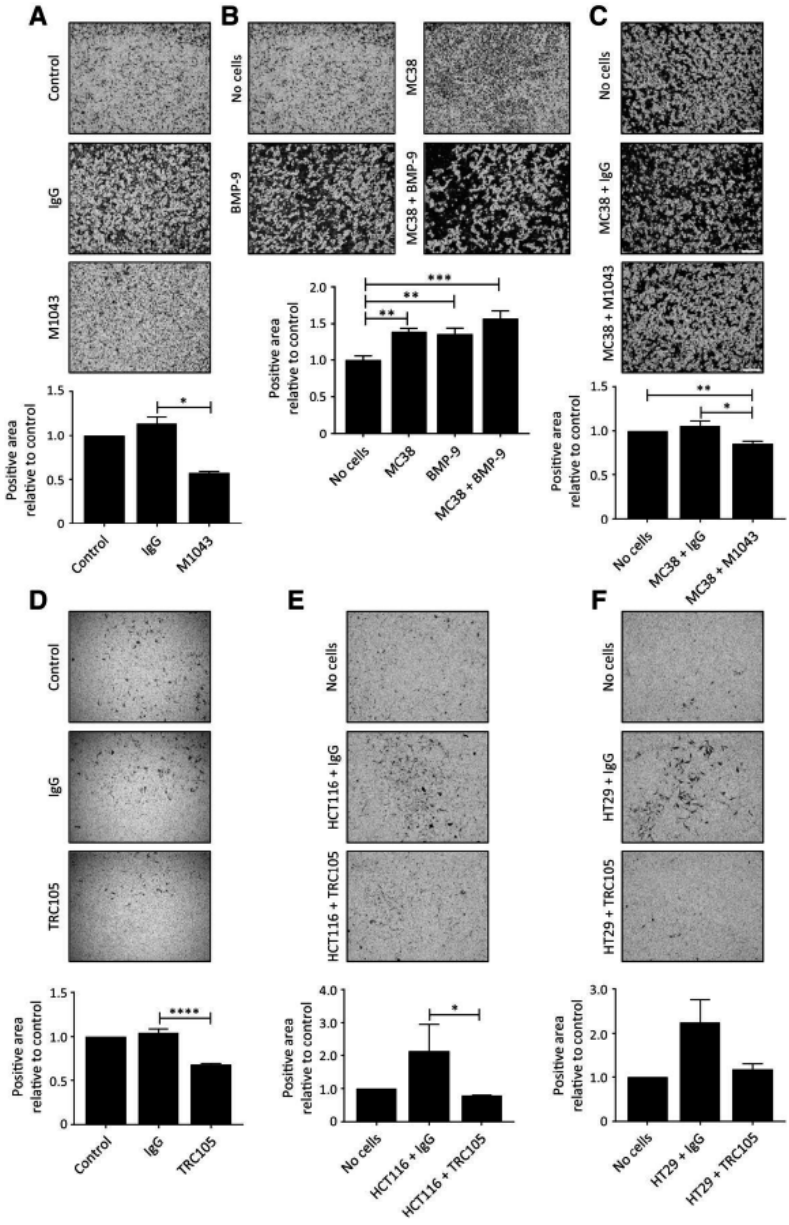

Figure 5. Endoglin targeting inhibits CAF invasion in vitro without affecting CAF morphology. A, M1043 significantly inhibited basal mouse CAF invasion. **B**, The presence of MC38 cells in the lower transwell compartment increased mouse CAF invasion to a similar extent as observed for BMP-9 stimulation. Addition of BMP-9 in the presence of MC38 did not further increase CAF invasion. **C**, Mouse CAF invasion toward MC38 mouse colorectal cancer cells was slightly, but significantly, reduced by M1043 when compared with IgG control. **D**, TRC105 significantly inhibited basal human CAF invasion. Human CAF invasion toward the human colorectal cancer cell lines HCT116 (**E**) or HT29 (**F**) was inhibited by TRC105 when compared with IgG control. All data represent mean of at least three independent experiments performed in triplicate. No cells, no tumor cells present in lower compartment. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. All graphs show mean + SEM of at least three independent experiments. Representative pictures for all conditions are shown at ×20 magnification.

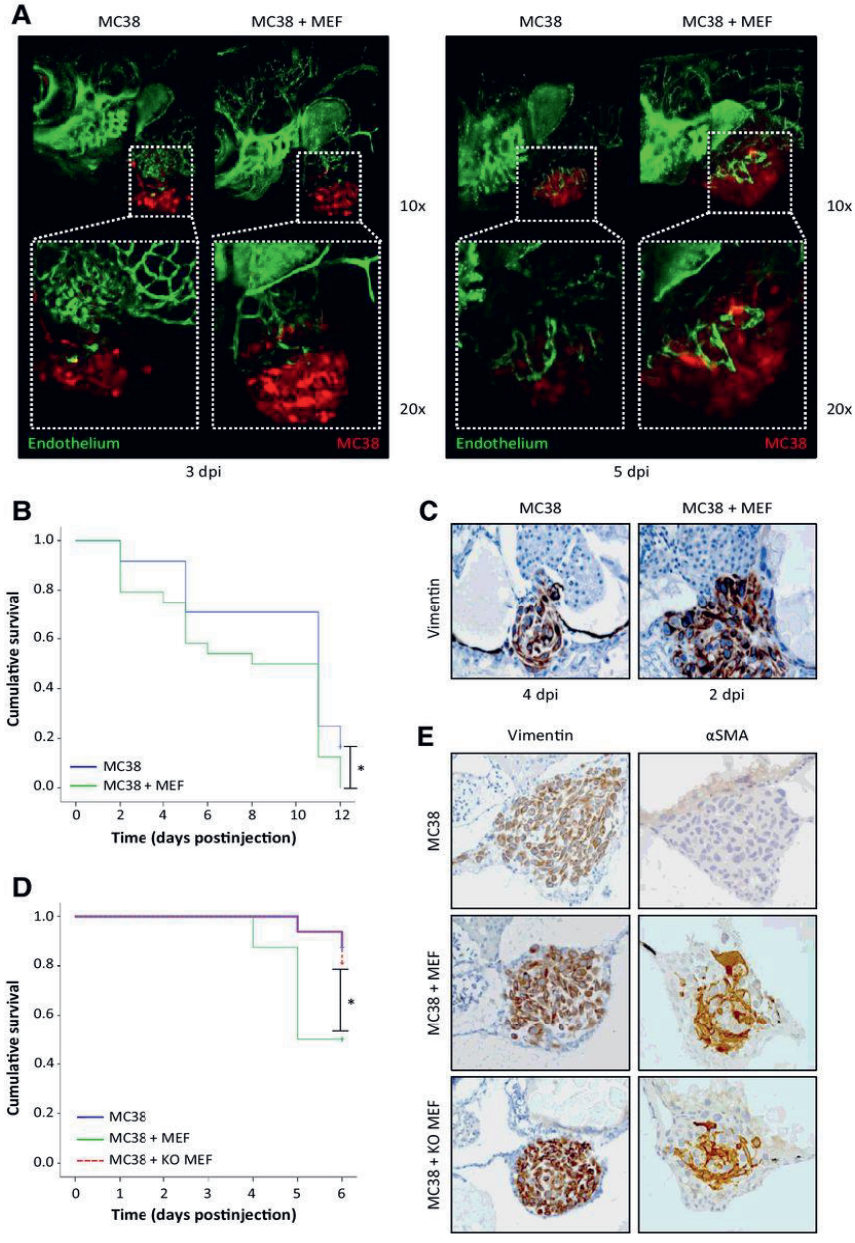

Figure 6. Endoglin-expressing CAFs reduce survival in a zebrafish model for colorectal cancer.
A, Injection of dTomato MC38 cells in the absence or presence of MEFs (MC38 + MEF) induced formation of solid tumors (red) and recruitment of vasculature (green) in zebrafish embryos. **B**, Coinjection of MC38 cells with MEFs decreased survival in zebrafish embryos when compared with MC38 alone (n = 24/group). **C**, IHC staining showed vimentin-expressing MC38 cells invading the zebrafish liver. Coinjection with MEFs accelerated liver invasion (2 dpi, right) compared with MC38 alone (4 dpi, left). **D**, Coinjection of MC38 with endoglin knockout (KO) MEFs (MC38 + KO MEF) resulted in similar fish survival when compared with injection of MC38 alone (n = 23/group). **E**, IHC staining of MC38 tumors for vimentin (MC38 cells) and αSMA (MEFs). *, P ≤ 0.05. dpi, days post injection.

Endoglin expression on fibroblasts promotes colorectal cancer liver invasion in zebrafish

To study the role of CAF-specific endoglin in tumor metastasis in a multicellular model, we developed a zebrafish model for colorectal cancer. Fluorescently labeled MC38 cells, in the presence or absence of MEFs (because endoglin deletion can be established in these cells, in contrast to CAFs), were injected in the heart cavity of zebrafish embryos and zebrafish were followed over time. Solid tumor-like structures were formed and induced angiogenesis (Fig. 6A). Coinjection of MC38 with MEFs significantly decreased fish survival (Fig. 6B), probably due to compromised liver function caused by tumor cell invasion. Histologic analysis revealed invasion of the vimentin-positive MC38 cells (MC38 cells do not express epithelial markers, like cytokeratin) into the liver in both experimental groups. However, coinjection with MEFs resulted in liver invasion as early as 2 days after injection, versus 4 days after injection when MC38 cells were injected alone, suggesting that the presence of MEFs accelerates this invasive process (Fig. 6C). To investigate endoglin dependency, MC38 cells were injected in combination with normal or endoglin KO MEFs. Tumor formation and angiogenesis were not affected (Supplementary Fig. S5). However, zebrafish survival markedly improved when endoglin KO MEFs were coinjected and resembled survival of zebrafish injected with MC38 alone (Fig. 6D). Integration of MEFs in the tumors of both coinjected groups was confirmed by staining for mouse-specific αSMA expression (Fig. 6E). These data indicate that endoglin expression on MEFs affects colorectal cancer cell invasion in zebrafish.

Endoglin targeting inhibits colorectal cancer liver metastasis in mice

Finally, we assessed therapeutic targeting of endoglin in an experimental mouse model for colorectal cancer liver metastasis. HT29 cells were injected in the spleen, alone or in combination with human CAFs. Mice were treated (Fig. 7A) and metastatic spread was monitored using bioluminescent imaging (BLI). HT29 cells express very low levels of endoglin, and in vitro proliferation of HT29 cells was not affected by TRC105 (Supplementary Fig. S6A and S6B). Therefore, as expected, TRC105 treatment did not affect metastatic spread in mice injected with HT29 cells alone (Supplementary Fig. S6C and S6D). In mice coinjected with HT29 and CAFs, however, TRC105 significantly reduced BLI signal from the liver (Fig. 7B). This indicates that TRC105 affects metastasis formation by directly targeting human CAFs. Metastatic lesions in the liver were visualized using ex vivo BLI upon termination of the experiment (Fig. 7C). IHC staining revealed no morphologic differences in liver metastases between groups (Fig. 7D). These data show that targeting endoglin on CAFs inhibits metastatic spread of HT29 colorectal cancer cells and imply CAFs as an additional target cell for TRC105 therapy.

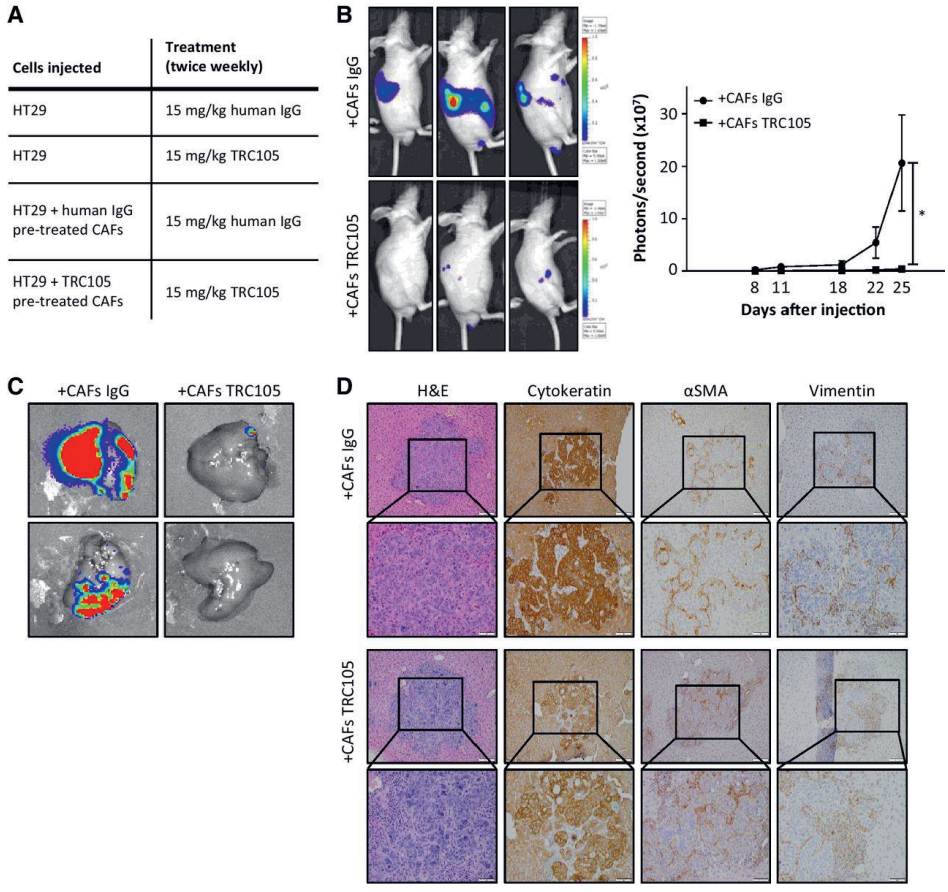

Figure 7. TRC105 inhibits CAF-mediated metastatic spread in a mouse model for liver metastasis. **A**, Experimental setup. Mice were injected with HT29 cells alone or in combination with human CAFs. Two days after injection, treatment with human IgG or TRC105 started. **B**, TRC105 treatment reduced metastatic spread to the liver in mice injected with HT29 and CAFs (+CAFs TRC105) as quantified by in vivo bioluminescence. Graph represents two independent experiments, 15 mice/group in total, showing mean ± SEM. **C**, TRC105 treatment decreased ex vivo bioluminescent signal in livers of mice coinjected with HT29 and CAFs. **D**, (Immuno)histologic analysis of liver metastasis with H&E and staining for cytokeratin (HT29), αSMA, and vimentin (CAFs). Magnification: ×100 and ×200. *, P ≤ 0.05.

DISCUSSION

In this study, we show that CAF-specific endoglin expression correlates with the development of metastatic disease in stage II colorectal cancer and endoglin-expressing CAFs are detected in metastatic lesions of patients with colorectal cancer. CAF-specific endoglin expression stimulates CAF invasion in vitro and tumor cell invasion and metastasis in a novel zebrafish model and in a murine model for colorectal cancer.

Endoglin is crucial for vascular development as underlined by embryonic lethality of endoglin knockout mice [12–14]. Our current study shows the importance of endoglin for fibroblast survival in vitro. Romero and colleagues reported the inability to culture primary prostate CAFs isolated from endoglin heterozygous mice, whereas CAFs from endoglin wild-type mice could easily be propagated in vitro (38). We observed that although NFs in vivo do not express endoglin, its expression is highly upregulated during cell culture. Because of its indispensability in vitro, neutralizing antibodies pose a useful tool to study the role of endoglin in culture, especially because CAF proliferation remains unaffected.

In endothelial cells, endoglin has been shown to be important for proliferation [39] and migration [40], and deletion of endoglin results in decreased Smad1 phosphorylation [41]. Recently, the crystal structure of BMP-9 bound to endoglin has been reported, revealing that endoglin is required to efficiently present BMP-9 to ALK1 [17] and induce downstream signaling. In our in vitro assays, endoglin was essential for BMP-9–induced Smad1 phosphorylation in CAFs and important for CAF invasion through collagen-I. Inhibition of ALK5 signaling by the ALK1/endoglin complex has been described in endothelial cells [42]. In accordance with this, we have recently reported increased Smad2 phosphorylation in endothelial cells upon TRC105 treatment [28], which alleviates the inhibitory function of this complex. Activation of ALK5 signaling results in decreased endothelial cell migration [42], which could pose an additional mechanism by which cell migration is decreased next to inhibition of endoglin signaling. However, increased Smad2 phosphorylation was not observed in CAFs upon TRC105 treatment, suggesting differences in TGF-β/BMP signaling between CAFs and endothelial cells, possibly by receptor abundance or expression of different type I receptors.

Colorectal cancer cells produce high levels of TGF-β [6], which could affect endoglin signaling by indirectly increasing BMP-9 expression in CAFs. In this article, we show that TGF-β stimulation does not affect BMP-9 expression in CAFs. In addition, Nolan-Stevaux and colleagues [19] and unpublished observations from our group showed that TRC105 does not inhibit binding of TGF-β to endoglin, further rendering observed effects on CAF invasion to be BMP-9 and most likely not TGF-β endoglin dependent.

In addition to signaling through ALK1, endoglin interacts with integrins, crucial for adhesion and migration of ECs [43]. In fibroblasts, interactions between ECM and integrins were shown to be important for cellular migration [44], but the role of endoglin in this interaction was not investigated. In our in vitro experiments, inhibiting endoglin function reduced CAF invasion through a collagen-I matrix, suggesting involvement of endoglin in cell–ECM interaction, although the underlying mechanism is yet unresolved.

The specific localization of endoglin-expressing CAFs at the invasive border of colorectal tumors suggests a role in tumor metastasis, a suggestion that is further strengthened by their detection in lymph node and liver metastases from patients with colorectal cancer. Recently, Labernadie and colleagues revealed that CAFs use heterotypic cadherin interactions to interact with tumor cells and physically pull these cells out of the tumor mass in order to induce tumor invasion [45]. Interactions of endoglin with VE-cadherin were reported in endothelial cells, in which VE-cadherin regulates cell migration [46]. Interactions of endoglin with other cadherins have not yet been reported but might be involved in CAF-mediated tumor invasion. The physical interaction of CAFs with tumor cells implies that CAFs could travel in a complex with tumor cells to metastatic sites. In accordance with this hypothesis, results from an experimental model for colorectal cancer metastasis showed that GFP-expressing CAFs were localized in liver metastases and increased the formation of these lesions [47]. In vivo experiments in a lung cancer model also showed stromal cells derived from the primary tumor in metastatic lesions [48]. These data combined with our observation that TRC105 inhibits liver metastasis by targeting endoglin on CAFs in vivo imply that metastatic spread could, at least in part, be regulated by endoglin.

Previously, in prostate cancer models, it was shown that although endoglin heterozygosity increased primary tumor growth, the number of metastases was lower than in wild-type mice [38]. In contrast, increased metastatic spread of pancreatic tumors and subcutaneous implanted lung cancer cells has been reported in, respectively, endoglin heterozygous or endothelial-specific endoglin KO mice [49]. Although these data contradict our findings, these studies investigated endoglin expression on endothelium and did not consider fibroblast-specific endoglin expression. Heterozygous endoglin deletion in vivo results in a phenotype resembling hereditary hemorrhagic telangiectasia, including increased vascular permeability [50, 51], possibly facilitating tumor cell intra- and extravasation and subsequent metastasis. Our current experiments specifically assessed the role of endoglin on CAFs. Moreover, unpublished data from our group showed that TRC105 treatment does not affect endothelial cell integrity in vitro. More in-depth studies using cell type-specific endoglin knockout mice have to be performed in order to unravel the exact contribution of endoglin on individual cell types in metastatic spread.

The fibrotic response is an important regulator of tumor progression and metastasis [52] and has been proposed as a prognostic factor in colorectal cancer [53]. Endoglin expression on fibroblasts has been reported during cardiac fibrosis and reduction of endoglin expression or endoglin targeting prevented cardiac fibrosis in vivo [54]. Interestingly, prostate tumors grown in endoglin heterozygous mice are not fibrotic and lack αSMA-expressing cells [38]. In line with these data, we have previously shown that TRC105 treatment reduced metastatic breast cancer spread in vivo,

which was accompanied by a decreased αSMA-positive stromal content [28]. Even though the αSMA content in the liver metastasis in vivo in this article was not different between groups, metastatic spread was reduced after TRC105 treatment, suggesting that these residual lesions managed to escape treatment. Moreover, we show here a correlation between high CAF-specific endoglin expression at the invasive tumor borders and worse metastasis-free survival in patients with stage II colorectal cancer, implying the involvement of this CAF subset in tumor invasion and metastasis. Analyses of different TCGA databases for colorectal cancer showed that endoglin is correlated to risk classification. Despite the fact that these databases provide valuable information, they do not distinguish between cell type-specific endoglin expression and specific localization, as reported in this study. Although we did not assess the relation between CAF-specific endoglin expression and fibrosis in this study, these data could imply that endoglin on CAFs contributes to peritumoral fibrosis as an adverse prognostic factor in colorectal cancer. In addition, it might suggest another potential therapeutic field for TRC105 for the treatment of fibrotic diseases.

In summary, the data presented here point to a crucial involvement of endoglin-expressing CAFs in colorectal cancer invasion and metastasis and could therefore be a potential therapeutic target. In addition, CAF-specific endoglin expression might be a novel prognostic factor in early-stage colorectal cancer. In a phase I study, TRC105 showed clinical efficacy on preexisting metastases in 2 patients [18]. Combined with our recently published data that adjuvant TRC105 treatment decreased metastatic spread in breast cancer [28], targeting endoglin on CAFs, in addition to the endothelium, could be a potent approach in preventing metastasis formation and underlines the potential of TRC105 being more than a classic antiangiogenic drug.

Disclosure of Potential Conflicts of Interest

C.P. Theuer has ownership interest (including patents) in TRACON Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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