

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

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Abstract

Background & aims

Cancer-associated fibroblasts (CAFs) are a major component of the tumor microenvironment in colorectal cancer (CRC). CAFs play an important role in tumor progression and metastasis, partly through the transforming growth factor- β (TGF- β) signaling pathway. We investigated

whether the TGF- β family co-receptor endoglin is involved in CAF-mediated invasion and metastatic spread.

Methods

CAF-specific endoglin expression was investigated in resection specimens from CRC patients using immunohistochemistry and related to metastases-free survival. *In vitro*, endoglin-mediated invasion was assessed in transwell invasion experiments, using primary CRC-derived CAFs. In a zebrafish model for CRC, involvement of CAF-specific endoglin expression on tumor cell invasion was investigated. Finally, effects on metastatic spread were assessed in a mouse model for experimental liver metastasis.

Results

CAFs specifically at invasive borders of CRC, express endoglin with increasing intensity through increasing stage. Additionally, endoglin-expressing CAFs were detected in lymph node and liver metastases from the same patients, suggesting a role in CRC metastasis formation. In stage-II CRC, CAF-specific endoglin expression at the invasive front positively correlated with metastasis-free survival. *In vitro* experiments revealed that endoglin is indispensable for bone morphogenetic protein (BMP)-9-induced signaling and fibroblast survival. CAF invasion *in vitro* was inhibited by targeting endoglin using the endoglin neutralizing antibody TRC105. In zebrafish, endoglin-expressing fibroblasts enhanced colorectal tumor cell infiltration into the liver and decreased survival. Finally, endoglin targeting, specifically on CAFs, with TRC105 decreased metastatic spread of CRC cells to the mouse liver.

Conclusions

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

Keywords

Endoglin, colorectal cancer, cancer-associated fibroblasts, metastasis, TRC105

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and accounts for 8,5% of all cancer deaths¹. Ninety percent of all cancer patients die because of metastatic spread to distant organs. Therefore, patients identified to be at risk to develop metastatic disease (stage-III/IV) receive adjuvant (chemo)-radiation therapy². Early stage patients are not adjuvantly treated, but a significant group still develops metastatic disease, emphasizing that better understanding of the mechanism underlying tumor metastasis is needed.

Next to malignant epithelial cells, the tumor stroma can make up to 50% of the tumor mass and is predictive for CRC patient survival³. The tumor stroma, or tumor microenvironment (TME), is composed of vascular endothelial cells, immune cells and cancer-associated fibroblasts (CAFs)⁴. CAFs interact with all other cells in the TME via direct cell-cell contacts and CAF-derived cytokines^{5, 6}. This leads to tumor progression and ultimately metastasis⁷. Therefore, CAFs are considered a potential target for novel cancer therapies.

Transforming growth factor- β (TGF- β) is capable of transdifferentiating resident fibroblasts into CAFs^{8, 9}, indicated by increased expression of α -smooth muscle actin (α SMA) and fibroblast activation protein (FAP)¹⁰. Endoglin is a co-receptor for TGF- β and bone morphogenetic protein (BMP)-9 which is highly expressed on the surface of activated endothelial cells and indispensable during embryogenesis¹¹⁻¹³. In endothelial cells, binding of TGF- β or BMP-9 to endoglin induces Smad1 phosphorylation¹⁴. Furthermore, endoglin microvessel density was correlated with tumor progression and metastases in CRC^{15, 16}. Consequently, the endoglin neutralizing antibody TRC105 is currently under clinical development as a novel anti-angiogenic therapy. TRC105 binds human endoglin with high affinity, prevents BMP-9 binding¹⁷ and induces antibody-dependent cell-mediated cytotoxicity^{18, 19}.

Although endoglin in endothelial cells has been extensively studied, expression on other cells in the TME is less known. Therefore, we have investigated the role of endoglin expression on CAFs. We showed that CAFs, specifically at invasive borders of colorectal tumors and in metastatic lesions, express endoglin. Next, we assessed regulation of endoglin expression in CAFs and its role in CAF invasion *in vitro*. The involvement of CAF-specific endoglin expression in tumor invasion was studied in a novel zebrafish model for CRC. Finally, in a mouse model for liver metastasis, we assessed the effects of therapeutically targeting endoglin on CAF-regulated metastatic spread to the liver. 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) and were used according to the guidelines of the Medical Ethical Committee of the LUMC. The first cohort consisted of 25 adenomas, 104 stage-II and 94 stage-III treatment naive CRC and the same number of adjacent normal tissue samples. The second cohort consisted of 31 patients of which resection specimens of the primary tumor, lymph node and liver metastases were present. Patient characteristics and >10 year follow-up were available.

Tissue analysis

Immunohistochemical and -fluorescent stainings were performed as described before^{20, 21}, using antibodies as shown in supplementary table S1. CAF-specific endoglin expression, was scored on a scale of 1 to 4 ($\leq 10\%$; 10-25%; 25-50% and $\geq 50\%$ positive) in a blinded manner by two independent observers. Pictures were obtained using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). Quantitative PCR analyses were performed as described before⁶ using primer sequences described in supplementary table S2.

Cell culture and signaling assays

Human CAFs and the mouse CRC cell line MC38²² were cultured in DMEM/F12, supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 50 $\mu\text{g}/\text{mL}$ gentamycin, 100 IU/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (PenStrep, all ThermoFisher). The immortalized HUVEC cell line ECRF²³ was cultured as described before²⁴. Human embryonic kidney 293 (HEK293T) cells were maintained in DMEM, supplemented with 10% FCS and PenStrep (all ThermoFisher). Primary human CAFs were isolated from non-necrotic parts of the tumors and normal fibroblasts (NFs) from adjacent healthy tissue as described before⁶. Tissues were cultured in complete DMEM/F12 with 2.5 $\mu\text{g}/\text{mL}$ Fungizone (ThermoFisher). Mouse CAFs were isolated from CRC tissue by culturing 5x5 mm pieces of tumor in DMEM/F12 as described above. For both human and mouse isolations, fibroblast-like cell outgrowth was observed after 7-10 days. Murine embryonic fibroblasts (MEFs) were obtained from E12.5 embryos as described before²⁵ from endoglin floxed mice²⁶. MEFs were

maintained in DMEM, supplemented with 10% FCS and PenStrep (all ThermoFisher). All cell cultures were monthly tested for mycoplasma contamination.

Constructs expressing human endoglin²⁷, 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. HEK293T cells were grown to 80% confluency and transfected with endoglin expressing plasmids using 1 mg/mL polyethylenimine (PEI; Polysciences Inc., Warrington, PA, USA).

For signaling assays, fibroblasts were seeded in six-well plates. Upon 90% confluency, cells were serum starved overnight in medium containing 40 µg/mL TRC105 (Tacon Pharmaceuticals, San Diego, CA, USA) or 40 µg/mL human IgG (BioXCell, West Lebanon, NH, USA) for human cells. Mouse fibroblasts were incubated in the presence of 40 µg/mL M1043 (Abzena, Cambridge, UK) or 40 µg/mL Rat IgG (BioXCell). Next day, cells were stimulated with either 5 ng/mL TGF-β3²⁸, 0.1 ng/mL BMP-9 (R&D systems) or 100 ng/mL BMP-6 (PeproTech, London, UK) for one hour. Cells were lysed in RIPA buffer, protein content was determined and western blot analysis was performed as described before²⁹. Membranes were incubated overnight with primary antibodies against endoglin, phosphorylated (p)Smad1 or pSmad2. Blots were stripped and reprobed with mouse anti-GAPDH or anti-actin antibodies (supplementary table S1) as loading control. Blots were developed using the Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad, Veenendaal, The Netherlands).

Invasion assays

One thousand HEK293T cells or 2500 CAFs were seeded on top of 0.6% agarose (Sigma, Zwijndrecht, The Netherlands) coated 96-well plates and left to form spheroids for 48h. Next, spheroids were collected and embedded in 1 mg/mL collagen-I matrix (Advanced BioMatrix, San Diego, CA, USA) containing 10% FCS. At 0, 24 and 48 hours, pictures were taken using a Zeiss Axiovert 200M microscope (Carl Zeiss BV, Sliedrecht, The Netherlands). Quantification of the invaded area was performed using Adobe Photoshop software.

For transwell invasion assays, the upper surface of 8.0 µm pore size ThinCert (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) 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- β 3. When invasion towards CRC cells was assessed, 2×10^5 HT29, HCT116 or MC38 cells were seeded in the lower well. 2.5×10^4 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, Zoeterwoude, The Netherlands), five pictures per insert were obtained at 2x 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 Image J software (National Institutes of Health, USA).

Zebrafish

Zebrafish were maintained according to standard methods approved by the Leiden University animal welfare committee. Two-day old Tg(fli1:GFP)³⁰ 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 34°C. Fluorescent imaging was performed using a Leica TCS SPE confocal microscope (Leica Microsystems, Son, The Netherlands) under sedation with 0.003% tricaine (Sigma, Zwijndrecht, The Netherlands). Confocal stacks were processed using Leica software. For survival analysis, embryos were injected and transferred to a 48-well culture plate and viability was monitored daily for 6-12 days.

Experimental metastasis model

Animal experiments were approved by the Dutch animal ethics committee. 20-weeks old Crl:CD-1Fox1nu male mice (Charles River, L'Arbresle Cedex, France) were injected intrasplenic with 5×10^5 HT29 cells expressing firefly luciferase under isoflurane anesthesia, either alone or combined with 10^5 CAFs. CAFs were pre-treated with 40 μ g/mL TRC105 or 40 μ g/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, Hopkinton, MA, USA). 25

days after tumor cell injection, mice were sacrificed and blood and tissue samples were collected.

Statistical analysis

Differences between two groups were calculated using Students' *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.

Results

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

To investigate endoglin expression in CRC, sequential slides of CRC tissues were stained for cytokeratin (epithelium), CD31 (endothelial cells), α SMA (CAFs) and endoglin. As previously described, endoglin is highly expressed on angiogenic endothelial cells, as shown by the overlap between CD31 and endoglin staining (Fig. 1A, white arrow heads). However, we also observed endoglin expression on CAFs at the invasive borders, as indicated by co-staining of endoglin and α SMA (Fig. 1A, black arrow heads). Endoglin expression by CAFs was further confirmed using immunofluorescent double staining (Fig. 1B and supplementary Fig. S1). Notably, fibroblasts in adjacent normal colonic tissue or CAFs at 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 CRC invasion and metastasis.

Exploring this hypothesis, we stained primary CRC, lymph node and liver metastases from the same patients for endoglin and α SMA (Fig. 2A). 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 throughout different CRC stages, we stained normal colonic tissue, polyps and stage-II and -III CRC patient tissues for α SMA and endoglin. Average scores for endoglin-expressing CAFs increased with tumor stage (Fig. 2B). To determine whether CAF-specific endoglin expression could be predictive for developing metastatic disease, we assessed the relation between endoglin expression and metastasis-free survival. In stage-II CRC, high CAF-specific endoglin expression significantly correlated with worse metastasis-free patient survival (Fig. 2C). In stage-III CRC, no relation between metastases-free survival and CAF-specific endoglin expression was observed (supplementary Fig. S2). These data indicate that CAF-specific endoglin expression is predictive for metastasis-free survival in stage-II CRC.

Normal fibroblasts and CAFs display similar receptor expression profiles in vitro

In order to characterize TGF- β signaling in CAFs, we isolated normal fibroblasts (NF) and CAFs from CRC patients. CAFs were isolated from CRC tissues and NF from adjacent normal mucosa (>10 cm from primary tumor) from four different patients and gene expression was assessed. Endoglin expression did not differ between NFs and CAFs from the same patient,

or between patients (supplementary table S3). Although NFs in tissue do not express endoglin (supplementary Fig. S3), during *in vitro* culturing endoglin expression is highly upregulated. Since endoglin can bind multiple TGF- β family members and mediate downstream signaling, expression of various TGF- β /BMP receptors was determined (supplementary table S3). No differences in expression levels were observed between patients or between NFs and CAFs from the same patient. Although platelet derived growth factor receptor (PDGFR) expression can be used to distinguish certain CAF subpopulations³¹, no clear distinction in PDGFR expression was observed in NFs or CAFs from these patients *in vitro* (supplementary table S3).

Endoglin expression on fibroblasts is indispensable for BMP-9-induced signaling

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, whereas ALK5 directs phosphorylation of Smad2/3. 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 fast and strong Smad1 phosphorylation, whereas TGF- β stimulation slightly affected Smad1 phosphorylation (Fig. 3B). 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. 3C). BMP-9 stimulation, however, did not induce ID-1 gene expression (Fig. 3C) nor Smad1 phosphorylation (Fig. 3D), confirming endoglin-dependency. Endoglin expression was unaffected by ligand stimulation (Fig. 3C and 3D). Mouse CAFs, highly expressing endoglin, showed similar gene expression results as observed in high endoglin-expressing human CAFs (Fig. 3E and 3F). Furthermore, BMP-9 induced strong Smad1 phosphorylation, whereas TGF- β increased Smad2 phosphorylation in these cells (Fig. 3F). Together, these results show that endoglin is crucial for BMP-9-induced signaling in both human and mouse CAFs.

Endoglin is required for in vitro CAF survival

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. Knockdown efficiency of the constructs at RNA and protein level was confirmed in endothelial cells (supplementary Fig. S4) and showed reduced endoglin expression of 40-90% compared to non-targeting control. Endoglin knockdown did not affect endothelial cell morphology or survival (Fig. 3G). When CAFs were transduced with endoglin shRNA constructs, proliferation ceased and cells started to adopt a senescent phenotype (Fig. 3G), progressing into cell detachment and death. This phenotype was confirmed in two CAFs and normal fibroblasts (data not shown), implying that endoglin is indispensable for CAF survival *in vitro*.

In order to interfere with endoglin signaling in CAFs we used the endoglin neutralizing antibody TRC105. 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. 3H). BMP-9 stimulation strongly increased Smad1 phosphorylation, which was abrogated by TRC105. BMP-6 induces endoglin-independent Smad1 phosphorylation, and was therefore unaffected by TRC105. In mouse CAFs, stimulation with BMP-9 strongly induced Smad1 phosphorylation (Fig. 3I). This was efficiently blocked by the mouse endoglin neutralizing antibody M1043, whereas TGF- β -induced Smad2 phosphorylation was unaffected (Fig. 3I). TRC105 also inhibited Smad1 phosphorylation, although to a lesser extent (data not shown). 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 regulates CAF invasion in vitro

In both endothelial and non-endothelial cells, different roles for endoglin in cell migration have been reported. Ectopic expression of endoglin in HEK293T cells enhanced cell invasion into a collagen-I matrix (supplementary Fig. S5). Spheroid diameter 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 to 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. To further confirm this, 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). Since endoglin knockdown is not possible in CAFs, we used murine embryonic fibroblasts (MEFs) isolated from endoglin *fl/fl* mouse embryos²⁶. To induce knock out (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. Endoglin mRNA levels were reduced by 90% (Fig. 4E and supplementary Fig. S6), and transwell invasion assays showed significantly reduced invasion by endoglin KO MEFs (Fig. 4F). These data demonstrate the importance of endoglin in CAF invasion.

Endoglin targeting reduces invasive properties of fibroblasts

To confirm that CAF invasion is dependent on ligand binding to endoglin, and not merely on its presence, invasive capacity of mouse CAF was assessed in the presence of M1043. M1043 treatment reduced basal mouse CAF invasion, whereas BMP-9- and TGF- β -induced invasion were inhibited to a similar level, albeit not statistically significant (Fig. 5A). Since interactions in the TME can be mediated by paracrine signaling, we assessed CAF invasion towards MC38 mouse CRC cells. First, we showed that MC38 stimulate CAF invasion to a similar extent as BMP-9, which could not be further enhanced by combining MC38 with BMP-9 (supplementary Fig. S7A). M1043 treatment significantly decreased MC38-induced CAF invasion, compared with an IgG control (Fig. 5B). In human CAFs, TRC105 decreased basal human fibroblast invasion (Fig. 5C). Since BMP-9 and TGF- β failed to increase CAF invasion in this case, TRC105 treatment showed similar effects as observed under basal conditions, although not statistically significant (Fig. 5C). In co-culture experiments, treatment with TRC105 significantly inhibited HCT116- and HT29-induced CAF invasion (Fig. 5D and supplementary Fig. S7B). Taken together, these experiments imply a substantial role for endoglin/BMP-9 signaling in CAF invasion *in vitro*.

Endoglin expression on fibroblasts promotes CRC 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 CRC. In short, fluorescently labeled MC38 cells, in the presence or absence of MEFs, were injected in the heart cavity of zebrafish embryos and fish were followed over time. Solid tumor-like structures were formed and induced angiogenesis (Fig. 6A). Co-injection of MC38 with MEFs significantly decreased fish survival (Fig. 6B), probably due to compromised liver function caused by tumor cell invasion. Histological analysis revealed the vimentin-positive MC38 cells invading into the liver in both groups, although liver invasion was observed at earlier time points in the co-injected group (Fig. 6C). To investigate endoglin-dependency, MC38 were injected in combination with normal or endoglin KO MEFs. Tumor formation and angiogenesis were not affected (supplementary Fig. S8). However, fish survival markedly improved when endoglin KO MEFs were co-injected and resembled survival of fish injected with MC38 alone (Fig. 6D). Integration of MEFs in the tumors of both co-injected groups was confirmed by staining for mouse-specific α SMA expression (Fig. 6E). These data indicate that endoglin expression on MEFs affects CRC cell invasion in zebrafish.

Endoglin targeting inhibits CRC liver metastasis in mice

Finally, we assessed therapeutic targeting of endoglin in an experimental mouse model for CRC 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). TRC105 treatment did not affect metastatic spread in mice injected with HT29 cells alone (data not shown). In mice co-injected with HT29 and CAFs TRC105 significantly reduced BLI signal from the liver (Fig. 7B), indicating that TRC105 affects metastasis formation by directly targeting CAFs. Metastatic lesions in the liver were visualized using *ex vivo* BLI upon termination of the experiment (Fig. 7C). (Immuno)histochemical staining revealed no morphological differences in liver metastases between groups (Fig. 7D). These data show that targeting endoglin on CAFs inhibits metastatic spread of HT29 CRC cells and imply CAFs as an additional target cell for TRC105.

Discussion

In this study, we show that CAF-specific endoglin expression is predictive for the development of metastatic disease in stage-II CRC and endoglin-expressing CAFs are

detected in metastatic lesions of CRC patients. CAF-specific endoglin expression stimulates CAF invasion *in vitro* and tumor cell invasion and metastasis in a novel zebrafish model and an *in vivo* model for CRC.

Endoglin is crucial for vascular development as underlined by embryonic lethality of endoglin knockout mice¹¹⁻¹³. 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*³². We observed that, although normal fibroblasts *in vivo* do not express endoglin, its expression is highly upregulated during cell culture. Despite its indispensability *in vitro*, the function of endoglin on CAFs can be further studied using neutralizing antibodies.

In our *in vitro* assays, endoglin was important for CAF invasion through collagen-I. In endothelial cells, endoglin has been shown to be important for proliferation³³ and migration³⁴ and deletion of endoglin resulted in decreased Smad1 phosphorylation³⁵. Recently, the crystal structure of BMP-9 bound to endoglin has been shown, revealing that endoglin is required to efficiently present BMP-9 to ALK1¹⁷. This confirms our findings that in CAFs endoglin is essential for BMP-9-induced Smad1 phosphorylation. Decreased CAF invasion upon endoglin targeting might also partly be explained by increased ALK5 signaling, due to alleviation of the inhibition by the ALK1/endoglin complex, which was described in endothelial cells³⁶. In accordance with this, we have recently observed increased Smad2 phosphorylation in endothelial cells upon TRC105 treatment²⁹. This phenomenon was not observed in CAFs, suggesting differences in TGF- β /BMP-signaling between CAFs and endothelial cells, possibly by receptor abundance or expression of different type I receptors. In addition to formation of signaling complexes endoglin also interacts with integrins, crucial for adhesion and migration of ECs³⁷. In fibroblasts, interactions between ECM and integrins were shown to be important for cellular migration³⁸, but the role of endoglin in this interaction was not investigated. In our *in vitro* experiments, blocking endoglin function reduced CAF invasion through a collagen-I matrix, suggesting involvement of endoglin in degrading ECM, although the underlying mechanism remains elusive.

The stimulatory role for endoglin in CAF invasion *in vitro* and its localization at the invasive border of colorectal tumors, suggests a role for endoglin-expressing CAFs in tumor metastasis. When we assessed lymph node and liver metastases from CRC patients, we

indeed observed the presence of endoglin positive CAFs in these lesions. Although contradictory findings have been published, co-migration of CAFs with CRC cells to the liver has been reported. Recently, Labernadie et al. 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 invasion³⁹. Endoglin interaction with VE-cadherin was reported in endothelial cells, in which VE-cadherin regulates cell migration⁴⁰. Interactions of endoglin with other cadherins have not been reported yet, but might be involved in CAF-mediated tumor invasion through endoglin. 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 CRC metastasis showed that GFP expressing CAFs were localized in liver metastases and enhanced the formation of these lesions⁴¹. Also, *in vivo* experiments in a lung cancer model showed stromal cells derived from the primary tumor in metastatic lesions⁴². Together with our observation that TRC105 inhibits specifically CAF-mediated liver metastasis *in vivo*, this implies that metastatic spread is, at least in part, 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 wild type mice³². 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 mice⁴³. Although this might seem contradictory to our findings, these studies investigated mostly endothelial endoglin expression and did not take fibroblast-specific endoglin into account. Heterozygous endoglin deletion *in vivo* results in a phenotype resembling hereditary hemorrhagic telangiectasia, including increased vascular permeability^{44, 45}, possibly facilitating tumor cell intra- and extravasation and subsequently 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*.

Interestingly, endoglin heterozygous prostate tumors presented as being non-fibrotic, and showed absence of α SMA expressing cells³². Moreover, we have previously shown that TRC105 treatment reduced metastatic spread, which was accompanied by a decreased α SMA-positive stromal content²⁹. These observations imply a relevant role for CAF-specific endoglin expression in relation to metastatic spread. The fibrotic response is an important

regulator of tumor progression and metastasis⁴⁶ and has been proposed as a prognostic factor in CRC⁴⁷. Regarding endoglin, increased expression has been shown during cardiac fibrosis. Reduced endoglin expression or endoglin targeting prevented cardiac fibrosis *in vivo* and overexpression of soluble endoglin, acting as a ligand trap, reduced cardiac fibrosis⁴⁸. These data combined with our observations that CAF-specific endoglin expression increases with increasing tumor stage, imply that endoglin could affect the fibrotic response and thereby further regulate tumor progression.

In patients, we showed a correlation between high endoglin expression on CAFs at the invasive tumor borders and worse metastasis-free survival in stage-II CRC, implying the involvement of this CAF subset in tumor invasion and metastasis. Although the overall score of endoglin expression on CAFs in the stage-III cohort was higher than for stage-II, we did not find a correlation with metastasis-free survival of these patients. Since lymph node metastases are already present in stage-III patients, higher CAF specific endoglin expression would be expected and even suggests that these patients might have been identified during earlier stages based on high CAF-specific endoglin expression.

In summary, the data presented here point to a crucial involvement of endoglin expressing CAFs in CRC invasion and metastasis and could therefore be a potential therapeutic target. Additionally, CAF-specific endoglin expression might be a novel prognostic factor in early stage CRC. In a phase I study, TRC105 showed clinical efficacy on pre-existing metastases in two patients¹⁸. Combined with our recently published data that adjuvant TRC105 treatment decreased metastatic spread in breast cancer²⁹, targeting endoglin on CAFs, in addition to the endothelium, could be a potent approach in preventing metastasis formation.

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Figure legends

Figure 1. CAF-specific endoglin expression in colorectal cancer

A. Immunohistochemical staining of CRC tissue for cytokeratin, CD31, α SMA and endoglin. White arrowheads indicate high endoglin expression on the vasculature (CD31+/endoglin+). Black arrow heads show endoglin-expressing CAFs (α SMA+/endoglin+). Asterisks; α SMA+ smooth muscle cells surrounding vasculature. B. Immunofluorescent staining for endoglin (red) and α SMA (green) indicates high vascular endoglin expression and co-localization of α SMA and endoglin on CAFs (left panel). CRC tissue containing endoglin-negative CAFs was used to show staining specificity (right panel). C. Immunohistochemical staining for endoglin (left) and α SMA (right) in normal colonic mucosa, tumor core and invasive border of the same CRC patient. Asterisk indicates endoglin-positive blood vessels.

Figure 2. CAF-specific endoglin expression at invasive border correlates to metastasis-free survival in stage-II CRC

A. Primary tumor, lymph node and liver metastases from the same CRC patient show endoglin expressing CAFs (black arrow heads). White arrow heads; endothelial endoglin expression. B. Endoglin expression on CAFs at the invasive border of CRC correlates to metastasis-free survival in stage-II CRC patients (n=140), scored as either high or low (exemplified in upper boxes). C. Average score of CAF-specific endoglin expression in healthy tissue, polyps, stage-II and -III CRC patients. * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 3. BMP-9-induced signaling in CAFs is endoglin-dependent

A. BMP-9 (0.1 ng/mL) stimulation of high endoglin expressing CAFs did not affect expression of PAI-1, but increased ID-1 expression. 5ng/mL TGF- β slightly increased ID-1 expression and highly stimulated PAI-1. Both ligands did not affect endoglin expression. B. BMP-9-stimulation induced high pSmad1, whereas TGF- β showed slight induction. Endoglin protein expression was unaffected. C. Low endoglin expressing human CAFs showed the same response to TGF- β and BMP-9 for PAI-1, whereas BMP-9 stimulation failed to induce ID-1 expression. Endoglin expression remained unaffected. D. Lack of BMP-9-induced pSmad1 in low endoglin expressing human CAFs. E. Mouse CAFs showed high induction of PAI-1 and ID-1 after stimulation with TGF- β or BMP-9, respectively. Endoglin expression was unaffected. F. BMP-9-induced pSmad1 in mouse CAFs, without effects on endoglin protein expression. G.

shRNA-mediated endoglin knock down leads to cell death in human CAFs, while ECRF were unaffected. H. TGF- β -induced pSmad2 and pSmad1 was independent of TRC105 in human CAFs. BMP-9-induced pSmad1 was abrogated by TRC105. Endoglin-independent pSmad1 induction by BMP-6 was unaffected by TRC105 treatment. I. In mouse CAFs, M1043 strongly decreased BMP-9-induced pSmad1. TGF- β increased pSmad2, which was unaffected by M1043. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. pSmad1: phosphorylated Smad1; pSmad2: phosphorylated Smad2

Figure 4. Endoglin regulates CAF invasive capacity *in vitro*

A. Low (F1) and high (F2) endoglin-expressing CAFs were assessed for invasive properties. B. F2 CAFs invaded collagen-I matrix more extensively than F1 CAFs. Representative pictures of two experiments performed in triplicate. C. Endoglin overexpression increased expression more than 1000-fold. Representative graph of three independent experiments. D. Endoglin overexpression significantly enhanced 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 KO MEFs (Endoglin KO). F. Endoglin KO significantly reduced MEF invasion in transwell invasion assays. Data represent mean of three independent experiments performed in triplicate. * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 5. Endoglin targeting inhibits CAF invasion *in vitro*

A. M1043 significantly inhibited basal mouse CAF invasion, and induced a trend to reduced BMP-9- or TGF- β -mediated invasion. B. Mouse CAF invasion towards MC38 mouse CRC cells was significantly reduced in the presence of M1043, when compared to IgG control. C. TRC105 significantly inhibited basal human CAF invasion. F. Human CAF invasion towards the human CRC cell line HT29 was reduced upon treatment with TRC105, when compared with IgG control. All data represent mean of at least three independent experiments performed in triplicate. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

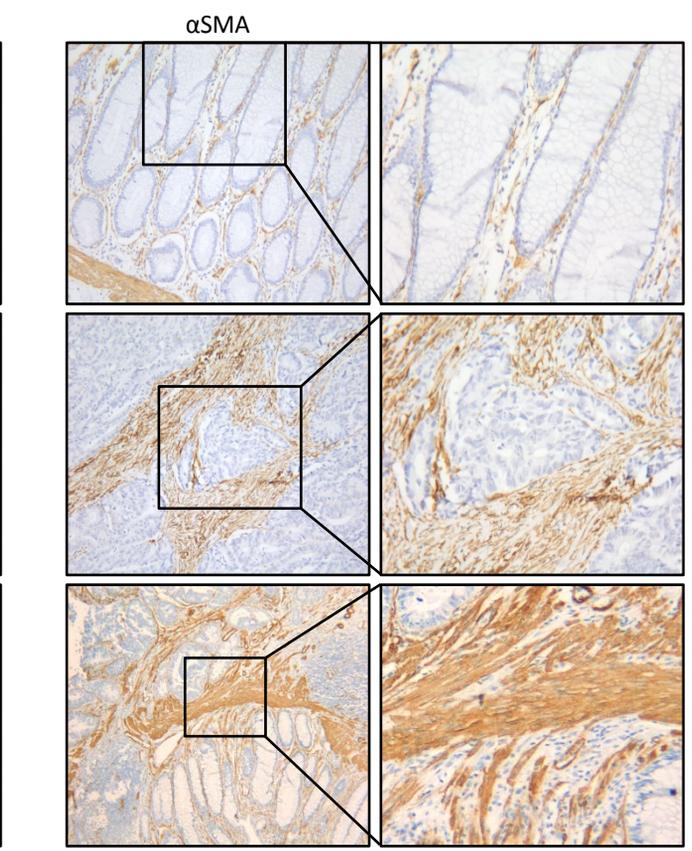
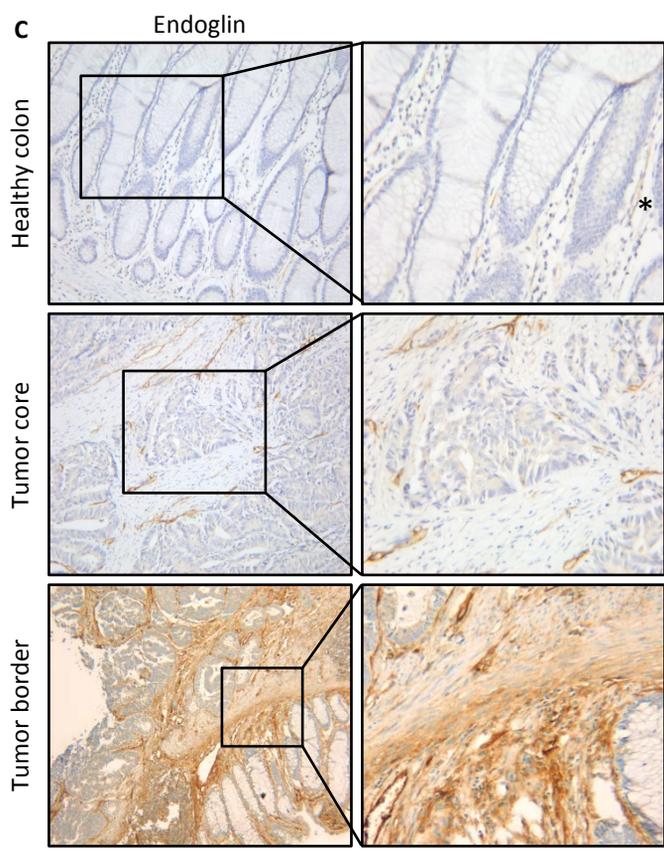
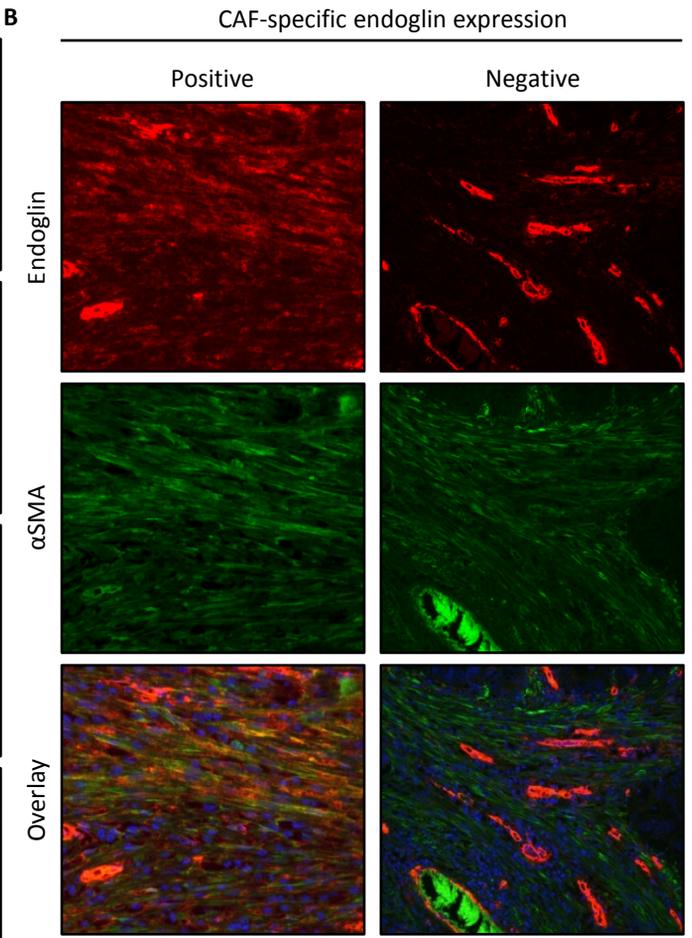
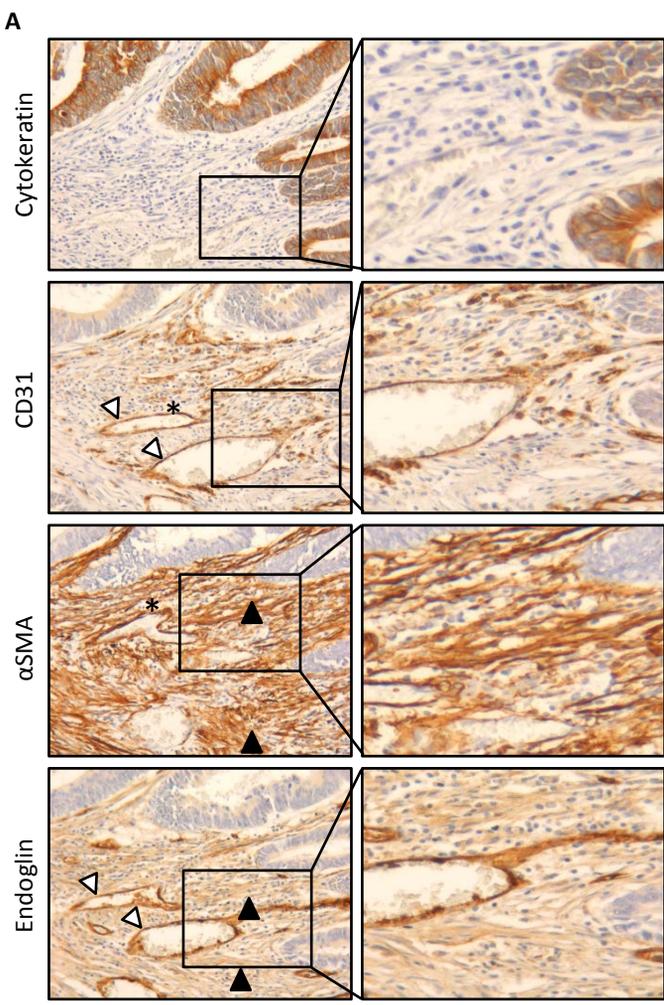
Figure 6. Endoglin-expressing CAFs reduce survival in a novel zebrafish model for CRC

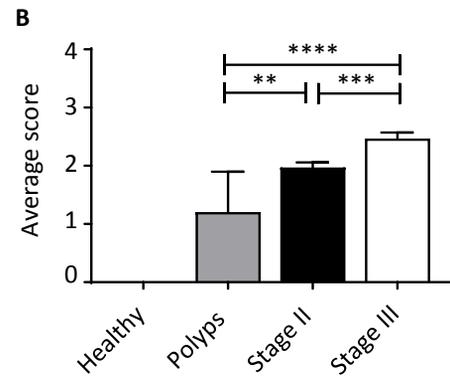
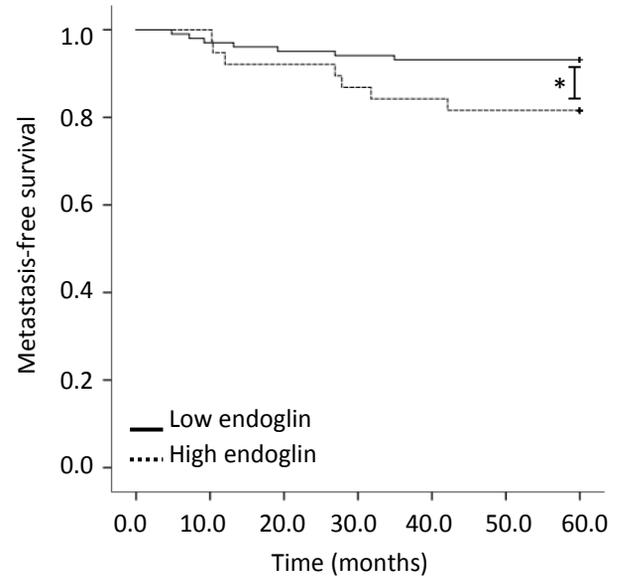
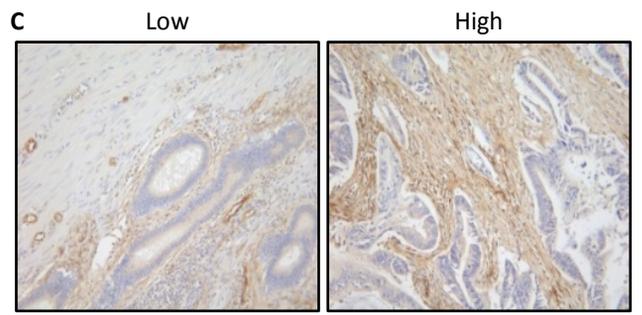
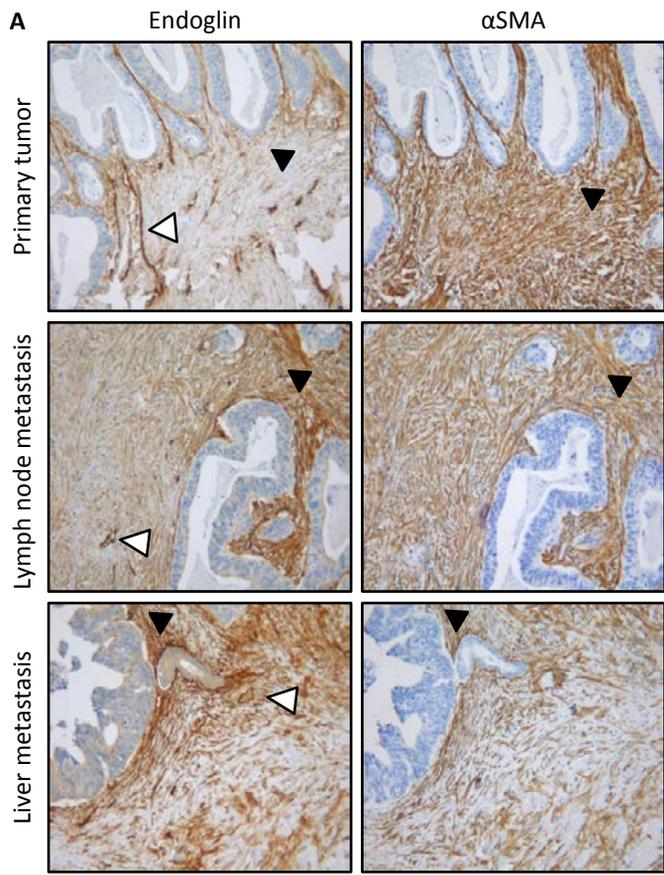
A. Injection of dTomato MC38 cells, in presence or absence of MEFs, induces formation of solid tumors (red) and recruitment of vasculature (green) in zebrafish embryos. B. Co-injection of MC38 cells with MEFs (MC38 + MEF) decreased survival in zebrafish embryos,

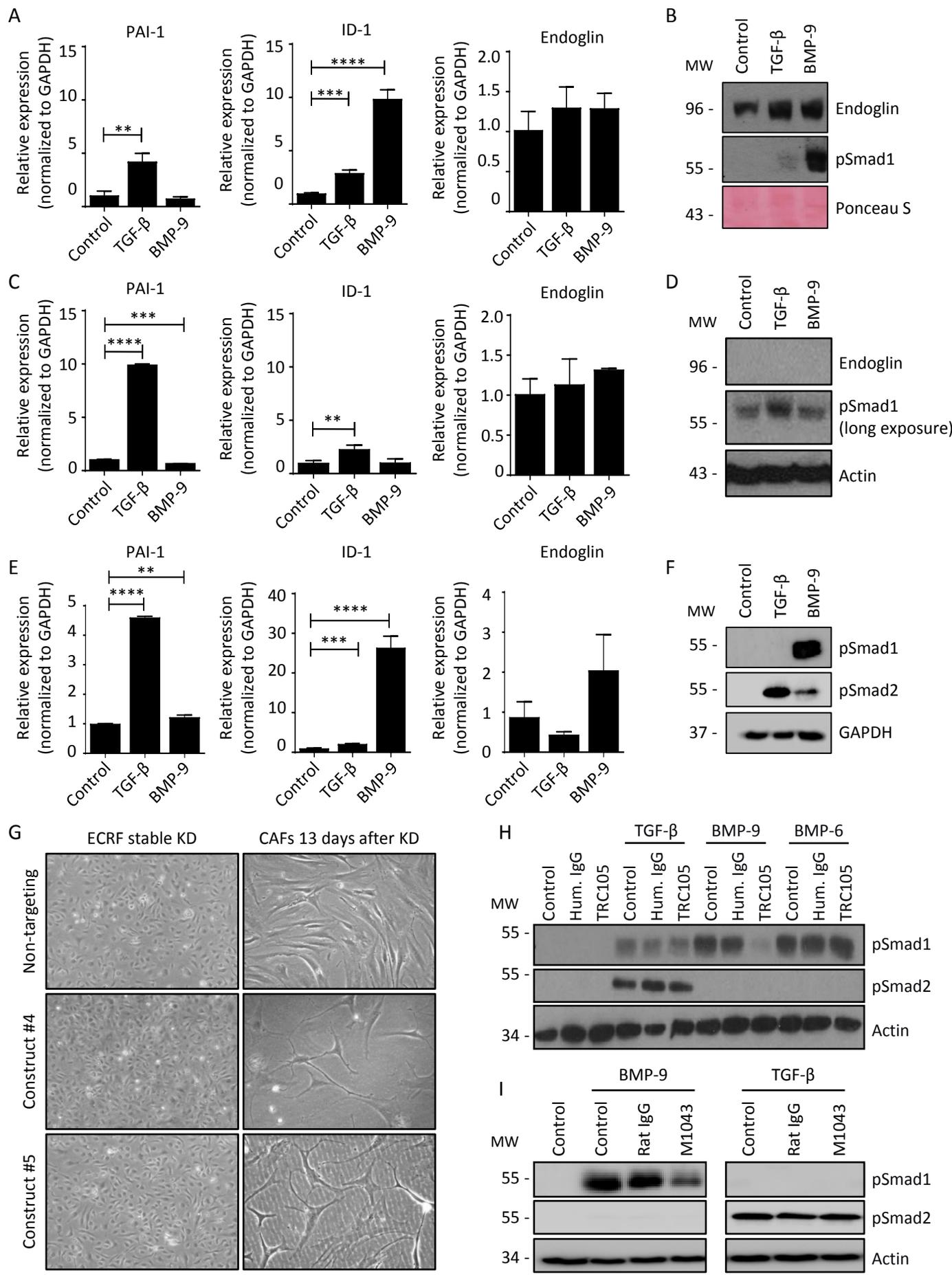
when compared to MC38 alone (n=24/group). C. Immunohistochemical staining showed vimentin-expressing MC38 cells invading the zebrafish liver. Co-injection with MEFs accelerated liver invasion (2 dpi, right panel) in comparison with MC38 alone (4 dpi, left panel). D. Co-injection of MC38 with endoglin KO MEFs resulted in similar fish survival when compared to injection of MC38 alone (n=23/group). E. Immunohistochemical staining of MC38 tumors for vimentin (MC38 cells) and α SMA (MEFs). * $p \leq 0.05$. dpi; days post injection.

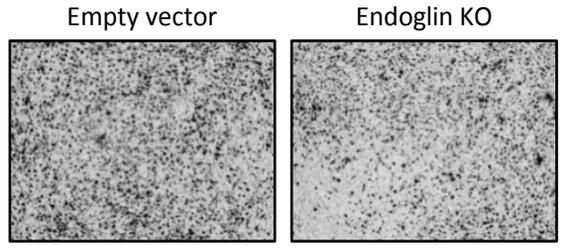
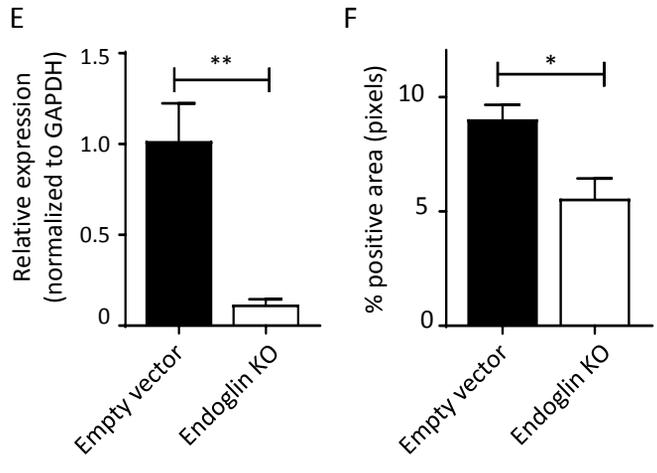
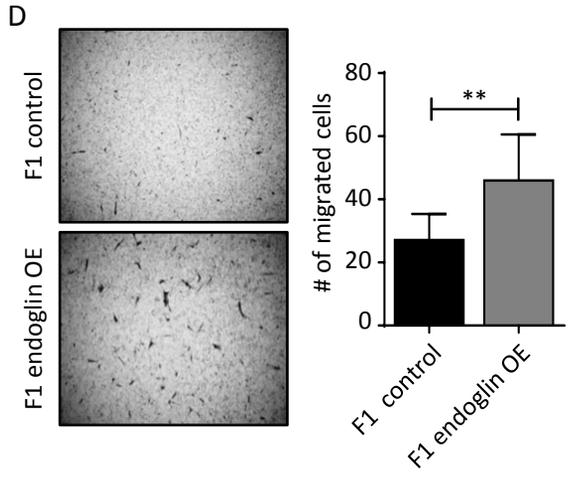
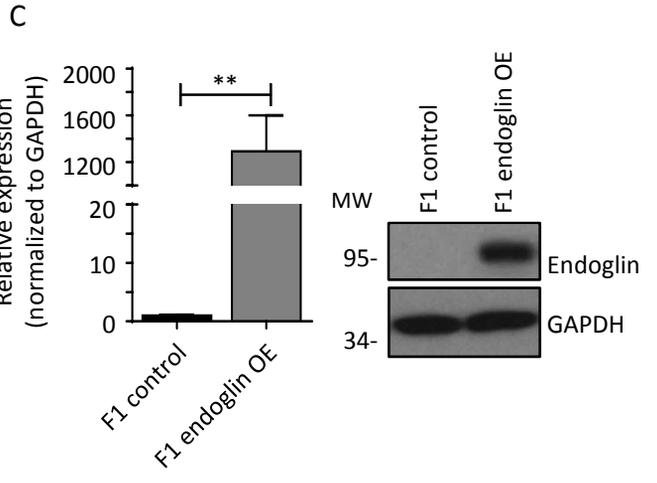
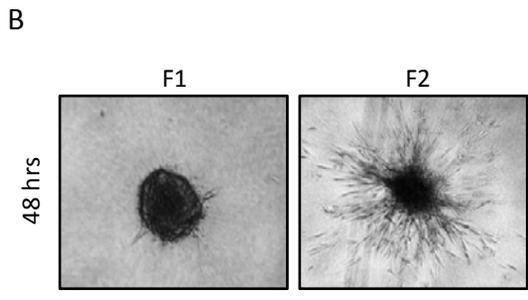
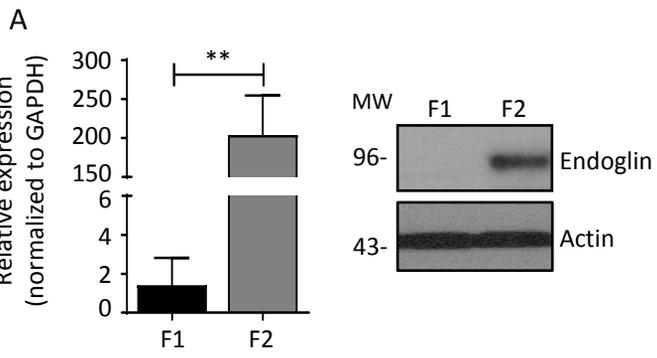
Figure 7. TRC105 inhibits CAF-mediated metastatic spread in a mouse model for liver metastasis

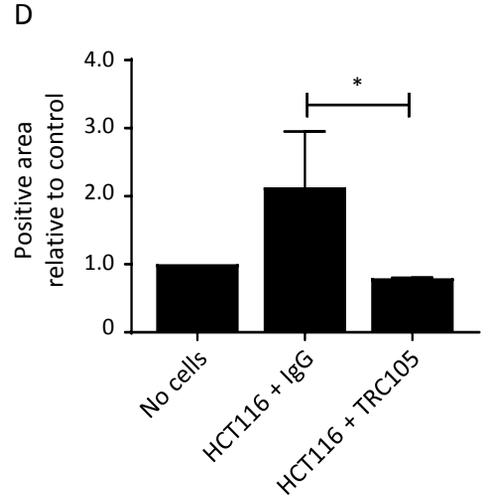
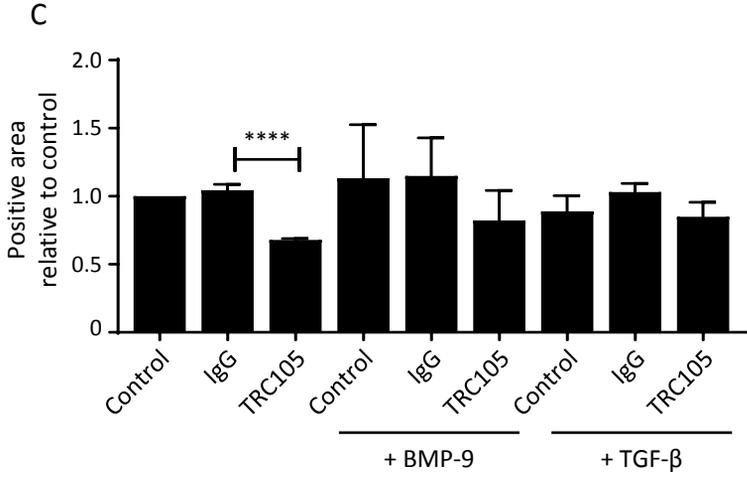
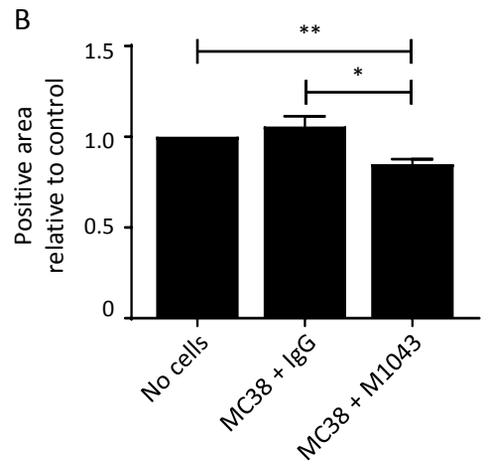
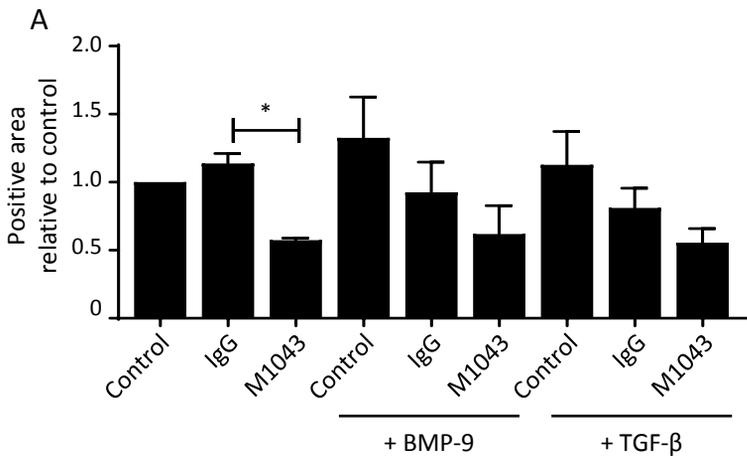
A. Experimental set-up. Mice were injected with HT29 alone, or in combination with CAFs. Two days after injection, treatment with human IgG or TRC105 started. B. *In vivo* BLI to follow metastatic spread and quantified at indicated time points. TRC105 treatment reduced metastatic spread to the liver in mice injected with HT29 and CAFs (+CAF TRC105). Graph represents two independent experiments, mean of 15 mice/group in total. C. TRC105 treatment decreased *ex-vivo* BLI signal in livers of mice co-injected with HT29 and CAFs. D. Histological analysis of liver metastasis with H&E and staining for cytokeratin (HT29), α SMA and vimentin (CAF). * $p \leq 0.05$.

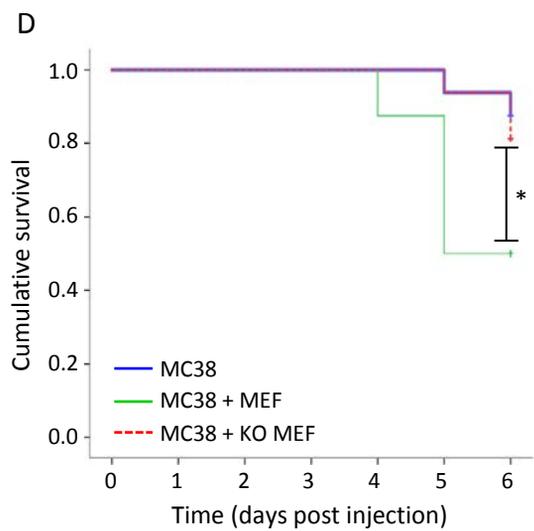
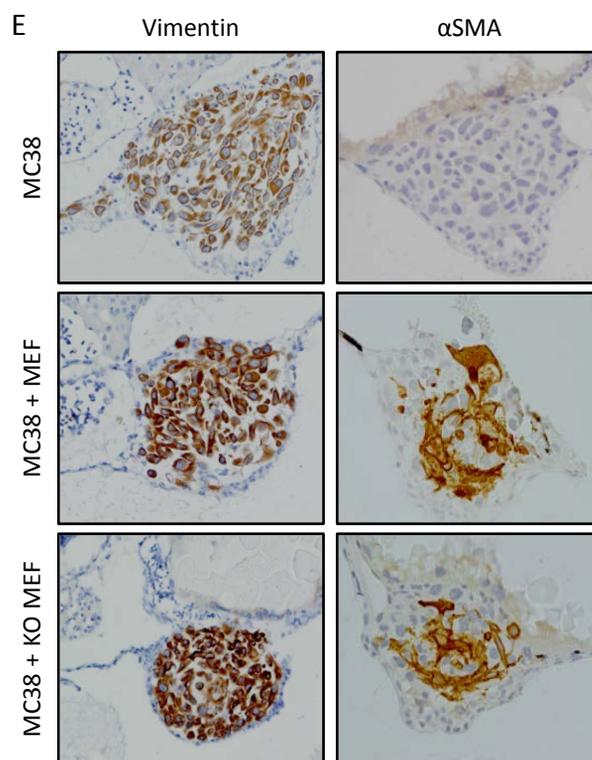
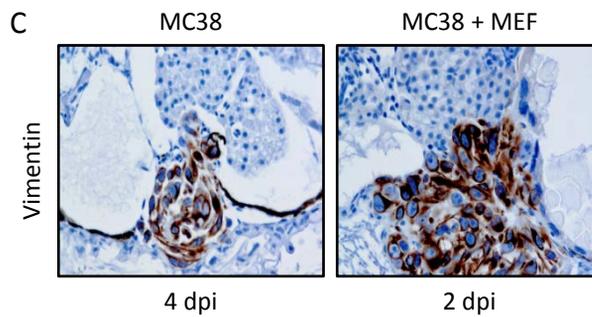
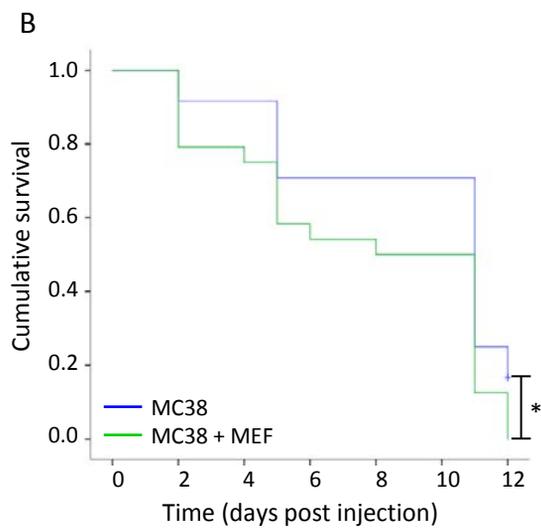
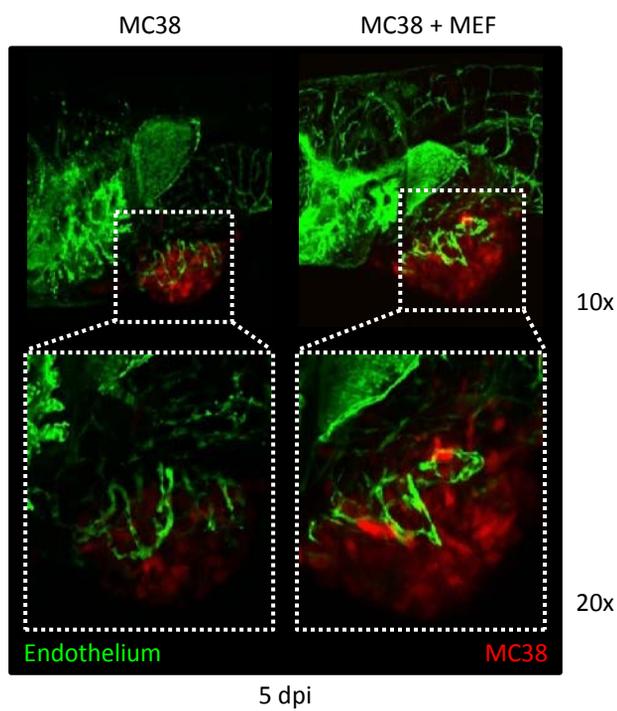
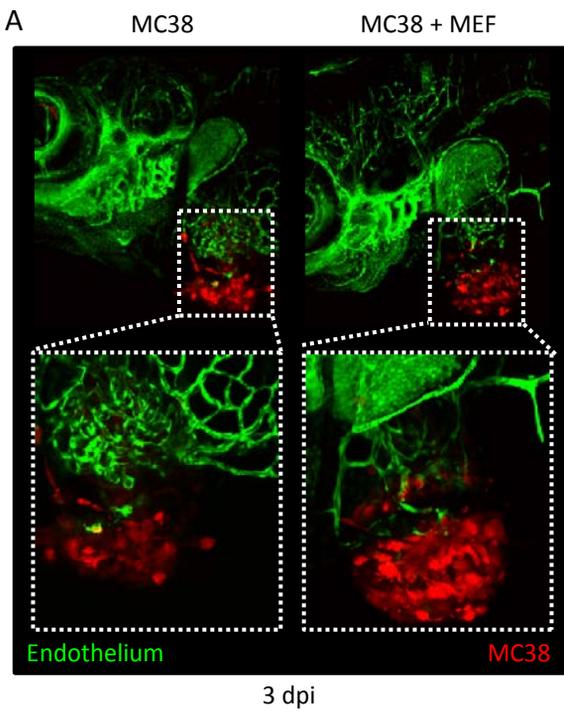






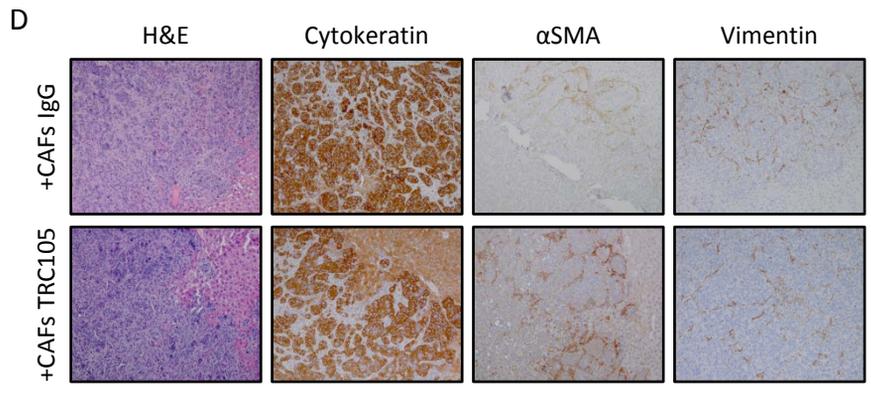
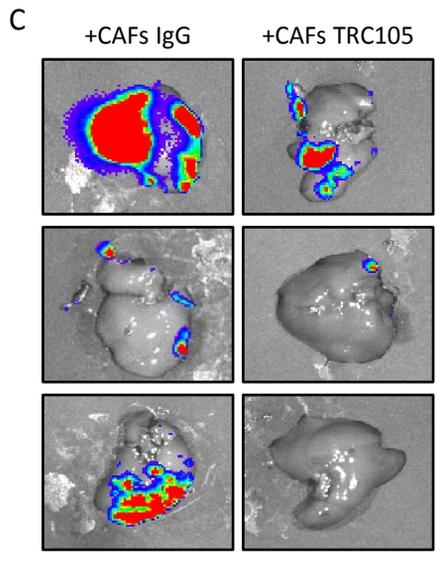
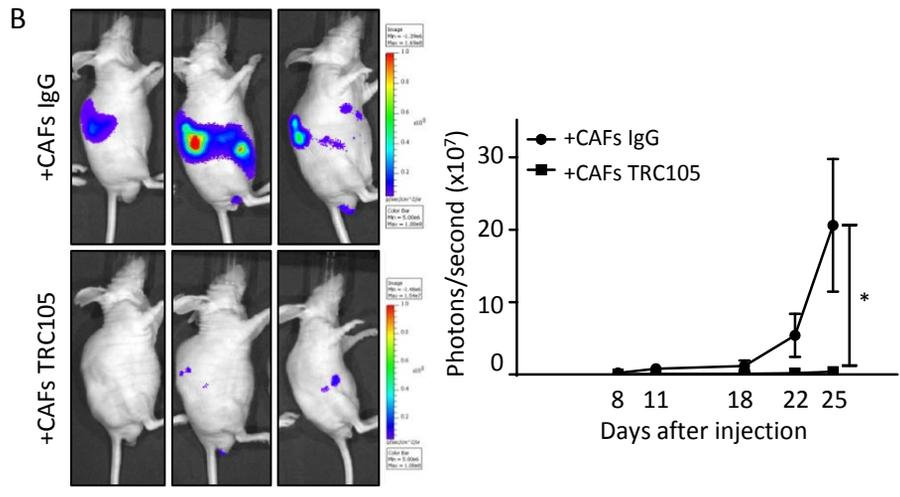


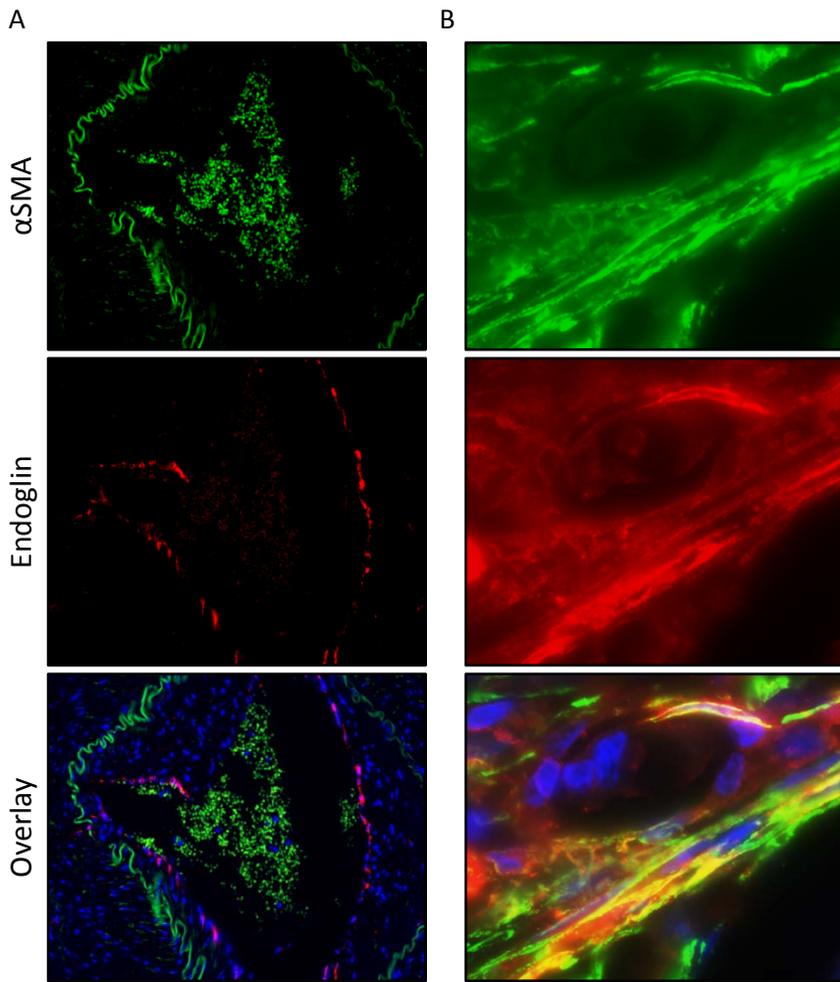




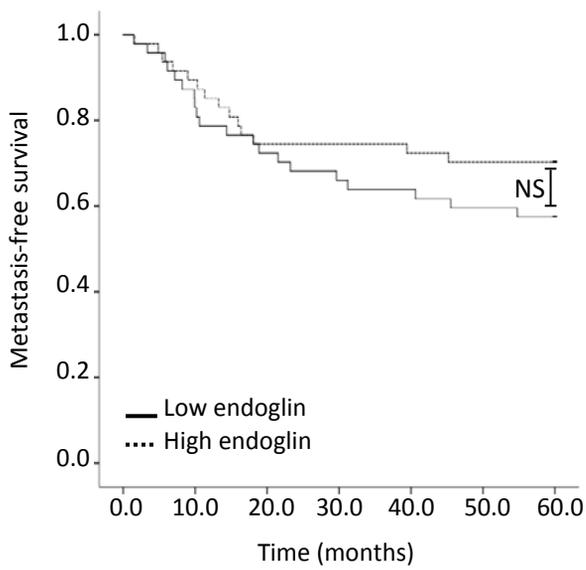
A

Cells injected	Treatment (twice weekly)
HT29	15 mg/kg human IgG
HT29	15 mg/kg TRC105
HT29 + human IgG pre-treated CAFs	15 mg/kg human IgG
HT29 + TRC105 pre-treated CAFs	15 mg/kg TRC105





Supplementary figure S1 A. α SMA expression (green) on smooth muscle cell layer surrounding a large vessel. Endoglin (red) is specifically expressed by angiogenic endothelial cells within the endothelial layer. B. High magnification of endoglin-expressing CAFs.

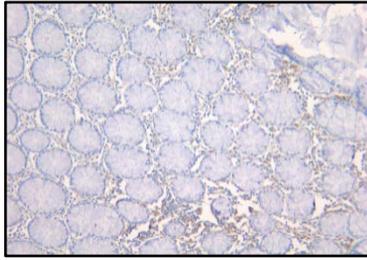
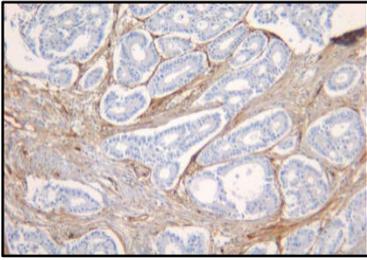


Supplementary figure S2 Endoglin expression on CAFs at the invasive border does not correlate with survival in stage-III CRC. When endoglin expression on CAFs at the invasive borders was related to distant metastasis-free survival, no correlation between the two variables was found.

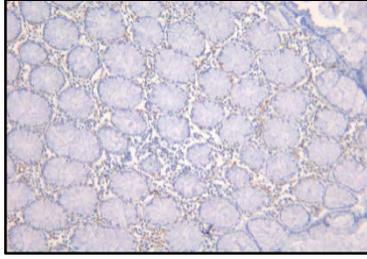
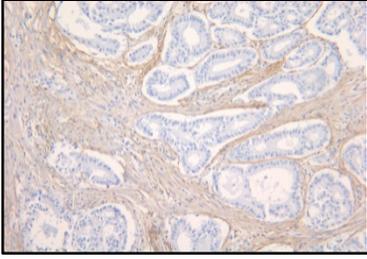
Tumor

Healthy

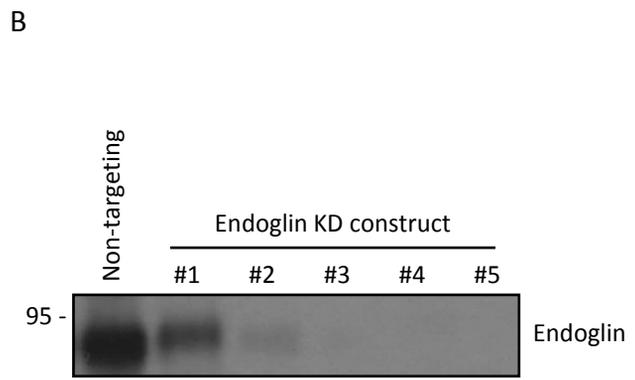
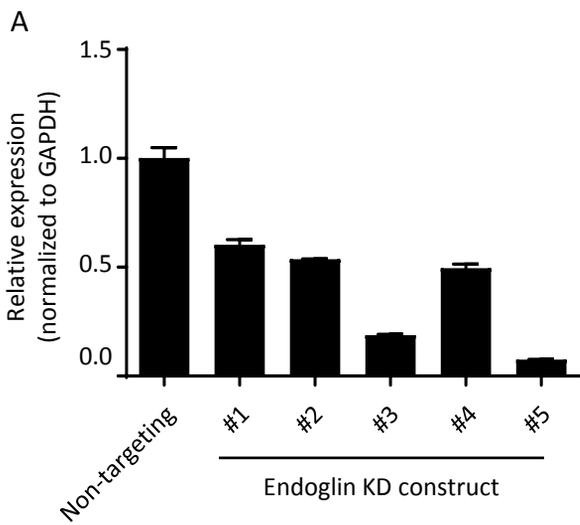
Endoglin



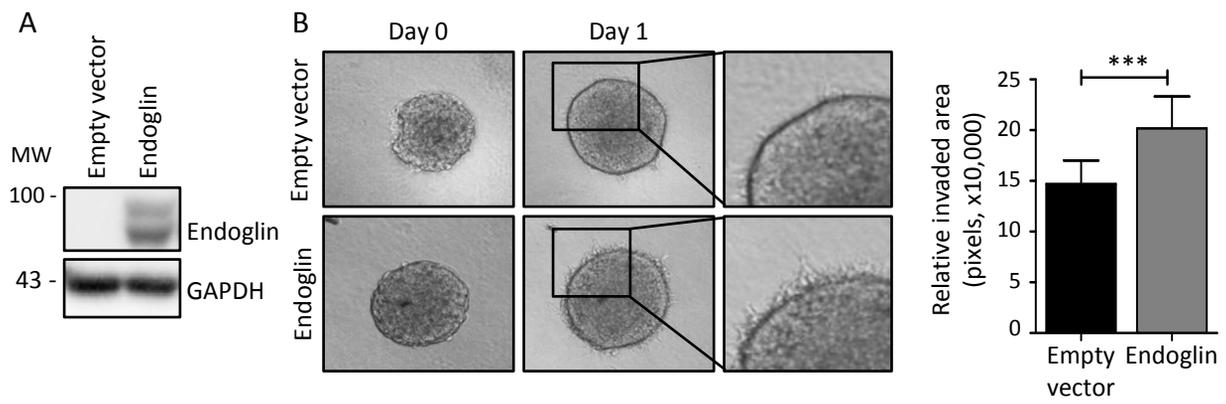
α SMA



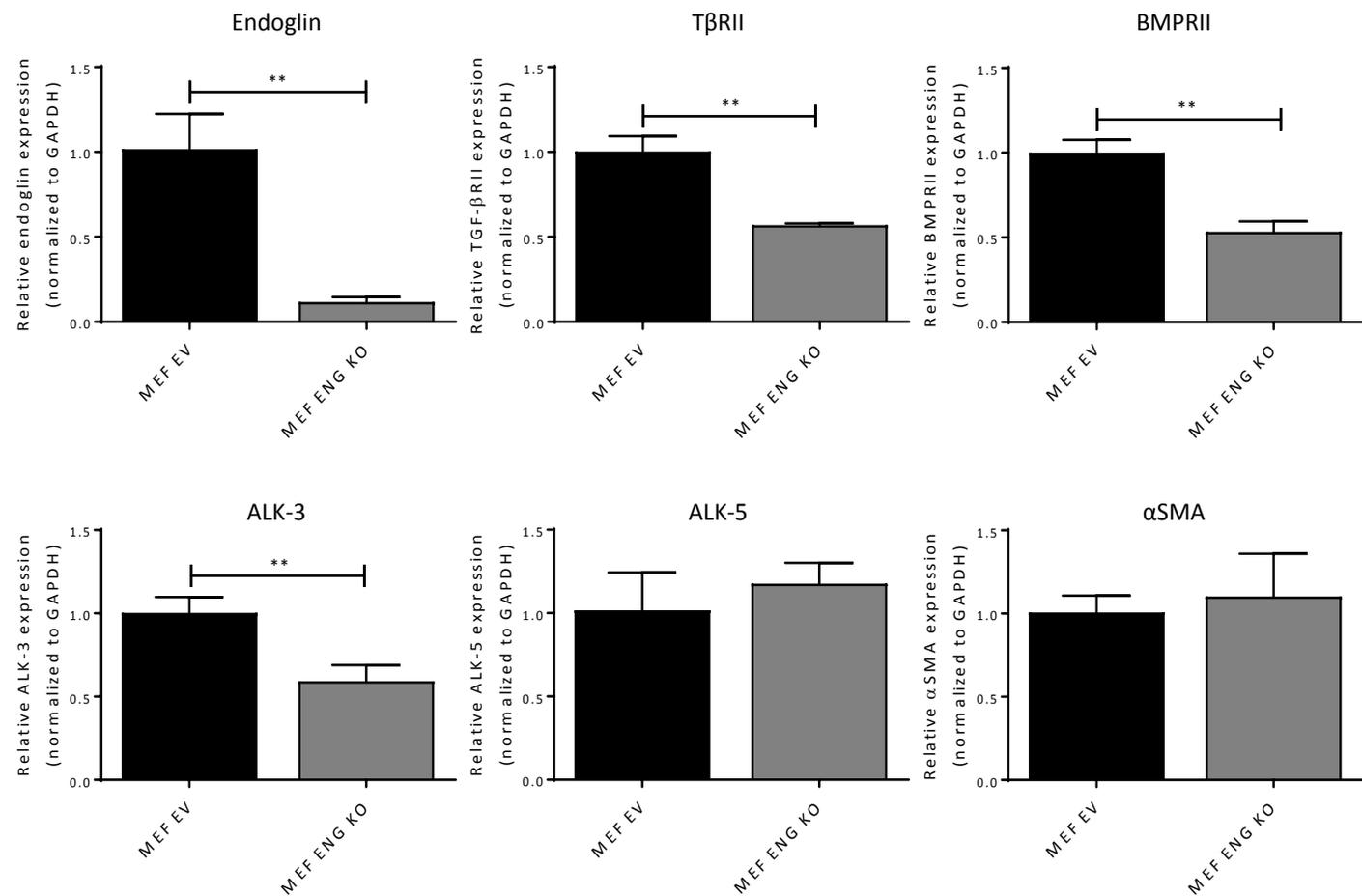
Supplementary figure S3 Fibroblasts in healthy colorectal tissue do not express endoglin. Healthy and tumor tissues from the same patient were stained for endoglin and α SMA to assess endoglin expression on (cancer-associated) fibroblasts. α SMA-positive fibroblasts in healthy colonic tissue did not show endoglin expression. Pictures are representative for all patient tissues discussed in this manuscript. Tissue from patient #4, characterized in Table 1.



Supplementary figure S4 Endoglin knock down in human endothelial cells. Short hairpin RNA constructs were introduced in human endothelial cells by lentiviral transduction. All constructs reduced endoglin expression at mRNA level with at least 40% (A), which was reflected at the protein level (B).

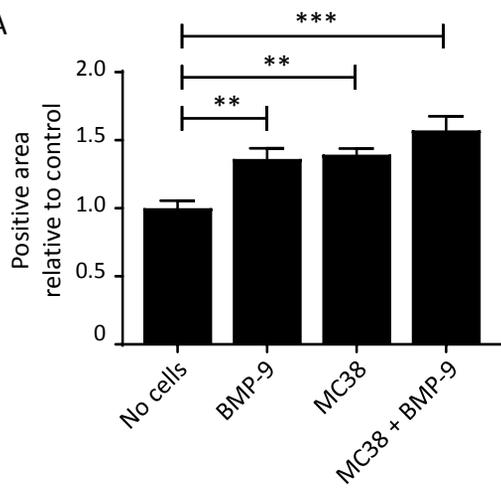


Supplementary figure S5 HEK293T cells were transfected to express endoglin (A) and left to form spheroids. B. Invasion of collagen-I matrix after 24 hours was increased in endoglin-expressing HEK293T.

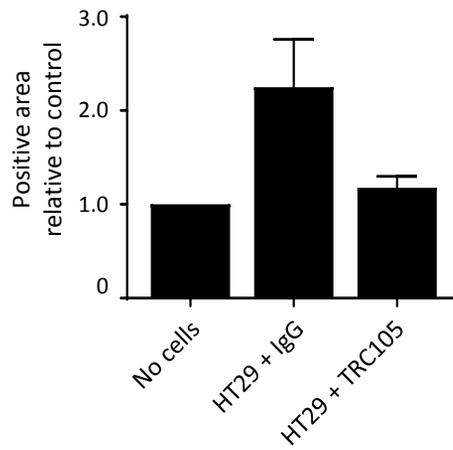


Supplementary figure S5 Characterization of TGF-β and BMP receptors in MEFs. After lentiviral transduction with a Cre recombinase expressing construct (ENG KO) or empty vector control (EV), cells were selected with puromycin. Effects of endoglin knock out on other receptors in TGF-β/BMP signaling were determined. Expression of endoglin, TGF-β type II receptor (TβRII), BMP type II receptor (BMPRII) and TGF-β type I receptor ALK-3 were significantly decreased upon Cre recombinase transduction. TGF-β type I receptor ALK-5 expression was not affected. αSMA levels did not change after Cre recombinase transduction.

A



B

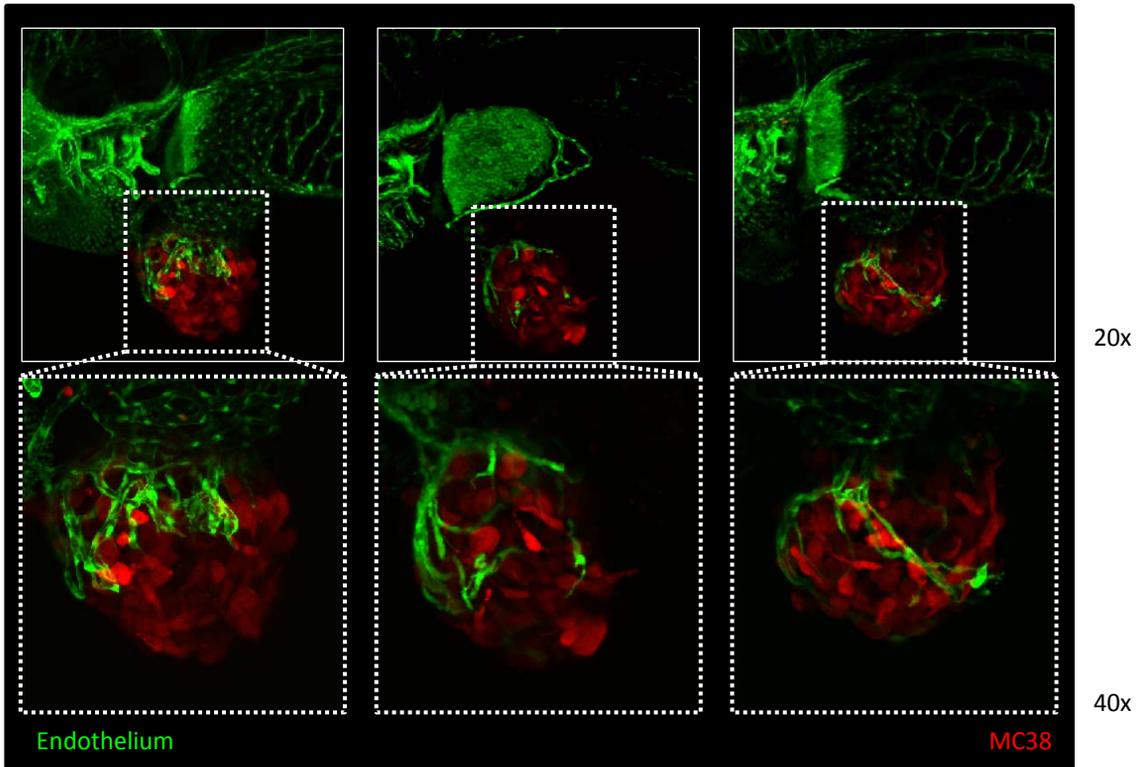


Supplementary figure S7 A. The presence of MC38 cells in the lower transwell compartment increased mouse CAF invasion to a similar extent as observed for BMP-9 stimulation. Addition of BMP-9 in the presence of MC38 did not further increase CAF invasion. **B.** The presence of HT29 human CRC cells strongly increased CAF invasion. Treatment with TRC105 decreased HT29-induced CAF invasion.

MC38

MC38 + MEF

MC38 + KO MEF



Supplementary figure S8 Primary tumor formation and angiogenesis did not differ between MC38 alone, MC38 co-injected with control MEFs or MC38 co-injected with endoglin KO MEFs.