MACC1 Induces Tumor Progression in Transgenic Mice and Colorectal Cancer Patients via Increased Pluripotency Markers Nanog and Oct4

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Abstract

Purpose: We have previously identified the gene MACC1 as a strong prognostic biomarker for colorectal cancer metastasis and patient survival. Here, we report for the first time the generation of transgenic mouse models for MACC1.

Experimental Design: We generated mice with transgenic overexpression of MACC1 in the intestine driven by the villin promoter (vil-MACC1) and crossed them with ApcMin mice (vil-MACC1/ApcMin).

Results: vil-MACC1/ApcMin mice significantly increased the total number of tumors (P = 0.0056). This was particularly apparent in large tumors (≥3-mm diameter; P = 0.0024). A detailed histopathologic analysis of these lesions demonstrated that the tumors from the vil-MACC1/ApcMin mice had a more invasive phenotype and, consequently, showed a significantly reduced survival time than ApcMin mice (P = 0.03). Molecular analysis revealed an increased Wnt and pluripotency signaling in the tumors of vil-MACC1/ApcMin mice. Specifically, we observed a prominent upregulation of the pluripotency markers Oct4 and Nanog in these tumors compared with ApcMin controls. Finally, we could also validate that Oct4 and Nanog are regulated by MACC1 in vitro and strongly correlate with MACC1 levels in a cohort of 60 tumors of colorectal cancer patients (r = 0.7005 and r = 0.6808, respectively; P = 0.0001 and P > 0.0002, respectively).

Conclusions: We provide proof of principle that MACC1-induced tumor progression in colorectal cancer acts, at least in part, via the newly discovered MACC1/Nanog/Oct4 axis. These findings might have important implications for the design of novel therapeutic intervention strategies to restrict tumor progression. Clin Cancer Res; 22(11); 2812–24. ©2016 AACR.

Introduction

Worldwide, colorectal cancer is one of the most common malignancies with more than 1 million cases diagnosed every year (1). Colorectal carcinogenesis is a multistep process associated with the accumulation of genetic alterations and mutations in oncogenes and tumor-suppressor genes (2). Prominent among these is the tumor-suppressor polyposis adenomatous coli (APC) gene, which is somatically mutated in more than 80% of sporadic colorectal tumors (3). Loss of APC function leads to inappropriate β-catenin stabilization, accumulation in the cytoplasm, and translocation to the nucleus, resulting in a constitutively active Wnt signaling. APC mutations, and consequent Wnt signaling activation, are regarded as the initiating event in colorectal cancer, playing also an important role in tumor progression (4–6).

MACC1 was identified by our group as a strong prognostic biomarker for colorectal cancer metastasis and patient survival (7). MACC1 induces proliferation, migration, and invasion of colorectal cancer cells in vitro and promotes tumor growth and metastasis in xenograft mouse models (7, 8). MACC1 was found significantly upregulated in primary colon cancer and organ metastases compared with normal tissues, indicating that the induction of MACC1 might occur at the crucial step of transition from the benign to the malignant phenotype (22, 23), cervical (24, 25), and lung cancer (26–28). Met was identified as the first transcriptional target of MACC1, suggesting that MACC1 might signal through the HGF–Met pathway to promote tumor growth and metastasis. In contrast to MACC1, Met was not prognostic for metastasis-free survival in colorectal cancer patients. Moreover, the combination of MACC1 and Met expression was not capable of improving the prognostic power of MACC1 alone (7). Therefore, one can anticipate that relevant target genes and/or pathways, other than Met, might be regulated by MACC1 and contribute to the tumor-promoting phenotype associated with MACC1 overexpression.

Considerable evidence has accumulated that suggests that tumors are maintained by dedicated stem cells (29–31).
Strikingly, MACC1 has appeared upregulated in the gene expression signature of intestinal stem cells in the mouse (29). However, a connection between MACC1 and stem cell signaling has never been established.

To genetically address the role of MACC1 in intestinal tumorigenesis and to further evaluate the molecular determinants underlying MACC1-associated phenotypes, we generated transgenic mouse models with intestine-specific overexpression of MACC1, vil-MACC1, and vil-MACC1/ApcMin. Our results show that MACC1 induces tumor progression in an Apc-mutated background. Intriguingly, our findings in cell culture, novel MACC1 transgenic mice as well as in tumors of colorectal cancer patients uncover the pluripotency markers Oct4 and Nanog as novel essential signaling mediators used by MACC1 to induce tumor progression.

Materials and Methods

Mice

A sequence containing a chimeric intron, the full-length cDNA of human MACC1, and a V5 tag was cloned into the p12.4kbVillin-DATG vector (kindly provided by Dr. Deborah Gumucio, University of Michigan, Ann Arbor, MI), which contains the mouse villin promoter (30). The p12.4kbVillin/int-MACC1-V5 plasmid was confirmed by sequencing. The transgene was isolated from the plasmid by (PmeI) digestion. The resulting founders and their progeny were genotyped by PCR of tail or ear-tag DNA using transgene-specific (genotyping) primers and mouse beta-globin primers as internal control (Supplementary Table S1). Four founder lines were established on a pure C57BL/6N genetic background [C57BL/6N-Tg(vil-MACC1); p12.4kbVillin-DATG; Lab code: Us]. Vil-MACC1 transgenic animals as well as colorectal cancer patients. This is the first time that the function of MACC1 is linked to pathways crucial in cancer stem cells. Based on the hypothesis that cancer stem cells are the main engine behind tumor progression and metastasis, these findings might have important therapeutic implications. Specifically, MACC1’s relevance as a biomarker might be further augmented by focusing on its expression in cancer stem cells. Furthermore, the connection of MACC1 with Oct4 and Nanog might offer novel options for therapeutic intervention.

Isolation of intestinal epithelial cells

Total intestinal epithelial cells (IEC) were isolated from the duodenum, jejunum, ileum, and colon by sequential incubation of intestinal tissue in 1 mmol/L DTT and 1.5 mmol/L EDTA solutions as described previously (32).

The sequential isolation of mouse small intestinal epithelial cells along the crypt–villus axis was performed as previously described (33), with minor modifications. Briefly, the small intestines were removed after sacrifice of the mice, open longitudinally, rinsed twice in ice-cold PBS and incubated at 37°C for 15 minutes in a citrate buffer containing 96 mmol/L NaCl, 1.5 mmol/L KCl, 27 mmol/L sodium citrate, 8 mmol/L KH2PO4, and 5.6 mmol/L Na2HPO4·pH 7.3. The tissues were then transferred to a Falcon tube containing 15 mL PBS buffer with 1.5 mmol/L EDTA (to remove calcium), 0.5 mmol/L DTT, and 1 mg/mL BSA, and placed in a shaking incubator (37°C; 180 rpm) for 10 minutes. The small intestines were removed from the first Falcon, gently shaken to remove adhering enterocytes, and transferred to a second Falcon containing 15 mL of PBS, EDTA, DTT, and BSA. This procedure was repeated for a total of nine consecutive incubation steps at 37°C, each step lasting 10, 6, 5, 5, 9, 10, 15, 25, and 30 minutes, respectively. The cells detached during each incubation were collected and immediately centrifuged at 0°C, 1,000 g for 3 minutes. The pellets were washed twice in ice-cold PBS and stored at –20°C.

Western blot analysis

For total protein extraction, cells were lysed with RIPA buffer [50 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1% Nonidet P-40; supplemented with protease and phosphatase inhibitor cocktail tablets; Roche Diagnostics] for 30 minutes on ice. Protein concentration was quantified with Bicinchoninic Acid Protein Assay Reagent (Pierce), according to the manufacturer’s instructions, and lysates of equal protein amount were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto OptiThran BA-S 85 nitrocellulose membranes (GE Healthcare). Membranes were incubated in blocking solution containing 5% non-fat dry milk for 1 hour at room temperature, followed by an incubation overnight at 4°C with primary antibodies: rabbit anti-MACC1 (polyclonal, Sigma-Aldrich, dilution 1:1,000), rabbit anti-Oct4 (polyclonal, Abcam ab19857, dilution 1:500), and mouse anti-β-actin (clone AC-74, Sigma-Aldrich, dilution 1:20,000). Subsequently, the membranes were incubated for 1 hour at room

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Figure 1.
Expression of the MACC1 transgene in vil-MACC1 mice. A, diagram of the MACC1 transgene. B, qRT-PCR analysis revealed transgenic MACC1 mRNA expression in the duodenum, jejunum, ileum, and colon, but not in the liver, lung, and kidney of 4 vil-MACC1 transgenic lines (T8892, T9757, T9760, and T9765). Data represent mean ± SEM (n = 4, per genotype). C, immunofluorescent staining with an anti-MACC1 antibody shows expression of MACC1 in the epithelial cells of the duodenum, jejunum, ileum, and colon, but not in the liver of vil-MACC1 mice. The depicted images are representative of WT (lower row) and vil-MACC1 (line T9757, upper row) mice with MACC1 stained in red (Cy3) and the nuclei stained in blue (DAPI). Scale bars, 100 μm. Transgenic MACC1 mRNA (D) and protein (E) were detected along the entire vertical axis [from villus tip (F1) to crypt (F10)] of the SI of vil-MACC1 transgenic mice (line T9757). mRNA data represent mean ± SEM (n = 2). A representative blot of two independent experiments is shown.
MACC1 overexpression increases tumor burden and reduces survival of vil-MACC1/ApcMin mice. A, the vil-MACC1/ApcMin mice showed a significant increase in the total number of intestinal tumors. B, the most prominent increase was observed for the bigger tumors (diameter ≥ 3 mm) in the SI. Quantification was performed by two independent researchers for vil-MACC1/ApcMin (n = 14) and ApcMin (n = 11) control mice at 25 ± 2 weeks of age. P values were calculated using an unpaired two-tailed Student t test with Welch’s correction. C, gross pictures and H&E stainings on representative sections of the ileum of three vil-MACC1/ApcMin and three ApcMin mice show the increase in the number and size of tumors in the former as compared with the latter. Scale bars: 2 mm. D, Kaplan-Meier survival analysis revealed that vil-MACC1/ApcMin mice (n = 38) have a significantly shorter survival as compared with ApcMin control mice (n = 32). The endpoint for survival was defined as the time of death or the time at which the mice were sacrificed due to severe tumor-related disease. Statistical analyses were evaluated by the P log-rank test.
temperature with secondary HRP-coupled antibodies (Promega and Thermo Scientific), developed with WesternBright ECL Western blotting detection kit (Advansta), and visualized in a FluorChem Q imager (Biozym). Immunoblotting for β-actin served as protein loading control.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was carried out according to standard protocols as described previously (34). Cell lysates were sonicated for 15 pulses at 40% output. Incubation with monoclonal V5-specific antibody (Invitrogen)
was performed overnight at 4°C. Immunoprecipitation of the DNA–protein complexes was performed with 50 µL 50:50 (v/v) Protein G Agarose (Invitrogen) for 2 hours at 4°C, followed by isolation of the DNA. Subsequent Nanog PCR produced 95-bp amplicons. For quantitative ChIP, the same procedure was used, followed by qRT-PCR analysis (see below). For sequences of ChIP primers, see Supplementary Table S3.

Microarray
Total RNAs extracted from the small intestinal tumors of Apc<sup>min</sup> and vil-MACC1/Apc<sup>min</sup> mice were reverse transcribed...
Figure 5.
MACC1 regulates the pluripotency markers Oct4 and Nanog. A, MACC1 mRNA expression in colorectal cancer cells following its ectopic overexpression or knockdown. B, upon ectopic overexpression of MACC1 in the colorectal cancer cells SW480 and Caco-2, an increase in the Oct4 mRNA expression levels was detected by qRT-PCR. (Continued on the following page.)
and labeled using the Ambion WT expression kit (Ambion) and the GeneChip WT Terminal Labeling Kit (Affymetrix), according to the manufacturer’s instructions, and hybridized to GeneChip Mouse Gene 2.0 ST Arrays (Affymetrix) that target a total of 35,240 transcripts. Arrays were scanned on a Affymetrix GeneChip Scanner 7G. After data normalization, genes with uniformly low expression were excluded from further analyses. Data were analyzed using GeneSpring GX software (Agilent). Genes with at least 1.5-fold change relative to Apc\textsuperscript{C0} control mice were selected.

**Tumor sphere formation assay**

For the sphere formation assay, single-cell suspensions were seeded in ultra-low attachment 6-well plates (Coming Inc.) in Advanced DMEM/F-12 (Gibco) supplemented with 1 x GlutaMAX (Gibco), 1 x Pen/Strep (PAA), 10 mmol/L HEPES (Sigma-Aldrich), 1 mmol/L N-Acetylcysteine (Sigma-Aldrich), 1 x N-2 Supplement (Gibco), 1 x B-27 Supplement (Gibco), 20 ng/mL hFGF basic/FGF2 (Cell Signaling Technology), 50 ng/mL hEGF (Sigma-Aldrich), 10 mmol/L Y-27632 dihydrochloride (Sigma-Aldrich) and 2.5 µg/mL Amphotericin B (Sigma-Aldrich). Per well, 2000 cells were seeded in 2 mL of media. Cells were incubated for 7 days without media change. After incubation, plates were gently shaken to dissociate cell aggregates and directly counted under a phase-contrast microscope. For counting, only spheres with a size of >50 µm and a clear round morphology were included.

**Statistical analysis**

All calculations and statistical analyses were performed with GraphPad Prism version 5.01 (GraphPad Software). For the comparison of two groups, an unpaired two-tailed Student\textit{t} test, an unpaired two-tailed Student\textit{t} test with Welch correction (unequal variances), and a Mann–Whitney test were used. For correlation analysis, nonparametric Spearman correlation was used. Kaplan–Meier analysis was used to plot overall survival, and differences in curves were analyzed by the log-rank test. \( P < 0.05 \) was considered statistically significant. All results shown represent mean ± SEM.

Cell culture, tissue preparation, histology and immunohistochemistry, RNA extraction, and qRT-PCR are described in Supplementary data.

**Results**

**Generation and characterization of vil-MACC1 mice**

We generated a transgenic mouse model that expresses MACC1 under the control of the villin promoter (vil-MACC1; Fig. 1A). The 12.4-kb mouse villin promoter was previously shown to drive transgene expression predominantly in the epithelial cells of the small intestine (SI) and colon (30). Overexpression of MACC1 in the IECs promoted tumor formation in vivo, as indicated by the higher rate of tumor incidence in vil-MACC1 mice (29/35, 83%) compared with Apc\textsuperscript{C0} littermate controls (1/35, 3%). The transgene construct was microinjected into fertilized mouse eggs, and the founder lines were analyzed for their MACC1 transgene expression (Supplementary Fig. S1A). All vil-MACC1 lines expressed transgenic MACC1 mRNA and protein in the whole horizontal axis of the intestine (duodenum, jejunum, ileum, and colon), but not in other organs such as the liver, lung, and kidney (Fig. 1B, 1C; Supplementary Fig. S1B). Transgenic MACC1 mRNA and protein were specifically targeted to the IECs of vil-MACC1 mice (Supplementary Fig. S1C and S1D). To characterize the transgene expression in the vertical axis of the intestine of these mice, we isolated successive fractions of IECs, from the villus tip (F1) to the crypt (F10). The highest levels of transgenic MACC1 were observed in the F1, with a continuous decrease toward fraction F10 (Fig. 1D and 1E). Importantly, we could still detect a significant amount of the transgene in fraction F10. This expression pattern is consistent with the endogenous expression of the villin gene (30), confirming that the MACC1 transgene is expressed in the entire vertical axis, including the cells of the crypts.

To investigate whether MACC1 overexpression in the IECs induced changes in the intestinal epithelium of vil-MACC1 mice, we analyzed hematoxylin and eosin (H&E) sections from the small and large intestine. At 3 months of age, vil-MACC1 mice showed a healthy intestinal mucosa without signs of abnormalities and with normal length (Supplementary Fig. S2A and S2B). We then aged the vil-MACC1 mice for at least 1 year (52–112 weeks, median 71 weeks) and analyzed the potential tumor development in these animals. We observed small adenomas in the SI of 2 out of 13 (15%) aged vil-MACC1 mice (Supplementary Fig. S2C), suggesting that these animals develop tumors at a very low rate and with long latency. The aged vil-MACC1 animals did not show signs of intestinal hyperplasia, as evidenced by the similar intestinal length when compared with littermate WT controls (Supplementary Fig. S2D).

**Overexpression of MACC1 in the IECs promoted tumor progression in vil-MACC1/Apc\textsuperscript{C6} mice**

It has been previously hypothesized that MACC1 induction might occur at the crucial step of transition from the benign (adenoma) to the malignant (carcinoma) phenotype in colorectal cancer (9,35). To genetically address this hypothesis, we crossed the vil-MACC1 transgenic mice with Apc\textsuperscript{C6} mice (36) to generate vil-MACC1/Apc\textsuperscript{C6} animals. The Apc\textsuperscript{C6} mice are a classical model for intestinal tumorigenesis, which develop an average of 30 to 40 tumors, predominantly in the SI and histologically classified as benign adenomas (32,33). The vil-MACC1/Apc\textsuperscript{C6} mice expressed transgenic MACC1 mRNA in the small and large intestines at a similar level as vil-MACC1 mice. They also expressed the transgenic mRNA in the intestinal crypts (Fig. S2C). Consistently, knockdown of MACC1 in Caco-2 and SW620 cells resulted in decreased levels of Oct4. Data represent mean ± SEM (n = 3). \( P \) values were calculated using an unpaired two-tailed Student\textit{t} test. C, Western blotting analyses in the same panel of cell lines revealed a correlation of Oct4 protein levels. D, the mRNA expression levels of Nanog showed an analogous trend compared with Oct4 across the panel of colorectal cancer cells. Data represent mean ± SEM (n = 5). \( P \) values were calculated using an unpaired two-tailed Student\textit{t} test. E, ChIP for binding of MACC1 to the Nanog promoter region. –173 to –79. Virtually no Nanog product was detectable in the SW480/e.v. cells and IgG controls. F, proposed model for a MACC1-mediated tumor- and metastasis promoting effect via Nanog. G, in a tumor sphere formation assay, SW480/MACC1 colorectal cancer cells displayed a significantly higher number of spheres, which were significantly bigger as compared with SW480/e.v. control cells. Data represent mean ± SEM (n = 6). \( P \) value was calculated using an unpaired two-tailed Student\textit{t} test. Scale bars, 100 µm.
tumors, but not in the liver (Supplementary Fig. S3A). Immunofluorescence staining revealed MACC1 transgenic protein in the epithelial cells of the SI and colon, as well as in tumor tissues of vil-MACC1/Apc\textsuperscript{Min} mice (Supplementary Fig. S3B). We evaluated the number and size of tumors formed in the SI and colon of vil-MACC1/Apc\textsuperscript{Min} and their Apc\textsuperscript{Min} littermate controls. At 6 months of age (25 ± 2 weeks), vil-MACC1/Apc\textsuperscript{Min} mouse had a significantly higher number of tumors in the whole intestine than Apc\textsuperscript{Min} controls (average of 73.57 ± 6.181 vs. 46.36 ± 6.358, respectively; $P = 0.0056$; Fig. 2A). A similar tendency was already observed at 4 months of age (17 ± 2 weeks), but without statistical significance (Supplementary Fig. S4A). The majority of the tumors were formed in the SI of both mouse models. Interestingly, we observed not only an increase in the total number but also in the size of those tumors in the vil-MACC1/Apc\textsuperscript{Min} mice (Fig. 2B), as illustrated in Fig. 2C. No difference was observed between Apc\textsuperscript{Min} and vil-MACC1/Apc\textsuperscript{Min} mice regarding the small (<1 mm diameter; $P = 0.2895$) tumors. The difference became evident for the intermediate-sized tumors (2 mm diameter; $P = 0.0448$), and was very clear for large tumors (>3 mm diameter; $P = 0.0024$). Consistent with the reported increased tumor burden in vil-MACC1/Apc\textsuperscript{Min} mice, we observed a significant decrease in their overall survival as compared with Apc\textsuperscript{Min} littermates (median survival was 23 and 26 weeks, respectively; $P = 0.03$; Fig. 2D).

A detailed histologic analysis of the tumors from both groups of animals revealed that the vil-MACC1/Apc\textsuperscript{Min} mice developed bigger tumors, which were highly proliferative, as evidenced by the Ki-67 staining (Fig. 3A–C, and 3I–N). The pathologic analysis demonstrated that the tumors from the vil-MACC1/Apc\textsuperscript{Min} mice had a more invasive phenotype, with destruction of the basement membrane and invasion of epithelial cells into the lamina propria (Fig. 3D, 3G, and 3I) compared with tumors of Apc\textsuperscript{Min} mice (Fig. 3H and 3f). Some of those tumors showed the presence of back-to-back glands (Fig. 3E) and budding, together with nuclear β-catenin accumulation (Fig. 3F and 3K).

It has been previously described that Apc\textsuperscript{Min} mice develop splenomegaly (39). Some studies reported a correlation between the severity of the tumorigenesis and the spleen size (39,40). Consistently, we observed a very strong positive correlation between the spleen size and the number of tumors in the vil-MACC1/Apc\textsuperscript{Min} and Apc\textsuperscript{Min} mouse (Supplementary Fig. S4B). We confirmed the development of splenomegaly in the Apc\textsuperscript{Min} mice, a phenotype that was even more prominent in the vil-MACC1/Apc\textsuperscript{Min} animals (Supplementary Fig. S4C and S4D).

Altogether, our results constitute robust evidence for the presence of an accelerated adenoma–carcinoma sequence in the vil-MACC1/Apc\textsuperscript{Min} mice, supporting the role of MACC1 on tumor progression in vivo.

The SI tumors of vil-MACC1/Apc\textsuperscript{Min} display increased Wnt and pluripotency signaling

To further investigate the molecular mechanism underlying MACC1-induced tumor progression, we performed a genome-wide mRNA expression analysis (Affymetrix microarrays) comparing the SI tumors from vil-MACC1/Apc\textsuperscript{Min} and Apc\textsuperscript{Min} mice. A total of 766 probes representing 760 genes were differentially expressed in the two groups (FC > 1.5; $P = 0.05$; Fig. 4A).

We then performed a pathway analysis to find relevant pathways associated with the tumors of vil-MACC1/Apc\textsuperscript{Min} mice. Prominently, we found “Wnt signaling and pluripotency” pathway with members significantly enriched in the list of upregulated genes in those mice (Supplementary Fig. S5). From the identified genes belonging to the “Wnt signaling and pluripotency” pathway, the majority were nucleus associated, suggesting a potential increase in the transcriptional activity of this pathway. To validate these findings, we analyzed the mRNA levels of some relevant genes in the SI tumors of both groups of mice by qRT-PCR. We found several Wnt-related genes upregulated in the tumors of vil-MACC1/Apc\textsuperscript{Min} mice compared with Apc\textsuperscript{Min} controls. Among those were different extracellular matrix modulators, including the urokinase plasminogen activator (PLAII), matrix metalloproteinase 7 (MMP7), and matrix metalloproteinase 9 (MMP9).

Additionally, we found the lymphoid enhancer–binding factor 1 (LEF1), the vascular endothelial growth factor A (VEGFA), and the tumor necrosis factor receptor superfamily, member 19 (TNFRSF19; also known as TROY) to be upregulated in the SI tumors of vil-MACC1/Apc\textsuperscript{Min} mice (Fig. 4B). TROY is a tissue-specific Wnt target gene, which is produced specifically by fast-cycling intestinal stem cells (41). This observation prompted us to analyze other stem cell–related genes that are also Wnt target genes. We found the leucine-rich repeat-containing G-protein–coupled receptor 5 (Lgr5) and CD44 to be expressed at similar levels in the tumors of both mouse groups. However, we observed a prominent upregulation of the pluripotency markers octamer-binding transcription factor 4 (Oct4; also known as POUSF1 (POU domain, class 5, transcription factor 1)) and Nanog in the SI tumors of vil-MACC1/Apc\textsuperscript{Min} mice, compared with Apc\textsuperscript{Min} controls (Fig. 4C). These results suggest that an activation of the pluripotency signaling might, at least in part, be responsible for the MACC1-induced tumor progression observed in the vil-MACC1/Apc\textsuperscript{Min} mice.

MACC1 regulates the pluripotency markers Oct4 and Nanog

We selected a panel of colorectal cancer cell lines with different endogenous levels of MACC1 and manipulated MACC1 levels through its ectopic overexpression or knockdown (Fig. 5A). Strikingly, we found that upon MACC1 overexpression in SW480 and Caco-2 cells, the mRNA levels of Oct4 and Nanog were significantly increased. Conversely, upon MACC1 knockdown in SW620 and Caco-2 cells, the mRNA levels of Oct4 and Nanog were significantly decreased (Fig. 5B and 5D). While we were not successful to assess the protein levels of Nanog in these cells, the levels of Oct4 show a corresponding trend in all manipulated cell lines (Fig. 5C). In order to elucidate how MACC1 might regulate the transcription of Oct4 and Nanog, we performed chromatin immunoprecipitation experiments. While no indications for a binding of MACC1 to the Oct4 gene promoter could be found, we did confirm a physical binding of MACC1 to the gene promoter of Nanog in MACC1-overexpressing colorectal cancer cells (Fig. 5E). This gives evidence to a model where MACC1 binds to the Nanog promoter, thereby inducing the expression of Nanog, which can in turn trigger the upregulation of Oct4 and confer other stem-like traits (Fig. 5F; ref. 42).
MACC1 expression strongly correlates with Oct4 and Nanog expression in colorectal cancer patients. A, a strong and statistically significant positive correlation was found between the mRNA levels of Oct4 and MACC1 in the tumors of 60 colorectal cancer patients. B, patients with high MACC1 mRNA expression had a significantly higher expression of Oct4 mRNA, as determined by qRT-PCR. C, correlation analysis revealed that the mRNA levels of MACC1 and Nanog are also positively correlated. D, the levels of Nanog mRNA were significantly higher in patients with high MACC1 mRNA expression. E, a strong positive correlation was also found for Oct4 and Nanog mRNA levels in the 60 colorectal cancer patients. MACC1, Oct4, and Nanog mRNA levels were determined by qRT-PCR, normalized to the house-keeping gene hGAPDH and calculated as fold of the calibrator. Correlation analysis was performed with nonparametric Spearman correlation. Classification of patients as low and high MACC1 expressers was performed using MACC1 median expression as cutoff. P values in B and D were calculated using a two-tailed Mann-Whitney test.
We performed a tumor sphere formation assay as a functional readout for stemness in SW480/MACC1 colorectal cancer cells. Cells were grown in serum-free, nonadherent conditions favoring survival and proliferation of stem/progenitor cells. Strikingly, the number and size of spheres formed after 1 week was significantly higher in the SW480/MACC1 cells, compared with SW480/e.v. control cells (Fig. 5F).

Furthermore, we analyzed the mRNA levels of Oct4 and Nanog in a cohort of 60 tumors from colorectal cancer patients and correlated them with MACC1 expression. These patients were diagnosed without distant metastases (stages I—III) at the time point of surgery, had no familial history of colon cancer, no tumor of the same or a second entity, were previously untreated and underwent surgical R0 resection. We found a very significant positive correlation between Oct4 and MACC1 mRNA levels ($r = 0.7005; P > 0.0001$) as well as between Nanog and MACC1 ($r = 0.6808; P > 0.0002$; Fig. 6A and 6C). Consistently, we observed significantly higher levels of Oct4 and Nanog mRNA in the group of patients with high MACC1 expression (Fig. 6B and 6D). Further, correlation of Oct4 and Nanog expression levels was also of statistical significance ($P < 0.0001$; Fig. 6E).

Discussion

The prognostic value of MACC1 for tumor progression and metastasis has been established in many different solid cancers, including colorectal cancer (10). However, it remains unclear whether MACC1 alone has the potential to induce neoplastic transformation in the normal intestinal epithelium. Here, we report the generation of the first MACC1 genetically engineered mouse models vil-MACC1 and vil-MACC1/ApcMin. MACC1 overexpression in an Apc-mutated background significantly exacerbates tumor formation and progression by increased expression of the pluripotency genes Oct4 and Nanog. These observations corroborate our initial hypothesis and allocate MACC1 to the transition of benign adenoma to malignant carcinoma in the adenoma–carcinoma model of colorectal cancer development. Several lines of evidence suggest that MACC1 regulates the pluripotency markers and Wnt target genes Oct4 and Nanog; the SI tumors of vil-MACC1/ApcMin mice have significantly higher levels of Oct4 and Nanog than those of ApcMin controls; manipulation of MACC1 levels in a panel of colorectal cancer cell lines induce a concomitant change in the expression levels of Oct4 and Nanog; the MACC1 mRNA levels strongly correlate with those of Oct4 and Nanog in a cohort of 60 tumors from colorectal cancer patients. Oct4 and Nanog are expressed mainly in embryonic stem cells and only in very low levels in normal adult tissues (42–44). However, an increasing number of studies demonstrate that both Oct4 and Nanog are aberrantly overexpressed in different types of cancer, contributing to the stem-cell phenotype of tumor cells (45,46). Consistently, it has been demonstrated that overexpression of Oct4 and Nanog in epithelial cells promotes tumorigenesis and metastasis (47,48). Furthermore, elevated expression of Nanog predicts poor prognosis in hepatocellular carcinoma and colorectal cancer (46,49). Therefore, one can hypothesize that the MACC1-induced tumor progression is mediated by an increased level of the pluripotency markers Oct4 and Nanog. Of note, similarly to what has been reported for Oct4 and Nanog, the levels of MACC1 are very low in normal adult tissues and aberrantly increased in tumor samples (7). Whether MACC1 is expressed during embryogenesis and plays a role in organ formation and/or development remains unclear. However, initial evidence demonstrated that MACC1 might be involved in the development of the craniofacial skeleton of zebra fish (50).

In conclusion, we report for the first time the generation and characterization of MACC1 genetically engineered mouse models, which provided strong evidence for the role of MACC1 in intestinal tumor progression. Furthermore, these novel transgenic mouse models constitute an important tool to unravel novel molecular mechanisms underlying MACC1-associated phenotypes. Thereby, we demonstrated that MACC1 induces the master regulators of pluripotency, Nanog and Oct4. We deduce that MACC1’s tumor and metastasis promoting effect might, in part, be mediated by its role in stem cell signaling via the newly discovered MACC1/Nanog/Oct4 axis. Additional experiments will further elucidate the function of MACC1 in the context of cancer stem cells. The interconnection of MACC1 with major regulators of pluripotency might offer novel options for therapeutic intervention strategies to restrict tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Lemos, M.S. Hardt, M. Juneja, U. Stein
Development of methodology: C. Lemos, M.S. Hardt
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Writing, review, and/or revision of the manuscript: C. Lemos, M.S. Hardt, M. Juneja, W. Haider, H. Bläker, U. Stein
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Acknowledgments

The authors thank Sanchita Ghosh, Gonçalo Regalo, Gabriele Born, Katerina Vlantis and Gabriele Hörmannsperger for technical assistance. They also thank Deborah Gumucio for providing the p12.4kbVillin-DATG vector and the lab members Dennis Kobelt, Mathias Dahlmann, Fabian Zincke, and Jessica Pahle for contributing to the revision process.

Grant Support

This work was supported by the Alexander von Humboldt Foundation (C. Lemos), the Wilhelm Sander Foundation (U. Stein and B. Jerchow), the Monika-Kützner foundation (U. Stein), the German Cancer Consortium (U. Stein, C. Voss, and M.S. Hardt) and the Predclinical Comprehensive Cancer Center (U. Stein and C. Lemos).

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Received June 16, 2015; revised November 3, 2015; accepted December 3, 2015; published OnlineFirst January 12, 2016.
MACC1 Induces Tumor Progression in Transgenic Mice

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