

ORIGINAL ARTICLE

SPON2, a newly identified target gene of MACC1, drives colorectal cancer metastasis in mice and is prognostic for colorectal cancer patient survival

F Schmid¹, Q Wang¹, MR Huska², MA Andrade-Navarro², M Lemm², I Fichtner², M Dahlmann¹, D Kobelt¹, W Walther¹, J Smith¹, PM Schlag³ and U Stein^{1,4}

MACC1 (metastasis associated in colon cancer 1) is a prognostic biomarker for tumor progression, metastasis and survival of a variety of solid cancers including colorectal cancer (CRC). Here we aimed to identify the MACC1-induced transcriptome and key players mediating the MACC1-induced effects in CRC. We performed microarray analyses using CRC cells ectopically overexpressing MACC1. We identified more than 1300 genes at least twofold differentially expressed, including the gene *SPON2* (*Spondin 2*) as 90-fold upregulated transcriptional target of MACC1. MACC1-dependent *SPON2* expression regulation was validated on mRNA and protein levels in MACC1 high (endogenously or ectopically) and low (endogenously or by knockdown) expressing cells. Chromatin immunoprecipitation analysis demonstrated the binding of MACC1 to the gene promoter of *SPON2*. In cell culture, ectopic *SPON2* overexpression induced cell viability, migration, invasion and colony formation in endogenously MACC1 and *SPON2* low expressing cells, whereas *SPON2* knockdown reduced proliferative, migratory and invasive abilities in CRC cells with high endogenous MACC1 and *SPON2* expression. In intrasplenically transplanted NOD/SCID mice, metastasis induction was analyzed with control or *SPON2*-overexpressing CRC cells. Tumors with *SPON2* overexpression induced liver metastasis (vs control animals without any metastases, $P=0.0036$). In CRC patients, *SPON2* expression was determined in primary tumors (stages I–III), and survival time was analyzed by Kaplan–Meier method. CRC patients with high *SPON2* expressing primary tumors demonstrated 8 months shorter metastasis-free survival (MFS) compared with patients with low *SPON2* levels ($P=0.053$). Combining high levels of *SPON2* and MACC1 improved the identification of high-risk patients with a 20-month shorter MFS vs patients with low biomarker expression. In summary, *SPON2* is a transcriptional target of the metastasis gene MACC1. *SPON2* induces cell motility *in vitro* and CRC metastasis in mice. In patients, *SPON2* serves as prognostic indicator for CRC metastasis and survival, and might represent a promising target for therapeutic approaches.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cause of cancer-related mortality in the Western world, mainly because of metastasis.¹ Patients with an early-stage primary tumor have a 5-year survival rate of ~90%, whereas the rate is reduced to 10% when distant metastases have formed.²

We identified the new gene *MACC1* (*metastasis associated in colon cancer 1*) as a prognostic marker for CRC metastasis.³ High expression of MACC1 in colorectal tumors, staged I, II and III, correlated in 78% of all cases with the development of metachronous metastasis. Patients with low MACC1 expression in their primary tumors had a metastasis-free 5-year survival time of 80% compared with only 15% of patients with high MACC1 levels.

Meanwhile, MACC1 has been established as a prognostic biomarker for CRC^{3–8} and a further variety of solid cancer such as the gastrointestinal tract (CRC, gastric, pancreatic), hepatobiliary, lung, ovarian, breast, renal, nasopharyngeal, esophageal cancers and glioblastomas.^{9–11} MACC1 expression correlated to tumor formation, metastases development and patient survival.

MACC1 regulates fundamental processes like proliferation, migration, invasion and dissemination in cell culture.^{3,9,12,13} Subcutaneous, orthotopic and intrasplenic transplantation of MACC1-expressing tumor cells induced tumor growth and liver metastasis in mice, short hairpin RNA (shRNA) acting on MACC1 decreased this metastasis formation.^{3,14} Furthermore, MACC1 was identified as a transcriptional regulator of the proto-oncogene MET, thereby inducing the metastatic phenotype of tumor cells via hepatocyte growth factor/MET signaling.^{3,15,16} MET is a key player in embryogenesis, angiogenesis, epithelial–mesenchymal transition, cell motility and, in particular, in metastasis formation.^{17,18} Thus, MACC1 and its target gene MET play a crucial role in CRC metastasis.

Here we aimed to identify the MACC1-induced transcriptome and thus novel transcriptional target genes of MACC1 by genome-wide expression screening. To the best of our knowledge, there are no MACC1-dependent CRC-specific transcriptome data available so far. For further analysis we focused on the gene *Spondin 2* (*SPON2*; alias M-spondin or DIL-1) that was ~90-fold upregulated in MACC1-overexpressing cells. *SPON2*, encoding an extracellular matrix protein, was previously identified by mRNA differential

¹Experimental and Clinical Research Center, Charité Universitätsmedizin Berlin and Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany;

²Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany; ³Charité Comprehensive Cancer Center, Berlin, Germany and ⁴German Cancer Consortium (DKTK), Heidelberg, Germany. Correspondence: Professor U Stein, Experimental and Clinical Research Center, Charité Universitätsmedizin Berlin and Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association, Robert-Rössle-Straße 10, Berlin 13125, Germany.

E-mail: ustein@mdc-berlin.de

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display screening of cancerous and noncancerous lung cells.¹⁹ A first report hinted to a potential role of SPON2 for CRC, showing its upregulation in carcinomas compared with adenomas.²⁰ However, there is no information available on the potential contribution of SPON2 to CRC progression, concerning its potential role in the development of metastasis and, even more crucial, for its potential use as a biomarker for prognosis of metastasis linked to CRC patient survival.

We demonstrate the MACC1-dependent SPON2 expression regulation as well as the binding of MACC1 to the gene promoter of SPON2. Furthermore, we show for the first time the potential of SPON2 itself for cell motility and proliferation in CRC cell culture, for metastasis formation in CRC xenografted mice and for metastasis-free survival (MFS) of CRC patients.

RESULTS

Identification of target genes of MACC1

We used CRC cells endogenously low in MACC1, SW480/vector, and CRC cells ectopically overexpressing MACC1, SW480/MACC1, for microarray analysis. A total of 1382 genes were significantly differentially expressed in MACC1-overexpressing cells vs SW480/vector cells. Out of these genes, 656 genes were more than twofold upregulated and 726 genes were more than twofold downregulated. Heat map representation shows the differentially expressed genes of duplicates of SW480/vector and SW480/MACC1 cells. The expression levels of the top 100 differentially regulated genes with a *P*-value of < 0.05 were normalized and hierarchically clustered, using the Qlucore Omics Explorer 3.0 software (Lund, Sweden; Figure 1a).

The already known transcriptional target gene of MACC1, MET, was one of the upregulated genes (\log_2 fold change = 3.03),³ but did not belong to the 79 most upregulated and 21 most downregulated genes, listed in Table 1. We categorized the differentially expressed genes into Gene Ontology terms related to biological processes and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways by using the DAVID Bioinformatic Resources 6.7 (Frederick, MD, USA). The ontology terms most significantly enriched in differentially expressed genes are regulation of cell viability, cell migration and cell motion (Table 2; Benjamini *P* < 0.05). These biological processes point to the contribution of MACC1 and its downstream targets to tumor progression, also underlining the role of MACC1 for metastasis formation. Out of 656 upregulated genes in the microarray analyses we finally chose the gene *SPON2* for further functional studies that was ~90-fold upregulated by MACC1 overexpression (fold change \log_2 = 6.49, adjusted *P*-value < 0.02).²¹

MACC1 regulates the expression of SPON2 in CRC cells

In SW480 CRC cells with low endogenous expression of MACC1, we detected only low levels of SPON2. Ectopic overexpression of MACC1 in these cells induced the mRNA expression of MACC1 and also of SPON2 to ~50-fold compared with SW480/vector control cells (Figure 1b, *P* < 0.001 and *P* = 0.02, respectively). In contrast to SW480 cells, SW620 cells show high endogenous expression of MACC1, and here, we determined high levels of SPON2. Knockdown of MACC1 by MACC1-shRNA in these cells not only resulted in reduced MACC1 expression but also significantly decreased the expression of SPON2 at mRNA level (Figure 1c, *P* = 0.05 and *P* = 0.03, respectively).

SW480/MACC1 cells showed a 4.21-fold higher MACC1 protein level (s.e.m. 0.33, *P* < 0.001) and a 2.01-fold higher SPON2 protein level (s.e.m. 0.66, *P* = 0.057). The knockdown of MACC1 in SW620 cells resulted in a 0.35-fold decrease of MACC1 protein level (s.e.m. 0.11, *P* = 0.002) and a 0.65-fold decrease of SPON2 protein level (s.e.m. 0.18, *P* = 0.028) as compared with their respective control cells.

Moreover, we analyzed the binding of MACC1 to the SPON2 gene promoter in SW480/MACC1 cells by chromatin immunoprecipitation (ChIP) analysis. We precipitated the V5-tagged MACC1 protein/DNA complex with an anti-V5 antibody and amplified SPON2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter regions by PCR. We demonstrate the binding of the MACC1 protein to the region –444 bp to –215 bp of the SPON2 promoter. No binding of the MACC1 protein to a GAPDH sequence was observed, used as a control (Figure 1d).

SPON2 promotes cell viability, migration, invasion and colony formation

In functional analyses we studied the impact of SPON2 expression on proliferation motility of CRC cells. First, we generated SW480/SPON2 cells 60-fold overexpressing SPON2 compared with the SW480/vector control cells (Figure 2a, *P* = 0.04). Moreover, we decreased the endogenous expression of SPON2 in SW620 cells by SPON2-shRNA. SW620/SPON2-shRNA cells demonstrated a 60% reduced SPON2 expression compared with the control cells (Figure 2a, *P* = 0.03). Furthermore, SW480/SPON2 cells showed a 12.4-fold higher SPON2 protein level (s.e.m. 5.0, *P* = 0.012), and the knockdown of SPON2 in SW620 resulted in a 0.73-fold decrease of SPON2 protein level (s.e.m. 0.13, *P* = 0.020) as compared with their respective control cells.

We studied the migratory potential of SW480/SPON2 (vs SW480/vector) and SW620/SPON2-shRNA (vs SW620/control-shRNA) by using Boyden chamber assays. Cells ectopically overexpressing SPON2 showed a 2.5-fold higher migratory ability than control cells (Figure 2b, *P* < 0.0001). Knockdown of SPON2 expression led to a 35% reduced cell migration (Figure 2b, *P* < 0.0001).

The influence of SPON2 on cell invasion was determined with matrigel-coated transwell chambers. SW480/SPON2 cells demonstrated a fourfold higher cell invasion than SW480/vector cells (Figure 2c, *P* < 0.0001). In contrast, knockdown of SPON2 in SW620 cells led to a 35% decrease in cell invasion compared with control cells (Figure 2c, *P* = 0.01).

The effect of SPON2 expression on directed migration was analyzed by wound healing assays (Figure 2d). SW480/SPON2 cells demonstrated an accelerated wound closure within 4 days compared with SW480 control cells. In contrast, knockdown of SPON2 expression in SW620 cells resulted in decelerated wound closure when compared with SW620 control cells. We also quantified the wound closure by measuring the wound width at days 0 and 4, calculated the closure rate relative to day 0 and normalized the wound closure to the respective control cells. SW480 cells overexpressing SPON2 closed the wound 2.69 times faster than SW480 cells transfected with the empty vector (s.d. 0.27; *P* = 0.0004). Knocking down SPON2 in SW620 cells caused a decrease in wound closure to 0.49 times compared with the cells transfected with control-siRNA (s.d. 0.24; *P* = 0.0258).

Next, we analyzed the impact of SPON2 on cell viability over a time period of 5 days by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. SW480/SPON2 cells had a significantly higher cell viability compared with SW480/vector control cells (Figure 2e, day 5: *P* = 0.048). SW620/SPON2-shRNA cells showed fewer viable cells than SW620/control-shRNA clones (Figure 2e, day 4: *P* = 0.039).

Furthermore, the impact of SPON2 on the ability of the cells to form colonies was determined. Single cells were seeded and cell colonies were counted after 10 days. SW480/SPON2 cells had a 1.5-fold higher ability to form cell colonies in comparison with control cells (Figure 2f, *P* = 0.045). Reduction of SPON2 expression in SW620 cells resulted in the formation of fewer colonies compared with controls (Figure 2f, *P* = 0.011).

In order to validate our findings in an additional CRC cell line, we included the cell line Colo205. Knockdown of MACC1 also

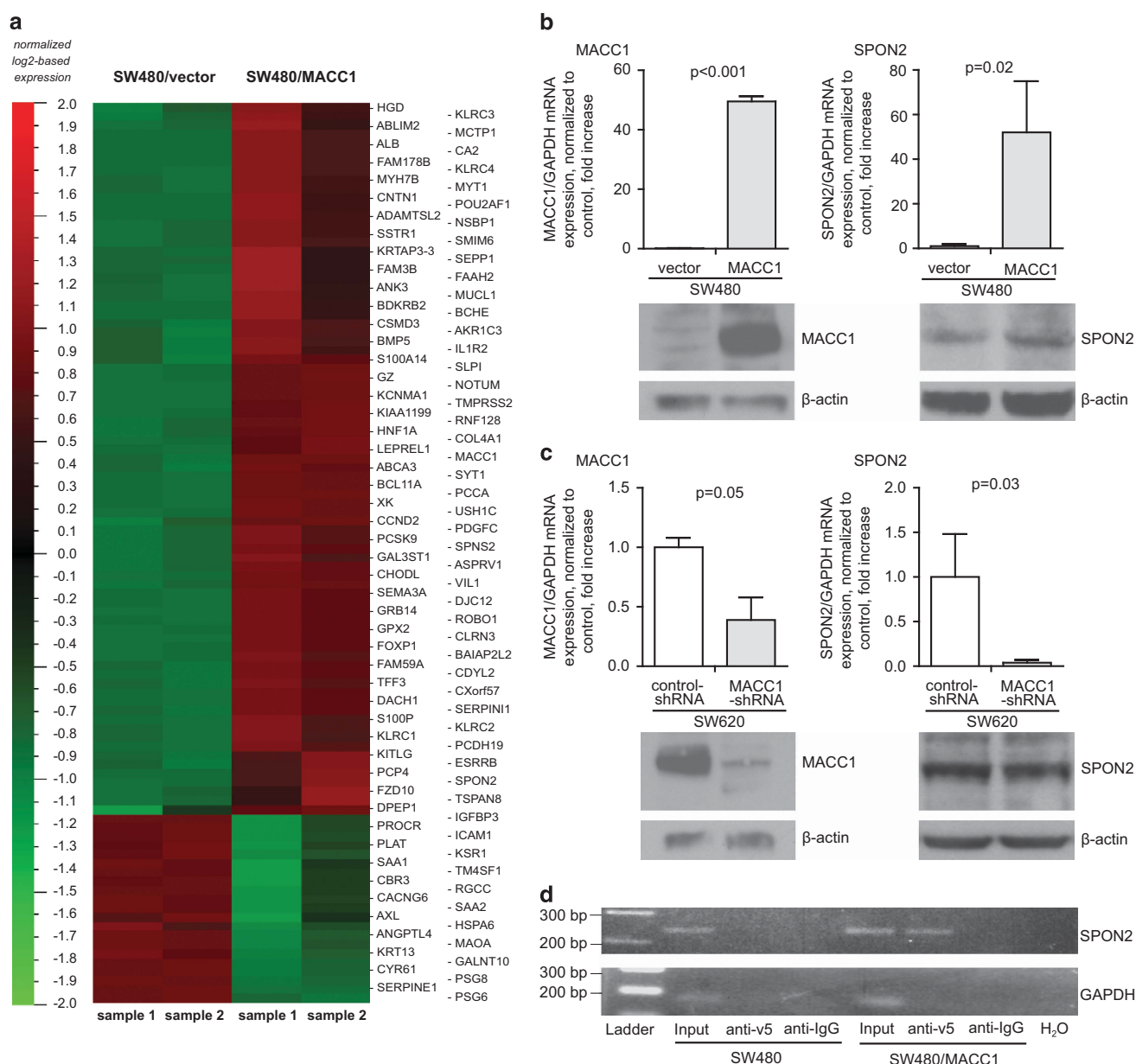


Figure 1. SPON2 is a target of MACC1. Identification of transcriptional target genes of MACC1 by microarray analysis. Total mRNA of SW480/vector and SW480/MACC1 cells was extracted, transcribed, labeled and hybridized onto a 4×44 K Whole Human Genome Microarray (Agilent Technologies). Arrays were carried out in duplicate. **(a)** Heat map representation of the top 100 differentially expressed (adjusted P -value < 0.05) genes of duplicates of SW480/vector and SW480/MACC1 cells. The expression levels of 79 up- and 21 down-regulated genes were normalized and hierarchically clustered, using the Qlucore Omics Explorer 3.0 software. **(b)** Ectopic overexpression of MACC1 induced the expression of SPON2 in SW480 cells at mRNA and protein levels compared with control cells (vector). MACC1 and SPON2 expression levels were determined by quantitative real-time reverse transcriptase-PCR (qRT-PCR; $P < 0.001$ and $P = 0.02$, respectively; normalized to GAPDH) and western blotting ($P \leq 0.001$ and $P = 0.057$, respectively; densitometry was based on protein of interest, normalized to loading control). Experiments were performed three times independently. P -values were calculated with the two-sided Student's t -test. **(c)** Knockdown of MACC1 with MACC1-shRNA decreased MACC1 and SPON2 mRNA ($P = 0.05$ and $P = 0.03$, respectively; normalized to GAPDH) and protein ($P = 0.002$ and $P = 0.028$, respectively; densitometry was based on protein of interest, normalized to loading control) expression in SW620 cells compared with control cells (control-shRNA). Experiments were performed three times independently. P -values were determined with the two-sided Student's t -test. **(d)** ChIP experiments. The V5-tagged MACC1 protein in SW480 and SW480/MACC1 cells was precipitated with an anti-V5-tag antibody. SPON2 promoter sequence (-444 bp to -215 bp) was amplified by PCR. PCR products were separated by gel electrophoresis. SPON2-specific promoter PCR products (229 bp) were observed in the input samples of SW480 and SW480/MACC1 cells (positive controls) and in the anti-V5 antibody-treated SW480/MACC1 cells. No PCR product was detected in the anti-V5 antibody-treated SW480 cells. PCR of samples treated with anti-IgG antibody did not show a PCR product (negative control). No PCR product was detected when amplifying a GAPDH sequence. Experiment was performed two independent times.

decreased SPON2 in Colo205/MACC1-siRNA cells compared with Colo205/control-siRNA cells (Supplementary Figures 1a and b; $P = 0.002$ and $P = 0.054$, respectively), thereby validating our findings obtained with SW620/MACC1-shRNA vs SW620/control-

shRNA (Figure 1c). Furthermore, knockdown of SPON2 in Colo205 cells ($P = 0.037$) resulted in reduced cell viability and colony formation ($P = 0.014$; Supplementary Figures 1c-e), as already observed for SW620/SPON2-shRNA cells (Figures 2e and f).

Table 1. List of the 100 most differentially expressed genes comparing SW480/vector and SW480/MACC1 cells by microarray (sorted by \log_2 fold change, with a filter for Benjamini–Hochberg (BH) corrected *P*-values < 0.05).

Gene symbol	\log_2 fold change	Adjusted P-value	Gene symbol	\log_2 fold change	Adjusted P-value
KLRC2	8.79	0.0144	SAA1	− 6.02	0.0407
KLRC4	8.17	0.0173	TM4SF1	− 5.74	0.0400
TFF3	8.08	0.0144	CACNG6	− 5.68	0.0302
KLRC1	7.81	0.0160	SAA2	− 5.57	0.0357
CLRN3	7.15	0.0144	IGFBP3	− 5.31	0.0284
ALB	6.99	0.0174	KSR1	− 5.19	0.0202
POU2AF1	6.85	0.0238	AXL	− 4.89	0.0491
CCND2	6.80	0.0144	CBR3	− 4.74	0.0400
GPX2	6.59	0.0144	PROCR	− 4.73	0.0286
PCDH19	6.58	0.0144	HSPA6	− 4.64	0.0294
ROBO1	6.58	0.0144	ANGPTL4	− 4.58	0.0173
DACH1	6.58	0.0144	KRT13	− 4.56	0.0159
HNF1A	6.54	0.0144	PSG8	− 4.53	0.0144
SPON2	6.49	0.0173	GALNT10	− 4.53	0.0144
BAIAP2L2	6.26	0.0144	RGCC	− 4.46	0.0383
DJC12	6.25	0.0144	PSG6	− 4.37	0.0144
XK	6.24	0.0144	SERPINE1	− 4.35	0.0144
S100P	6.23	0.0144	ICAM1	− 4.35	0.0248
MYT1	6.17	0.0206	CYR61	− 4.27	0.0144
KLRC3	5.83	0.0268	PLAT	− 4.27	0.0339
SEMA3A	5.83	0.0144	MAOA	− 4.25	0.0182
AKR1C3	5.74	0.0188			
BCHE	5.70	0.0300			
MYH7B	5.68	0.0211			
TSPAN8	5.62	0.0317			
MUCL1	5.62	0.0352			
CHODL	5.57	0.0144			
FAM3B	5.55	0.0450			
VIL1	5.54	0.0144			
SMIM6	5.53	0.0185			
SPNS2	5.49	0.0144			
KIAA1199	5.44	0.0144			
MCTP1	5.38	0.0185			
GAL3ST1	5.27	0.0149			
HGD	5.26	0.0274			
BDKRB2	5.24	0.0328			
CNTN1	5.22	0.0242			
GZ	5.21	0.0144			
PCSK9	5.17	0.0144			
ABCA3	5.17	0.0144			
PCP4	5.15	0.0190			
ASPRV1	5.14	0.0144			
SERPINI1	5.13	0.0144			
IL1R2	5.09	0.0268			
SEPP1	5.05	0.0456			
FOXP1	5.04	0.0144			
CA2	4.95	0.0182			
USH1C	4.90	0.0144			
CXorf57	4.88	0.0144			
RNF128	4.87	0.0144			
CDYL2	4.87	0.0144			
PDGFC	4.85	0.0144			
TMPRSS2	4.82	0.0144			
CSMD3	4.76	0.0182			
KCNMA1	4.72	0.0144			
BCL11A	4.69	0.0144			
LEPREL1	4.59	0.0144			
FAAH2	4.57	0.0484			
FZD10	4.57	0.0356			
DPEP1	4.55	0.0431			
COL4A1	4.55	0.0144			
ESRRB	4.53	0.0212			
KRTAP3-3	4.53	0.0425			
SSTR1	4.53	0.0229			
NSBP1	4.48	0.0237			
BMP5	4.45	0.0234			

Table 1. (Continued)

Gene symbol	\log_2 fold change	Adjusted P-value	Gene symbol	\log_2 fold change	Adjusted P-value
GRB14	4.41	0.0144			
MACC1	4.41	0.0144			
SLPI	4.41	0.0144			
ADAMTSL2	4.39	0.0245			
SYT1	4.36	0.0144			
NOTUM	4.35	0.0144			
PCCA	4.33	0.0144			
ANK3	4.28	0.0403			
ABLIM2	4.26	0.0322			
S100A14	4.25	0.0159			
KITLG	4.25	0.0173			
FAM178B	4.24	0.0192			
FAM59A	4.19	0.0149			

Table 2. Genes differentially expressed in MACC1-overexpressing cells categorized into biological processes

Biological processes (GOTERM_BP_FAT)	Genes	P-value	Benjamini
Regulation of cell development	34	3.5×10^{-8}	1.1×10^{-4}
Regulation of neuron differentiation	23	4.2×10^{-6}	6.7×10^{-3}
Positive regulation of developmental process	35	1.4×10^{-5}	1.5×10^{-2}
Regulation of nervous system development	27	2.5×10^{-5}	2.0×10^{-5}
Regulation of cell motion	27	2.7×10^{-5}	1.7×10^{-2}
Vasculature development	32	2.8×10^{-5}	1.5×10^{-2}
Blood vessel development	31	4.3×10^{-5}	2.0×10^{-2}
Blood vessel morphogenesis	28	4.8×10^{-5}	1.9×10^{-2}
Regulation of neurogenesis	24	5.0×10^{-5}	1.8×10^{-2}
Regulation of cell proliferation	70	1.1×10^{-4}	3.6×10^{-2}
Regulation of cell morphogenesis	20	1.2×10^{-4}	3.4×10^{-2}
Regulation of cell migration	23	1.8×10^{-4}	4.8×10^{-2}
Regulation of cell morphogenesis involved in differentiation	14	3.1×10^{-4}	7.4×10^{-2}

SPON2 induces liver metastasis formation in xenografted mice SW480/vector_Luc and SW480/SPON2_Luc cells were intrasplenically injected into nonobese diabetic/severe combined immune deficient (NOD/SCID) mice. On day 62, the spleen tumor was palpable and mice were killed. Spleens and livers were removed and pictures of the organs were taken followed by *ex vivo* bioluminescence imaging of the isolated organs (Figure 3a). All mice, either transplanted with SW480/vector_Luc ($n=9$ mice) or with SW480/SPON2_Luc cells ($n=8$ mice), developed a spleen tumor. No animal transplanted with SW480/vector_Luc cells formed distant liver metastases. However, five out of eight mice transplanted with SW480/SPON2_Luc cells developed liver metastases.

We also measured the bioluminescence signals of the spleens (site of transplantation) and of the livers (site of metastasis) *ex vivo*. Thereby, we clearly confirmed the development of up to four liver metastases in 5 out of 8 mice transplanted with SW480/SPON2_Luc cells, whereas in all nine mice transplanted with SW480/vector_Luc cells the liver was free of metastases (Figure 3b, $P=0.0036$).

In order to further analyze the development of liver metastases in the mice induced by the human SPON2-overexpressing cells, we performed immunohistochemical staining for human cytokeratin-19 in liver cryosections of mice transplanted with SW480/vector_Luc or SW480/SPON2_Luc cells. Micrometastases were clearly identified in those mice transplanted with SPON2-

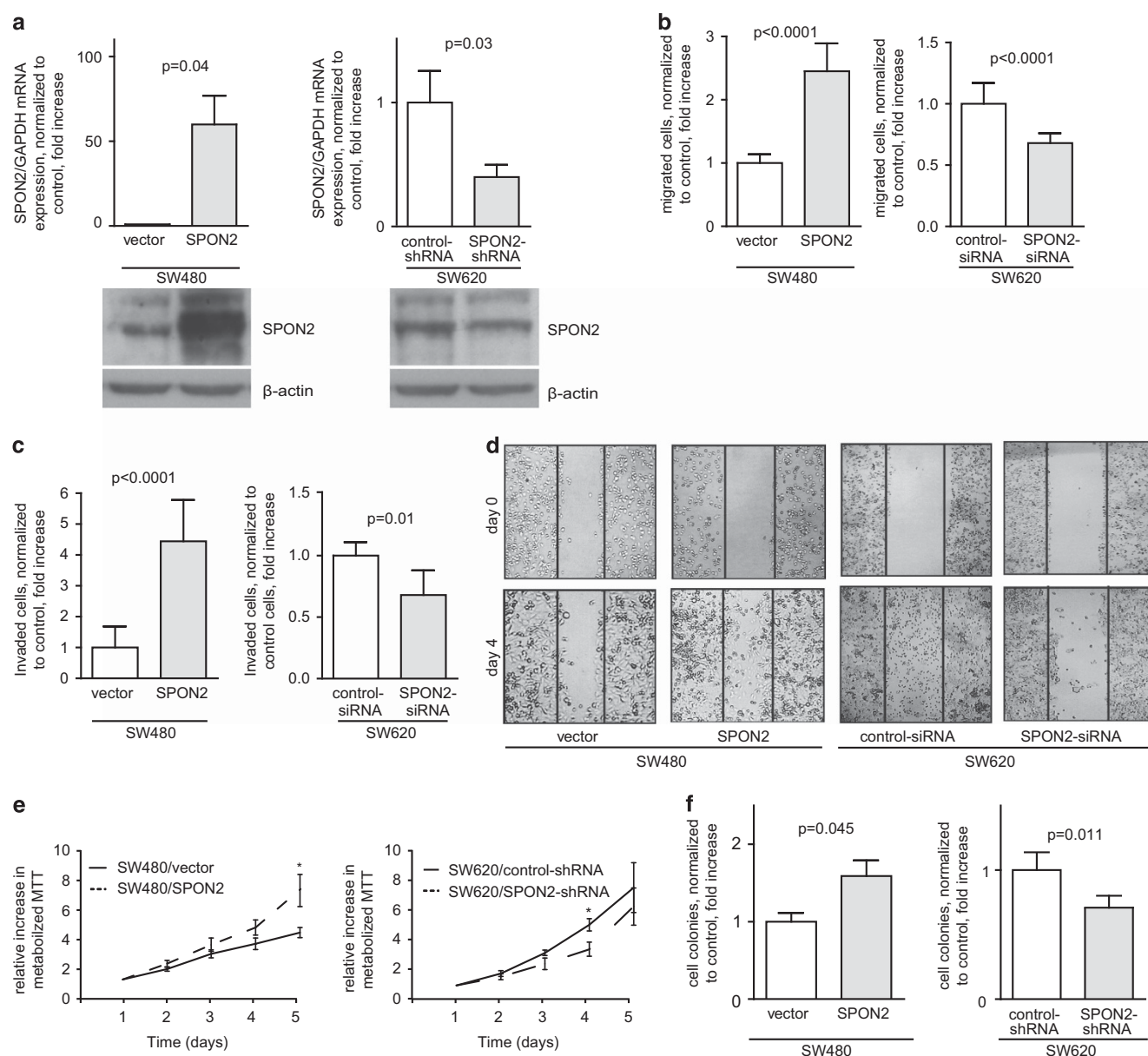


Figure 2. SPON2 induces motility and cell viability of CRC cells. **(a)** SPON2 mRNA expression was increased 60-fold in SW480/SPON2 cells vs SW480/vector controls ($P=0.04$; one representative clone is shown). SPON2 mRNA expression was reduced to 40% by SPON2-shRNA in SW620 cells ($P=0.03$; one representative clone is shown). SPON2 protein levels increased in SW480/SPON2 cells vs SW480/vector cells and decreased in SW620/SPON2-shRNA vs SW620/control-shRNA ($P=0.012$ and $P=0.02$, respectively; densitometry was based on protein of interest, normalized to loading control). Gene expression was determined in three independent experiments by quantitative real-time reverse transcriptase-PCR (qRT-PCR) and western blotting. P -values were calculated with the two-sided Student's t -test. **(b)** Cell migration. Transwell chambers were used for migration assays. SW480/SPON2 cells had a higher migration rate compared with control cells ($P<0.0001$). SW620/SPON2-siRNA cells showed a reduced migration ($P<0.0001$). Assays were carried out three independent times. P -values were calculated with the two-sided Student's t -test. **(c)** Cell invasion. Invasion assays were performed by using matrigel-coated transwell chambers. SW480/SPON2 cells had a higher ($P<0.0001$) and SW620/SPON2-siRNA cells had a lower cell invasion ($P=0.01$) compared with the respective control cells. Assays were performed three times. P -values were calculated with the two-sided Student's t -test. **(d)** Directed cell migration. SW480/SPON2 cells closed the wound (set at day 0) faster ($P=0.0004$), SW620/SPON2-siRNA cells closed the wound slower ($P=0.0258$) compared with the respective control cells. Wound closure was documented until day 4. A second wound healing experiment showed the same effect (not shown). **(e)** Cell viability MTT assay. SW480/SPON2 cells showed higher cell viability ($P=0.048$, day 5) and SW620/SPON2-shRNA cells showed lower cell viability ($P=0.039$, day 4) compared with the respective control cells. Assays were repeated three times. P -values were calculated with the two-sided Student's t -test. **(f)** Colony formation. Single cells were seeded into soft agar plates. SW480/SPON2 cells formed more cell colonies after 10 days ($P=0.045$), and SW620/SPON2-shRNA cells formed fewer colonies than the respective control cells ($P<0.011$). Colony formation assays were performed three independent times. P -values were calculated with the two-sided Student's t -test.

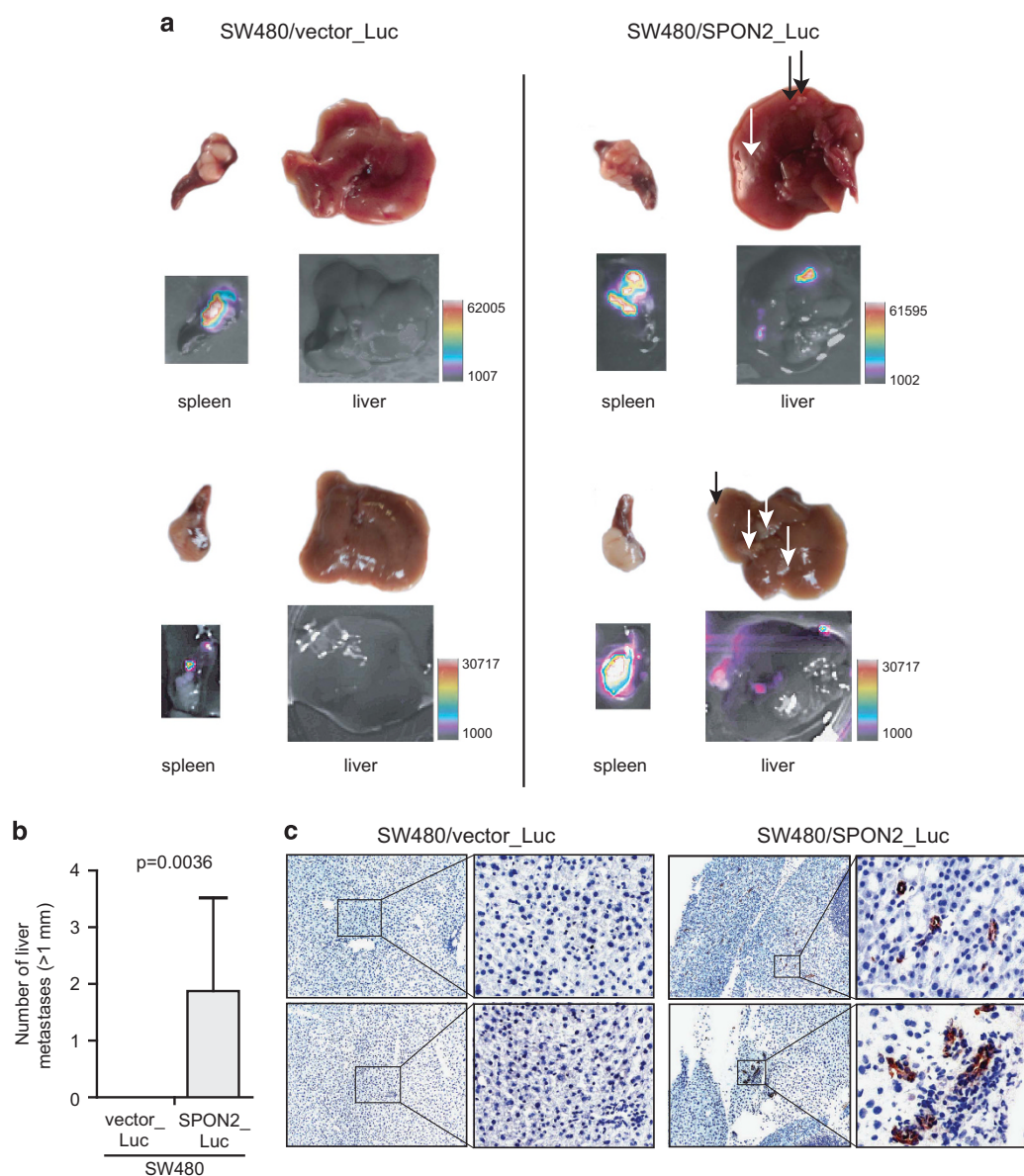


Figure 3. SPON2 drives metastasis formation *in vivo*. **(a)** NOD/SCID mice were intrasplenically transplanted with SW480/vector_Luc ($n=9$) or SW480/SPON2_Luc ($n=8$) cells. Mice were killed after 62 days, spleens and livers were removed and documented. Images of two representative animals per group are shown. Images and *ex vivo* bioluminescence documentations confirmed the development of liver metastases in SW480/SPON2_Luc mice as indicated by arrows in the visible light images and by bioluminescence signals in *ex vivo* bioluminescence pictures. **(b)** SW480/vector_Luc cells did not metastasize into the liver of any of the mice. Five out of 8 mice with SW480/SPON2_Luc cells developed liver metastases ($P=0.0036$). SW480/SPON2_Luc cells developed on average 1.8 liver metastases per liver (liver metastases with a size >1 mm were counted). P -value was calculated with the two-sided Student's t -test. **(c)** Immunohistochemistry of mice liver sections for human cytokeratin-19 identified micrometastases exclusively in the SW480/SPON2_Luc group. Two representative examples per group are shown.

overexpressing cells, whereas no micrometastases were seen in the livers of control mice (Figure 3c).

SPON2 expression is higher in metachronously metastasizing colorectal tumors and correlates with shorter MFS of CRC patients. We evaluated the expression of SPON2 in 60 nontreated human colorectal tumors of stages I–III, that is, not distantly metastasized at the time point of analysis. We performed microdissection and quantitative real-time reverse transcriptase–PCR using the RNA from these tumor cell populations. The SPON2 expression was higher in colorectal tumors that later, metachronously, developed distant metastases compared with those that did not (Figure 4a, $P=0.12$). Tumors that metachronously metastasized showed a

median relative SPON2 expression of 13.92 (SPON2 mRNA expression/GAPDH mRNA expression/% calibrator) compared with a median relative SPON2 expression of 9.21 (SPON2 mRNA expression/GAPDH mRNA expression/% calibrator) in tumors without any formation of metastases.

Furthermore, we evaluated SPON2 as a potential prognostic marker for patient survival. We performed Kaplan–Meier survival analysis based on the SPON2 expression in the primary tumors staged I–III. CRC patients with high SPON2 expression ($n=42$) in their primary tumors had a significantly shorter MFS time compared with low SPON2 expressing patients ($n=18$). The mean 5-year MFS was 48.4 months (s.d. 3.1 months) for the low SPON2 group vs 40.8 months (s.d. 4.4 months) for the group with high

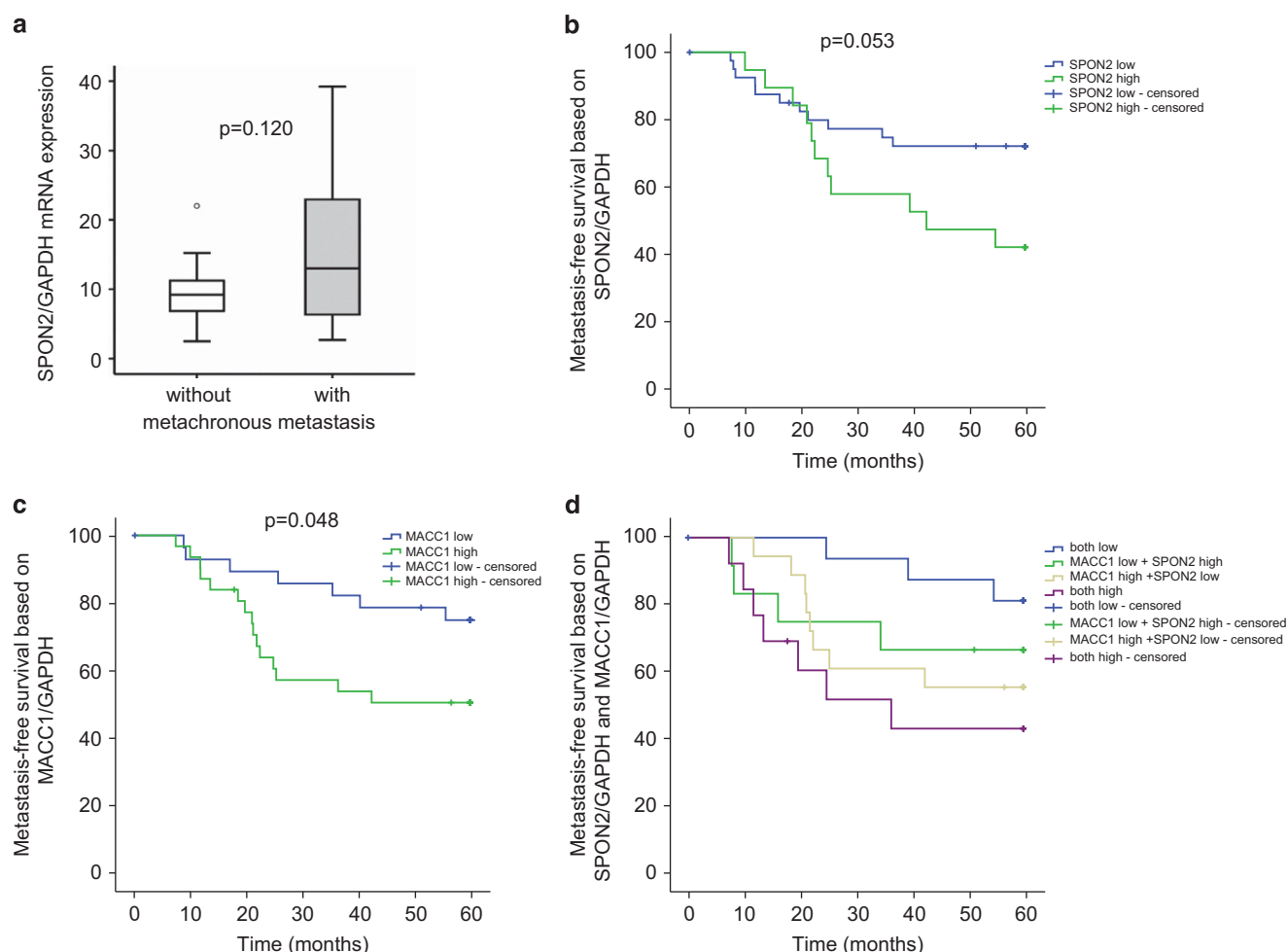


Figure 4. SPON2 expression correlates to MFS of CRC patients. **(a)** SPON2 expression in colorectal primary tumors ($n=60$, stages I–III) was determined by quantitative real-time reverse transcriptase–PCR (qRT–PCR). Tumors with metachronous metastasis had a higher SPON2 mRNA expression compared with tumors without metachronous metastasis. The box plots show the median (black line in the box), the 25th and 75th percentile (box) and 95% confidence interval (error bars) for each group of samples. The P -value was determined by Mann–Whitney U -test. **(b–d)** MFS of patients ($n=60$, stages I–III) within 5 years after diagnosis depending on the relative gene expression level of SPON2 (**b**, $P=0.053$), MACC1 (**c**, $P=0.048$) and the combination of SPON2 and MACC1 (**d**). Cutoffs were calculated by receiver operating characteristic (ROC) analysis and curves were performed by Kaplan–Meier method. P -values were calculated with log-rank test.

SPON2 expression (Figure 4b, $P=0.053$). Thus, the mean 5-year MFS rate for patients with high SPON2 expression in their tumors was 8 months shorter than for patients with low SPON2 expressing tumors.

We also did a combinatorial analysis of SPON2 expression together with MACC1 expression in order to test whether the patient prognosis based on one marker alone, SPON2 or MACC1, can be improved when both markers were combined. For this comparison, we used MACC1/GAPDH data from our cohort using the same tumor samples (Figure 4c).¹³ Patients with high MACC1 expression ($n=31$) had a mean 5-year MFS of 40.6 months (s.d. 3.7 months) compared with low MACC1 expressing patients ($n=29$) with 51.7 months (s.d. 3.1 months), and showed a 11-month shorter MFS compared with the MACC1 low expressing patients.

Based on this combination, four subcohorts could be defined. Patients with MACC1 low and SPON2 low in their primary tumors showed best MFS and a mean 5-year MFS of 56.2 months (s.d. 2.4 months; $n=17$). Patients with MACC1 low and SPON2 high had a mean 5-year MFS of 45.6 months (s.d. 6.1 months; $n=12$). Patients in the group of MACC1 high and SPON2 low showed a mean 5-year MFS of 43.7 months (s.d. 4.5 months; $n=18$). Patients with MACC1 high and SPON2 high demonstrated the worst MFS

and mean 5-year survival of 36.4 months (s.d. 6.2 months; $n=13$). Thus, patients in the MACC1-high/SPON2-high group had a 20-month shorter mean 5-year MFS than patients in MACC1-low/SPON2-low group.

Taken together, by combining the expression data of MACC1 with SPON2, the prognosis for CRC patients MFS could be improved. Thus, the inclusion of SPON2 as a marker for CRC patient survival is of benefit to identify high-risk CRC patients.

DISCUSSION

Metastasis is the main cause of death in CRC.^{1,2} The newly identified gene *MACC1* is a metastasis inducer and a promising biomarker for CRC metastasis.^{3,9} Here we report the identification and characterization of a new transcriptional target gene of MACC1, SPON2. SPON2 was highly upregulated in MACC1 ectopically overexpressing CRC cells, and was downregulated in CRC cells with high endogenous MACC1 expression following MACC1 knockdown. Binding of the MACC1 protein to the SPON2 promoter region –444 bp to –215 bp was demonstrated. In cell culture, SPON2 overexpression induced cell proliferation, migration, invasion and colony formation, whereas SPON2 knockdown resulted in reduced proliferative, migratory and invasive abilities.

In mice, transplantation of CRC cells overexpressing SPON2 led to the formation of liver metastases. In patients with CRC in stages I–III, not yet distantly metastasized at the time point of analysis, high expression levels of SPON2 in the primary tumors correlated with metachronous development of metastases, and with significantly shorter MFS time, compared with patients with low SPON2-expressing primary tumors. Combination of SPON2 with MACC1 expression data improved the identification of CRC patients at high risk.

Genome-wide expression analysis revealed more than 1300 genes that were twofold or more differentially expressed in MACC1-overexpressing cells. Clustering of these differentially expressed genes into biological processes revealed mechanisms associated with tumor development and progression, regulation of cell proliferation and cell migration. Genes upregulated by MACC1 belong to gene families like the killer cell lectin-like receptor subfamily C (KLRC), the ADAM metalloproteinase with thrombospondin type 1 motif family (ADAMTS), the sema domain, immunoglobulin domain (Ig), short basic domain, secreted, semaphorin family (SEMA) and the protein phosphatase 1 (KIAA) family. Some of them such as ADAMTS metalloproteinases are often deregulated in human cancers and their overexpression is associated with tumor progression and metastasis formation.²² Others such as the class of semaphorins can either promote or inhibit metastasis formation depending on the tumor entity.^{23,24}

Here, we focused on the gene *SPON2* that was 90-fold upregulated in MACC1-overexpressing cells. *SPON2*, known as spondin 2, M-spondin or DIL-1, is an extracellular matrix protein. It was previously identified by mRNA differential display screening of cancerous and noncancerous lung cells.¹⁹ For CRC, *SPON2* was found to be upregulated in carcinomas compared with adenomas.²⁰ However, the impact of *SPON2* for tumor progression and metastasis formation and, importantly, for its use as biomarker for prognosis of metastasis linked to CRC patient survival has not been addressed so far.

We previously reported that MACC1 binds to the specificity protein 1 (SP1) binding site in the *MET* promoter.¹⁶ Based on this, Galimi *et al.*²⁵ postulated MACC1 target genes using bioinformatics approaches. They identified putative target genes using motifs in the promoter regions similar to the *MET* promoter motifs with one or several SP1 site(s) and an activating protein 2 (AP2) site, also considering the distance of these sites. The final list of 129 putative target genes of MACC1 includes 10, being also differentially expressed in our microarray analysis; upregulated: GTF2L repeat domain containing 1 (GTF2IRD1), frizzled family receptor 10 (FZD10), alveolar soft part sarcoma chromosome region, candidate 1 (ASPSCR1), uridine-cytidine kinase 1-like 1 (UCKL1), Usher syndrome 1C (autosomal recessive, severe; USH1C), chromatin accessibility complex 1 (CHRAC1), EPS8-like 3 (EPS8L3) and RAD23 homolog A (*S. cerevisiae*; RAD23A); downregulated: nasal embryonic LHRH factor (NELF) and phosphatidic acid phosphatase type 2C (PPAP2C). Further efforts are needed to verify these genes as target genes of MACC1. *SPON2* was included in a first selection of target genes based exclusively on the presence on SP1 sites. Although the *SPON2* promoter region harbors two SP1 sites, it lacks an AP2 site. This suggests that MACC1 might interact with the *SPON2* promoter via a mechanism/protein complex different from those with the *MET* gene promoter.

Wang *et al.*²⁶ overexpressed MACC1 in a gastric cell line of Asian origin (BCG-823) and identified 33 upregulated and 24 downregulated genes by microarray technology. However, neither the most upregulated nor the most downregulated genes of this study were modulated in MACC1-overexpressing CRC cells, suggesting tumor-specific and/or origin-specific effects induced by MACC1.

Here we show that *SPON2* is regulated by MACC1 and promotes cell viability, migration and invasion of CRC cells. It is known that *SPON2* serves as a ligand for integrins.²⁷ Mice lacking *SPON2*

expression exhibit severely impaired recruitment of neutrophils and macrophages. Integrins increase tumor cell migration, invasion, proliferation and survival by regulating the localization of matrix metalloproteinases, activating the focal adhesion kinase pathway and controlling the expression of cell cycle genes (for example, Cyclin D1), thereby affecting cell proliferation.²⁸ Furthermore, dendritic cells when lacking *SPON2* expression²⁹ demonstrated reduced expression of Rho GTPases (rho family, small GTP binding protein Rac1 and 2, RAC1 and RAC2) known to promote cell motility by modifying the actin cytoskeleton and by regulating the polymerization of actin.³⁰ The activation of Rho GTPases by *SPON2* could be a crucial mechanism in tumor cells.

Based on SuperPaths (pathcards.genecards.org) where *in silico* data from different sources are mined and analyzed, connections of *SPON2* to different pathways were given, for example, to the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (Jaccard similarity score 0.58 and 0.61 respectively). This pathway is important for MACC1 function, as shown in our very first publication describing MACC1 as a biomarker in colon cancer.³ The analysis of this pathway concerning its importance for *SPON2* expression and function could be the next step pointing also to intervention strategies, for example, mitogen-activated protein kinase pathway inhibitors, interfering with MACC1 and *SPON2* in parallel.

In prostate cancer xenografted mice, Parry *et al.*³¹ demonstrated reduced tumor growth by application of anti-*SPON2* antibody. However, the impact of *SPON2* on metastasis formation *in vivo* has not been studied so far. To address this important issue, we transplanted *SPON2*-overexpressing CRC cells intrasplenically. Here we provide the first evidence for induction of metastasis formation by *SPON2* in xenografted mice.

In human tissues, *SPON2* is higher expressed in ovarian, prostate and gastric tumors as compared with the respective normal tissues.^{32–38} In particular, for prostate and ovarian cancers *SPON2* is used as a diagnostic biomarker, also via detection in patient blood. In CRC, *SPON2* was also upregulated compared with adenomas.⁷ However, the prognostic aspect of *SPON2* was not addressed so far. In our colorectal tumor panel, *SPON2* was higher expressed in those primary tumors that later, metachronously, developed distant metastases compared with non-metastasizing tumors. Remarkably, the MFS time of CRC patients with high *SPON2* expression in their primary tumors was 8 months shorter than of patients with low *SPON2* expression. Although these data certainly have to be validated in independent patient cohorts, they point to a potential exploitation of *SPON2* for prognosis of CRC.

During the revision of our paper, a paper by Zhang *et al.*³⁹ was published on upregulation of *SPON2* for prediction of poor survival of colorectal carcinoma patients. The authors demonstrate the upregulation of *SPON2* in CRC tissues using quantitative real-time reverse transcriptase-PCR and data mining of public Oncomine microarray data sets. By using data mining of the Oncomine microarray gene expression data sets, they evaluated CRC patients without or with distant metastasis and found a significant correlation of *SPON2* expression with respect to metastasis ($P=0.046$). In addition, by tissue microarray-immunohistochemistry, they evaluated CRC patients without or with metastasis and found a significant correlation of *SPON2* with respect to metastasis ($P=0.039$). Kaplan–Meier analysis revealed that upregulated *SPON2* predicted poor prognosis of CRC patients. The authors stated that *SPON2* could be an independent diagnostic and prognostic biomarker of colon cancer. Thus, these findings of Zhang *et al.*³⁹ are perfectly in line with the data we presented concerning *SPON2* as a prognostic biomarker for CRC and underline the clinical importance of our findings.

Interestingly, combining the expression of MACC1 and *SPON2* improved the prognosis for CRC patients and allowed to identify a high-risk group for shorter MFS showing a 20-month shorter mean

5-year MFS. Thus, the inclusion of SPON2 as a marker for CRC patient survival is of advantage for CRC patients' prognosis. Although we demonstrated the transcriptional regulation of SPON2 by MACC1, and thereby a dependency of one marker on the other, the improvement of the prognosis when combining both markers points to additional regulators of SPON2.

Taken together, this is the first report identifying SPON2 as a target of the metastasis gene MACC1, thereby providing the new interconnection between MACC1 and SPON2. We demonstrate that SPON2 induces cell motility and proliferation of CRC cells in cell culture and liver metastasis formation in mice, thereby underlining that the metastasis inducer MACC1 regulates genes able to induce metastasis by themselves. Furthermore, SPON2—alone or in combination with MACC1—might serve as novel prognostic biomarker for CRC metastasis and CRC patient survival, and might represent a promising target for intervention strategies toward CRC metastasis restriction.

MATERIALS AND METHODS

Genome-wide expression analysis

Differential gene expression was studied with DNA microarrays to identify genes that are differentially regulated in SW480/vector compared with SW480/MACC1 cells. The total number of samples analyzed was four: two replicates for cells overexpressing MACC1 and two replicates for cells that were transfected with an empty control vector. For this, two different passages of each cell clone, SW480/vector and SW480/MACC1, were harvested and total RNA was isolated with the RNeasy Mini Kit (Qiagen, Düsseldorf, Germany; including DNA digestion). RNA quality was determined (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) and samples with an RNA integrity number >9.0 were used for microarray analyses. For each sample, 950 ng of RNA was labeled by using the Quick Amp Labeling Kit (Agilent Technologies) and hybridized overnight onto a 4×44K Whole Human Genome Microarray (Agilent Technologies). Microarrays were washed and scanned with the Gene-Chip Scanner 3000 (Agilent Technologies). Arrays were performed in duplicate.

We performed clustering of the all raw and normalized expression values as part of our standard quality control pipeline that is part of Bioconductor's 'arrayQualityMetrics' package (Boston, MA, USA). This is mainly used to look for outlier arrays, and includes clustering of the arrays, plotting of the distribution of expression values for each array (boxplots and kernel density plots) and plots of the variance mean dependence. No arrays were excluded from the analysis.

In both cases (raw and normalized) the arrays clustered by MACC1 or control status. The distribution of raw expression values for all arrays was comparable even before performing normalization. In addition, we did minimal nonspecific filtering of the probes to remove those that are consistently of low quality, have expression below background or are control probes, and then performed additional clustering and principal component analysis. The arrays clustered by MACC1 or control status, and in the principal component analysis the control and MACC1 arrays separated in the first principal component, suggesting that the major source of variance in the data is due to the difference in MACC1 expression (and downstream effects). More than twofold differentially expressed genes with a calculated false discovery rate of $P < 0.05$ were chosen for further analysis.

Heat map representation of the top 100 differentially regulated genes of duplicates of SW480/MACC1 and SW480/vector was performed. The expression levels of the top 100 differentially regulated genes were normalized and hierarchically clustered using the Qlucore Omics Explorer 3.0 software (Qlucore).

Differentially expressed genes were categorized with the Functional Annotation Tool of DAVID Bioinformatic Resources 6.7 into biological processes and KEGG pathway.^{40,41}

The microarray data were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (geo.ncbi.nlm.nih.gov).

Cell lines and cell clones

Cell lines SW480, SW480/vector, SW480/MACC1, SW480/SPON2, SW480/vector_Luc, SW480/SPON2_Luc, SW620, SW620/control-shRNA, SW620/SPON2-shRNA, Colo205, Colo205/control-siRNA and Colo205/SPON2-siRNA cells were grown in RPMI-1640 medium (PAA Laboratories, Freiburg, Germany) containing 10% fetal bovine serum (PAA Laboratories) and were

cultured in a humidified atmosphere with 5% CO₂. Authentication of all CRC cells was performed by short tandem repeat genotyping. The short tandem repeat genotype was consistent with published genotype. Cells were free of mycoplasma.

SW480 cells were transfected with the plasmids pcDNA3.1 or pcDNA3.1/SPON2 by using Fugene HD (Roche, Mannheim, Germany) and treated twice a week with 1 mg/ml G418 (PAA Laboratories), thereby generating SW480/vector and SW480/SPON2 clones. For *in vivo* experiments, SW480/vector and SW480/SPON2 clones were additionally transfected with a pcDNA3.1/Luciferase plasmid containing a puromycin resistance gene. Clones SW480/vector_Luc and SW480/SPON2_Luc were cultured with 1 µg/ml puromycin (Clontech, Takara Bio Europe SAS, Saint-Germain-en-Laye, France). Firefly luciferase activity assays were carried out with the Steady-Glo Luciferase Assay System (Promega, Mannheim, Germany). SW620 cells were stably transfected with the plasmid pSil2/SPON2-shRNA (SW620/SPON2-shRNA) and were treated twice a week with 1 mg/ml G418 (PAA Laboratories). SW620 as well as Colo205 cells were also transfected with control-siRNA or SPON2-siRNA (end concentration 50 nM, Applied Biosystems, Foster City, CA, USA) using Oligofectamine (Invitrogen, Carlsbad, CA, USA).

RNA isolation and complementary DNA synthesis

Total RNA was isolated (Universal RNA Purification Kit, Roboklon, Berlin, Germany) and 50 ng RNA was reverse transcribed (20 µl reaction: 1× RT Buffer, 1 U RNase inhibitor, 10 mM MgCl₂, random hexamers, 250 µM pooled dNTPs and 2.5 U MuLV reverse transcriptase; all products from Applied Biosystems). The reaction was carried out for 15 min at 42 °C, 5 min at 99 °C and subsequent cooling at 4 °C for 5 min (T300 thermocycler, Biometra, Göttingen, Germany).

Quantitative real-time reverse transcriptase-PCR

Quantification of gene expression was performed using GoTaq qPCR Master Mix (Promega) and was normalized to the housekeeping gene GAPDH. PCR was carried out in duplicate in a final volume of 10 µl in 96-well plates, including preincubation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 7 s, annealing at 60 °C for 10 s and elongation at 72 °C for 5 s. PCR specificity was tested in the final melting curve (increase of the temperature from 65 to 95 °C, 0.1 °C/s). Data were analyzed using the LightCycler 480 Software release 1.5.0 SP3 (Roche).

Sequences of primers used for quantitative real-time reverse transcriptase-PCR are listed in Table 3.

Protein extraction, western blotting and immunohistochemistry

5×10⁵ Cells were lysed with 100 µl RIPA-Buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, protease inhibitor cocktail, Roche). Protein concentrations were measured with Coomassie Plus (Bradford) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Next, 50 µg of protein was separated (NuPAGE Novex 10% Bis-Tris gel, Invitrogen) and was transferred by semi-dry electroblotting onto a Hybond C Extra nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). Membrane was blocked, incubated overnight with anti-human MACC1 (1:1000; Sigma (Taufkirchen, Germany), Prestige Antibodies HPA 020103), anti-human SPON2 (1:250; Santa Cruz, Dallas, TX, USA, sc166867, A-9) or anti-human β-actin (1:10 000; Sigma, Ac-19, A1978) antibody and incubated for 1 h with anti-rabbit horse-radish peroxidase (HRP)-conjugated (1:10 000; Promega) or anti-mouse-IgG HRP-conjugated (1:10 000, Pierce, Waltham, MA, USA) antibody. Membrane was incubated with ECL solution (100 mM Tris-HCl,

Table 3. Sequences of primers used for qRT-PCR and ChIP

Gene	Primers	Sequence 5'–3'
MACC1	Primer fwd	TTCTTTTGATTCTCTCCGGTGA
	Primer rev	ACTCTGATGGGCATGTGCTG
SPON2	Primer fwd	TGGTCTCGTTTGTGGTGC
	Primer rev	GGAGGACGTTATCTCGGTCA
	Promoter primer fwd	ATGGCTTGCAAAATGCCCTCTG
	Promoter primer rev	ATTAGAGCCAGCGTGCTACAC
GAPDH	Primer fwd	GAAGATGGTGATGGGATTTC
	Primer rev	GAAGGTGAAGGTCGGAGT

Abbreviations: ChIP, chromatin immunoprecipitation; fwd, forward; qRT-PCR, quantitative real-time reverse transcriptase-PCR; rev, reverse.

0.025% w/v luminol, 0.011% w/v para-hydroxycoumaric acid, 10% v/v dimethyl sulfoxide, 0.004% v/v H₂O₂, pH 8.6). Protein bands were detected using CL-XPosure films (Pierce). Experiments were carried out three independent times.

Immunohistochemistry was performed as previously described.⁴² Briefly, cryosections of mouse liver tissues were fixed, endogenous peroxidase was inactivated and membranes were permeabilized. After blocking, cells were incubated with rabbit anti-human-specific cytokeratin-19 antibody (1:50; Acris Antibodies, Herford, Germany, DB103-0.2, E16-L). Detection was performed using a HRP-coupled anti-rabbit antibody (1:300, Abcam, Cambridge, UK) and diaminobenzidine as substrate. Counter staining was done using hemalum.

Chromatin immunoprecipitation

ChIP was carried out using the EZ-ChIP Kit (Millipore, Darmstadt, Germany). The V5-tagged MACC1 protein was precipitated with 10 µg anti-V5-tag antibody (Life Technologies, Carlsbad, CA, USA, R960-29). SPON2 promoter sequence (for primer sequences see Table 3) was amplified by PCR using the PWO-Master (Roche) according to the manufacturer's protocol. PCR products were separated by gel electrophoresis and bands were visualized and documented. The ChIP assay was performed independently two times.

Cell viability, migration, wound healing, invasion and colony formation assays

For cell viability assays, 5×10^3 cells were seeded into a 96-well plate. MTT (Sigma) was added (end concentration 0.5 mg/ml), incubated for 2.5 h at 37 °C and crystallized MTT was resolved in dimethyl sulfoxide. Plate was measured at 560 nm with the Infinite M200 Pro Reader (Tecan, Männedorf, Switzerland). The absorption values measured in the MTT assay were normalized both to initial absorption at day 0 (to avoid the impact of fluctuations in cell seeding) and to the background signal of cell-free medium in each plate. The assay was repeated three independent times, each in quadruplicate.

Migration assays were carried out by using transwells with 12 µm pores (Millipore). 2.5×10^5 Cells were seeded into the upper chamber. After 24 h, migrated cells in the lower chamber were harvested and counted 10 times with Neubauer chambers. Each experiment was performed three times, each in triplicate.

For wound healing assays, 4×10^5 SW480/vector and SW480/SPON2 cells were seeded into 6-well plates and were grown to form subconfluent monolayers. After 24 h, a wound of ~300 µm width was inflicted by cell scraping. The culture medium was exchanged to remove free-floating cells. The closure of the wound within the scrape line was inspected microscopically on a daily basis for 4 consecutive days. Monitoring and documentation of the progress of wound closure were carried out with microphotographs of $\times 10$ magnification taken with the Leica DM IL light microscope (Leica Microsystems, Wetzlar, Germany). The wound healing experiment was performed two independent times, each in duplicate. Quantification of wound closure was performed by measuring the wound width at days 0 and 4. Closure rate was calculated relative to day 0, and by normalizing the wound closure to the respective control cells.

For invasion assays transwell chambers (Millipore) were coated with 1:3 diluted matrigel (BD Biosciences, Heidelberg, Germany). 5×10^5 Cells were seeded in triplicate into the upper chamber. After 72 h, cells in the lower chamber were counted 10 times. The invasion assay was performed three times, each in triplicate.

Colony formation was assessed using 6 cm dishes that were coated with a layer containing 5% w/v agarose in RPMI-1640 medium with 10% fetal bovine serum. A second layer was added containing 4×10^4 single cells in 0.33% w/v agarose and RPMI-1640 medium with 10% fetal bovine serum. Plates were incubated for 10 days at 5% CO₂ at 37 °C. Every 3 days, 2 ml of fresh medium was added. The colony formation was inspected microscopically on a daily basis for 10 consecutive days. Monitoring and documentation of the progress of colony formation were carried out with microphotographs of $\times 40$ magnification taken with the Leica DM IL light microscope (Leica Microsystems). Only cell colonies that consisted of more than four cells were counted. The colony formation assay was performed three independent times, each in duplicate.

In vivo analysis of metastasis formation

All experiments were performed in accordance with the United Kingdom Coordinated Committee on Cancer Research (UKCCCR) guidelines and approved by the responsible local authorities (State Office of Health and

Table 4. Characteristics of CRC patients

Characteristics of the patients	Number (%)
European	60 (100%)
Male	37 (62%)
Female	23 (38%)
Age < 60 years	22 (37%)
Age > 60 years	38 (63%)
Tumor stages T1 and T2	22 (37%)
Tumor stages T3 and T4	38 (63%)
Without lymph node metastasis	40 (67%)
With lymph node metastasis	20 (33%)
UICC-stage I	17 (28%)
UICC-stage II	23 (38%)
UICC-stage III	20 (33%)
Without metachronous metastasis	38 (63%)
With metachronous metastasis	22 (37%)

Abbreviations: CRC, colorectal cancer; UICC, Union for International Cancer Control.

Social Affairs, Berlin, Germany). Sample size calculation was carried out using the program G*power (Heinrich-Heine University, Düsseldorf, Germany), based on $P=0.05$ and a power of 0.8 (actual power was 0.8138335). The random assignment of mice to groups was done by two independent researchers. 3×10^6 SW480/vector_Luc or SW480/SPON2_Luc cells in 50 µl phosphate-buffered saline were intrasplenically transplanted into 6–8-week-old female NOD/SCID mice: for SW480/vector_Luc cells $n=9$ mice; for SW480/SPON2_Luc cells $n=8$ mice (the technician was blinded, the investigator was not blinded for subsequent analyses).

Mice were killed on day 62 (spleen tumor was palpable and gave a strong bioluminescent signal). Spleens and livers were removed, documented and liver metastases with a size > 1 mm were counted. In addition, organs were incubated with luciferin (Promega) and *ex vivo* bioluminescence signals were measured with the VisiLux Imager (Visitron Systems, Puchheim, Germany). Overlay pictures were performed with MetaMorph Software (Visitron Systems).

Patient tissues

Sixty primary colorectal tumors were obtained (approved by Charité Ethics Committee, Charité University Medicine Berlin, Germany). Informed consent was obtained from all subjects. Characteristics of the patients are shown in Table 4. Patients with colorectal adenocarcinomas were classified to stages I–III (without distant metastases at the time of diagnosis). Patients were previously untreated, did not have a history of familial colon cancer, did not suffer from a second tumor of the same or a different entity and underwent surgical R0 resection. Twenty-two patients developed metastases in the following 5 years. MFS was calculated from the date of histopathological diagnosis until the date when the development of distant metastases was observed.

Tumors were shock frozen in liquid nitrogen immediately after surgery. Cryosections were performed and stained with hematoxylin and eosin. Tumor cell areas were evaluated and marked. Tumor cells were micro-dissected and RNA was extracted by using TRIzol reagent (Invitrogen).

Statistics

All adjusted P -values that are part of the microarray analysis were adjusted for multiple testing using the Benjamini and Hochberg method.²¹ Statistical analysis was performed with GraphPad Prism version 5 (La Jolla, CA, USA) or PASW Statistics version 18 (Armonk, NY, USA). The comparison of two groups was done by two-sided Student's t -test. Normal distribution for *in vivo* experiments was analyzed using D'Agostino and Pearson omnibus normality test and Shapiro–Wilk normality test. To calculate sample size, we assumed no equal variation, as control animals do not show metastasis. The experimental data met the assumptions. Significance of box plots was tested by Mann–Whitney U -test. The cutoff value of the SPON2 expression was determined by receiver operating characteristic analysis. The Kaplan–Meier method was used to estimate cumulative survival rates, and differences in survival rates were assessed using the log-rank test. $P \leq 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

FS and US conceived and designed the experiments and wrote the manuscript. FS and JS performed the *in vitro* experiments. FS, QW, MRH and MAA-N analyzed the microarray data. FS, ML and IF designed and performed the *in vivo* studies. MD, DK and WW evaluated the experimental data and contributed to the revised version of the manuscript. PMS evaluated the clinical data. All authors have read and approved the final manuscript.

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