Identification of MAGI1 as a tumor-suppressor protein induced by cyclooxygenase-2 inhibitors in colorectal cancer cells

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Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the prostaglandin synthesis pathway, is overexpressed in many cancers and contributes to cancer progression through tumor cell-autonomous and paracrine effects. Regular use of non-steroidal anti-inflammatory drugs or selective COX-2 inhibitors (COXIBs) reduces the risk of cancer development and progression, in particular of the colon. The COXIB celecoxib is approved for adjunct therapy in patients with Familial adenomatous polyposis at high risk for colorectal cancer (CRC) formation. Long-term use of COXIBs, however, is associated with potentially severe cardiovascular complications, which hampers their broader use as preventive anticancer agents. In an effort to better understand the tumor-suppressive mechanisms of COXIBs, we identified MAGUK with Inverted domain structure-1 (MAGI1), a scaffolding protein implicated in the stabilization of adherens junctions, as a gene upregulated by COXIBs in CRC cells and acting as tumor suppressor. Overexpression of MAGI1 in CRC cell lines SW480 and HCT116 induced an epithelial-like morphology; stabilized E-cadherin and β-catenin localization at cell–cell junctions; enhanced actin stress fiber and focal adhesion formation; increased cell adhesion to matrix proteins and suppressed Wnt signaling, anchorage-independent growth, migration and invasion in vitro. Conversely, MAGI1 silencing decreased E-cadherin and β-catenin localization at cell–cell junctions; disrupted actin stress fiber and focal adhesion formation; and enhanced Wnt signaling, anchorage-independent growth, migration and invasion in vitro. MAGI1 overexpression suppressed SW480 and HCT116 subcutaneous primary tumor growth, attenuated primary tumor growth and spontaneous lung metastasis in an orthotopic model of CRC, and decreased the number and size of metastatic nodules in an experimental model of lung metastasis.

Collectively, these results identify MAGI1 as a COXIB-induced inhibitor of the Wnt/β-catenin signaling pathway, with tumor-suppressive and anti-metastatic activity in experimental colon cancer.

Keywords: COX-2; Wnt signaling; metastasis; colorectal cancer; tumor suppressor

Introduction

Colorectal cancer (CRC) development is a multistep process (Ilyas et al., 1999). Activation of the Wnt/β-catenin pathway is considered as a critical initiating event in the majority of human CRCs (Fodde and Brabletz, 2007). The Wnt/β-catenin pathway is negatively regulated by the adenomatous polyposis coli (APC) tumor-suppressor protein, which targets β-catenin to proteosomal degradation, and by the cell–cell adhesion molecule E-cadherin, which sequesters β-catenin to the cell membrane. The Wnt/β-catenin pathway can be activated through inhibition of β-catenin degradation or loss of E-cadherin, thereby causing the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus. In the nucleus, β-catenin forms a complex with the T-cell factor/lymphoid enhancer-binding factor-1 (TCF/LEF1) transcription factor to control gene expression (Clevers, 2006). Familial adenomatous polyposis patients have APC mutations preventing β-catenin degradation, and develop multiple intestinal polyps progressing to CRC at an early age. At least one APC allele is mutated in about 60% of sporadic CRCs and somatic β-catenin mutations are found in 50% of CRCs with wild-type (WT) APC alleles, further emphasizing the importance of the Wnt/β-catenin pathway in CRC development (Fodde and Brabletz, 2007).

A significant increase in cyclooxygenase-2 (COX-2, also known as PTGS2) levels occurs during colorectal carcinogenesis (Gupta, 2001). Epidemiological studies showed that regular intake of non-steroidal anti-
inflammatory drugs (for example, aspirin) or selective COX-2 inhibitors (COXIBs, for example, celecoxib) decreases the risk of developing CRC (Wang and Dubois, 2006). Celecoxib (2 × 400 mg/day) was shown to significantly reduce the number of colorectal and duodenal polyps in familial adenomatous polyposis patients (Steinbach et al., 2000), and is now approved as adjuvant therapy to decrease the risk of CRC development in familial adenomatous polyposis patients (Bertagnolli et al., 2009). Long-term use of high doses of COXIBs as chemopreventive strategy in sporadic CRC, however, is not recommended because of the elevated risk of potentially severe cardiovascular complications (Cuzick et al., 2009).

The Membrane-Associated Guanylate Kinase (MAGUK) family member with Inverted domain structure-1 (MAGI1) consists of six PSD95/DiscLarge/ZO-1 (PDZ) domains, a guanylate kinase domain and two WW domains flanked by two PDZ domains (Dobrosotskaya et al., 1997). MAGI1 localizes to cell–cell contacts and acts as a scaffold molecule to stabilize cadherin-mediated adhesions and to recruit molecules at cell–cell contacts in endothelial and epithelial cells (Laura et al., 2002). Through its PDZ domains MAGI1 associates with a variety of PDZ-binding molecules such as N-methyl-D-aspartate receptors (Hirao et al., 1998), β-catenin (Ide et al., 1999), brain specific angiogenesis inhibitor 1 (BAI-1) (Mino et al., 2000), mNET1 (Dobrosotskaya, 2001), phosphatase and tensin homologue (PTEN) (Kotelevets et al., 2005) and β-catenin (Dobrosotskaya and James, 2000; Kawajiri et al., 2000). In epithelial cells MAGI1 localizes at adherens junctions in complex with β-catenin/ E-cadherin (Dobrosotskaya and James, 2000; Kawajiri et al., 2000) as well as at tight junctions (Ide et al., 1999). MAGI1 suppresses the invasiveness of Madin-Darby Canine Kidney (MDCK) cells by recruiting PTEN to cell–cell contacts and decreasing phosphatidylinositol-3-OH kinase signaling (Kotelevets et al., 2005). TRIP6 has been identified as a binding partner of MAGI1b in epithelial cells that promotes MDCK invasiveness through the activation of nuclear factor-κB and Akt (Chastre et al., 2009). Taken together, MAGI1 is an important molecule for the stabilization of cadherin-mediated cell–cell interactions and the suppression of invasiveness in non-transformed epithelial cells.

In an effort to identify COXIBs-regulated genes affecting cancer progression, we have identified MAGI1 as COXIB-induced gene in CRC cells. MAGI1 promotes E-cadherin and β-catenin recruitment to the membrane, and suppresses TCF/LEF1 transcriptional activity. It induces a cohesive epithelial cell phenotype, decreases the migration and invasiveness of CRC cell lines in vitro, and inhibits experimental CRC growth and metastasis in vivo.

**Results**

**COX-2 inhibition increases MAGI1 expression in CRC cells**

Using a microarray-based screen in human umbilical vein endothelial cells treated with the COXIB celecoxib, we have recently identified genes regulated by celecoxib. A total of 105 genes were downregulated with >2-fold difference and 47 genes were upregulated with >2-fold difference (false discovery rate (FDR) <0.05). MAGI1 mRNA was among the induced transcripts (FDR = 0.0047) (J Zaric et al., unpublished data). As COX-2 promotes CRC (CRC) formation and progression (Gupta, 2001), and COXIBs have protective effects, we set up to test whether celecoxib modulated MAGI1 expression in CRC cells. Celecoxib treatment increased MAGI1 mRNA and protein levels in the SW480, HCT116, SW680, T84 and HT29 human CRC-derived

![Figure 1](http://doc.rero.ch)
cell lines in vitro (Figure 1a and Supplementary Figure S1a). MAGI1 was abundant in the DLD1 cell line (Stolfi et al., 2008) expressing only trace amounts of COX-2 protein and mRNA (Supplementary Figures S1b and S1c), and no increase was observed in response to celecoxib (Supplementary Figure S1a). Treatment of SW480 and HCT116 cells with NS-398, another COX-2-specific inhibitor, and ibuprofen, a pan-COX-1/2 inhibitor, also increased the MAGI1 protein level (Figure 1b). Silencing of COX-2 expression in SW480 cells using three different COX-2 short-hairpin RNAs (shRNAs) resulted in increased MAGI1 expression (Figure 1c). SW480 and HCT116 cells express mostly the prostaglandin-E2 (PGE2) receptor EP4 (Supplementary Figure S1d), and treatment of SW480 cells with the stabilized PGE2 analog, 16,16 dimethyl PGE2, resulted in a decrease of MAGI1 protein and mRNA levels (Figure 1d and Supplementary Figure S1e). SW480 and HCT116 cells were used for subsequent experiments.

To obtain evidence that COX-2 also regulates MAGI1 expression in vivo, we injected SW480 cells subcutaneously into Swiss nu/nu mice and when tumors were palpable we fed the mice either with a celecoxib-supplemented diet or a conventional diet. Celecoxib treatment significantly reduced tumor growth (Supplementary Figure S2a), and in derived tumors (Figure 1a and Supplementary Figure S1a), and increased MAGI1 levels (Supplementary Figure S2b).

From these results, we conclude that the MAGI1 mRNA and protein are induced by celecoxib in CRC cell lines in vitro and in derived tumors in vivo, and that PGE2 regulates MAGI1 expression.

**MAGI1 promotes an epithelial morphology and inhibits the migration, invasion and adhesion-independent growth of CRC cells**

In order to investigate the effects of MAGI1 in CRC cells, we generated SW480 and HCT116 cells overexpressing MAGI1 or with silenced MAGI1 (Supplementary Figure S3). Parental SW480 cells consist of a mixture of adherent and loosely attached, rounded cells, often overgrowing as cell aggregates (Figure 2a, top row). MAGI1-overexpressing SW480 cells acquired a flattened, epithelial-like morphology, whereas MAGI1-silenced cells acquired a round shape and grew in clusters. Staining for F-actin and paxillin showed cortical actin, stress fibers and paxillin-positive focal adhesions in SW480 WT cells. MAGI1-overexpressing cells have mostly stress fibers and well-formed focal adhesions, whereas MAGI1-silenced cells had no stress fibers, some cortical actin and no paxillin-positive focal adhesions (Figure 2a, middle and lower rows). In MAGI1-overexpressing SW480 cells, E-cadherin localized predominantly at cell-cell junctions, whereas in MAGI1-silenced cells E-cadherin localization at cell-cell contacts was largely lost (Figure 2b). MAGI1 overexpression reduced SW480 anchorage-independent colony formation in an agarose assay (Figure 2d). MAGI1 overexpression or silencing had minor but significant effects on cell growth in two-dimensional conditions (Supplementary Figure S4b).

These results show that MAGI1 reverses the in vitro cell-autonomous traits of malignancy in CRC cells, including morphology, motility, invasion and anchorage-dependent growth.

**MAGI1 promotes integrin-mediated cell adhesion and signaling**

To address whether MAGI1 might modify cell adhesion to extracellular matrix proteins, we performed short-term in vitro adhesions assays. MAGI1 overexpression enhanced and MAGI1 silencing decreased SW480 adhesion to collagen-I, fibronectin and laminin (Figure 3a). Enhanced adhesion was not associated with increased cell-surface integrin expression and could be prevented by using anti-integrin function-blocking antibodies (Supplementary Figures S5a and S5b). MAGI1 overexpression in SW480 cells enhanced the phosphorylation of focal adhesion kinase (FAK), extracellular signal-regulated kinase-1/2 (ERK1/2) and Akt (Figures 3b and c), three kinases activated by out-side-in integrin signaling (Cabodi et al., 2010). Increased FAK, ERK1/2 and Akt phosphorylation was transient and no longer detectable 60 min after adhesion.

These results show that MAGI1 enhances integrin-mediated cell adhesion and out-side-in signaling.

**MAGI1 inhibits Wnt/β-catenin signaling in CRC cells**

Activation of the Wnt/β-catenin pathway is considered as an initiating event in human colorectal cancerogenesis (Clevers, 2006). As β-catenin was shown to bind to MAGI1 in adherens junctions in epithelial cells (Dobrosotskaya and James, 2000), we speculated that MAGI1 expression levels might modulate the Wnt/β-catenin pathway. To address this issue, we first analyzed the effect of MAGI1 on the activity of the β-catenin-regulated TCF/LEF1 transcriptional complex using the TCF/LEF1 luciferase reporter system TOPFlash/FOP-Flash. MAGI1 overexpression in SW480 decreased TCF/LEF1 transcriptional activity, whereas MAGI1 silencing increased the same (Figure 4a). Consistent with these results, MAGI1 overexpression reduced axin-2 and c-Myc levels, two β-catenin/TCF/LEF1 target genes, whereas MAGI1 silencing increased their expression (Figures 4b and c and Supplementary Figure S6). In MAGI1-overexpressing SW480 cells β-catenin was predominantly located at the cell membrane, whereas in MAGI1-silenced cells it was diffuse in the cytoplasm (Figure 4d), consistent with the modulation of the Wnt/β-catenin pathway by MAGI1.

Taken together, these results show that MAGI1 inhibits Wnt/β-catenin signaling in CRC cells.

**MAGI1 overexpression suppresses tumor cell growth and spontaneous lung metastasis**

Next, we tested whether changes in MAGI1 levels might modulate SW480 and HCT116 tumor growth and...
Figure 2 MAGI1 modulates morphology, migration, invasion and anchorage-independent growth. (a) Phase-contrast microscopic images (upper row), and immunostaining of F-actin (phalloidin, middle row) and paxillin (paxillin) of WT, MAGI1-overexpressing and MAGI1-silenced SW480 cells. Nuclei were counterstained with 4,6-diamidino-2-phenylindole. MAGI1 overexpression induced a flattened epithelial morphology, actin stress fiber and paxillin-positive focal adhesion formation, whereas MAGI1 silencing caused cell rounding and aggregation, and loss of actin stress fibers and focal adhesions. (b) Confocal immunofluorescence imaging showing that in SW480 cells E-cadherin localized both at cell junctions and the cytoplasm. MAGI1 overexpression promoted localization at cell–cell junctions, whereas MAGI1 silencing disrupted E-cadherin localization at cell–cell contacts. (c) Effect of MAGI1 overexpression silencing on the cell migration (left panel) and invasion (right panel) of serum-starved SW480 cells. The results represent the average number of cells per field ± s.d. counted on the lower side of the insert membrane. *P<0.001 compared with the WT (n = 3). (d) SW480 WT, SW480-overexpressing and SW480-silenced cells grown in soft agar. MAGI1 silencing favors anchorage-independent growth of SW480 cells, whereas MAGI1 overexpression inhibits the same. The quantitative results are expressed on the right as mean value of triplicate determinations ± s.d. *P<0.05 compared with the WT (n = 3). MAGI1, MAGI1-overexpressing; MAGI1, MAGUK with Inverted domain structure-1; MAGUK, Membrane-Associated Guanylate Kinase; shMAGI1, MAGI1-silenced; shNS, non-silenced control; WT, wild type.
progression by injecting WT, MAGI1-silenced or MAGI1-overexpressing SW480 and HCT116 tumor cells subcutaneously into immunodeficient mice. MAGI1 overexpression strongly suppressed tumor growth, whereas silencing of MAGI1 resulted in a modest increase in tumor size as compared with that of control tumors (Figures 5a and b). Histological analyses of the primary tumors, lungs and livers showed no evidence of local invasion or metastasis (data not shown).

To test whether MAGI1 overexpression was essential for the tumor-suppressive effects of celecoxib, we injected mice with SW480 cells non-silenced or silenced for MAGI1 expression and treated one cohort of mice with celecoxib. MAGI1-silenced cells were slightly but significantly ($P < 0.04$) less sensitive to the effect of celecoxib (Figure 5c). This suggests that celecoxib acts through MAGI1-dependent and MAGI1-independent mechanisms. One possible MAGI1-independent mechanism is inhibition of angiogenesis (Gupta and Dubois, 2001). Indeed, microvascular density was decreased in celecoxib-treated tumors, regardless of the MAGI1 status (Supplementary Figure S7a).

As subcutaneous transplantation models are not well suited for studying metastasis, we interrogated the role of MAGI1 on metastasis formation using an orthotopic implantation model (Cespedes et al., 2007). This model produces metastases in almost all clinically relevant sites occurring in human patients: regional and occasionally distal lymph nodes, liver, lungs and peritoneum. Luciferase-expressing WT, MAGI1-overexpressing and MAGI1-silenced SW480 cells were implanted orthotopically in the cecum of NOD/SCIDcγ-null mice (NSG) mice. Luminescence detection showed a 1-log increase in the signal from in MAGI1-silenced SW480 tumors as compared with that from SW480 WT tumors, and a 3-log decrease in the signal from MAGI1-overexpressing SW480 tumors as compared with that from WT tumors (Figure 5d and Supplementary Figure S7b). SW480 tumors metastasized to the lungs as detected by the luminescence signal in the thorax region starting week 4 (Figure 6a). MAGI1 overexpression consistently suppressed the luciferase signal fully in the lungs, whereas MAGI1 silencing enhanced the same, however, with great variability (Figure 6a). Histological analysis of lungs, liver and mesenteric tissue confirmed the presence of metastases in the lungs and mesenterium of mice bearing WT and MAGI1-silenced tumors, whereas liver was metastasis-free (Figure 6b and data not shown).

In conclusion, SW480 tumor transplantation experiments showed that MAGI1 overexpression suppresses primary tumor growth, metastasis seeding and metastasis growth.
Discussion

Increased colorectal mucosal levels of COX-2 and PGE\textsubscript{2} are observed in colon adenoma and CRC, and are implicated in colorectal carcinogenesis (Gupta, 2001). Two distinct, complementary tumor-promoting effects of COX-2 have been reported. First, COX-2 induces modifications of the tumor stroma, promoting tumor progression (Gupta and Dubois, 2001). For example, COX-2 promotes tumor angiogenesis by stimulating the expression of vascular endothelial growth factor in tumor and stromal cells (Tsujii et al., 1998), enhancing vascular endothelial growth factor mitogenic activity in endothelial cells (Jones et al., 1999) and $\alpha_v\beta_3$-dependent activation of Rac-1 and Cdc42 (Dormond et al., 2001). Second, COX-2 directly stimulates tumor cell survival, proliferation, migration and invasion in conjunction with oncogenic pathways. For example, activation of HER2/HER3 heterodimers in CRC cells induces COX-2 expression and PGE\textsubscript{2} production (Vadlamudi et al., 1999), which transactivates the epidermal growth factor receptor and activates ERK, resulting in increased cell migration and proliferation (Pai et al., 2002; Shao et al., 2003). COX-2 also cross-talks with the Wnt pathway (Wang et al., 2004). PGE\textsubscript{2} treatment of mice harboring a heterozygous APC mutation (APC min), a murine model of familial adenomatous polyposis, accelerates polyt formation (Sonoshita et al., 2001). PGE\textsubscript{2} was subsequently found to induce the dephosphorylation of $\beta$-catenin resulting in increased nuclear translocation and TCF/LEF1 activation through activation of the phosphatidylinositol-3-OH kinase/Akt pathway and inhibition of glycogen synthase.

Figure 4 MAGI1 overexpression inhibits Wnt signaling. (a) Measurement of TCF/LEF1 transcriptional activity using the TOPFlash/FOPFlash assay in SW80 cells. MAGI1 silencing enhanced TOPFlash activity, whereas MAGI1 overexpression decreased the same. *$P<0.001$ compared with WT ($n=3$). (b) Real-time RT–PCR analysis showing that MAGI1 silencing enhances axin-2 mRNA level whereas MAGI1 overexpression decreases the same. The results represent the mean values $\pm$ s.d. of fold difference relative to the axin-2 level in WT cells normalized to GAPDH levels. *$P<0.001$ compared with the WT. (c) Left panel: Western blot analysis showing that MAGI1 overexpression represses $\beta$-Myc expression in SW480 cells, whereas MAGI1 silencing induces the same. Right panel: Western blot analysis of SW680 and HCT116 cells showing that MAGI1 reduces $\beta$-Myc expression. Actin immunoblotting shows equivalent protein loading. (d) Confocal immunofluorescence images of SW480 cells showing that MAGI1 overexpression promotes $\beta$-catenin localization at cell-cell junctions whereas MAGI1 silencing disrupts the same. WT, wild type; MAGI1, MAGI1-overexpressing; shMAGI1, MAGI1-silenced; shNS, non-silenced control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAGI1, MAGUK with Inverted domain structure-1; MAGUK, Membrane-Associated Guanylate Kinase; RT–PCR, reverse transcription–PCR; TCF/LEF1, T-cell factor/lymphoid enhancer-binding factor-1.
Consistent with such a mechanism, COX-2 inhibition by celecoxib reduced polyp formation (Swamy et al., 2006). Furthermore, COX-2 itself is a Wnt target (Howe et al., 1999). Expression of Wnt-activating, mutant β-catenin induces COX-2 expression, whereas inhibition of the Wnt pathway by the APC protein suppresses COX-2 expression in CRC cell lines (Araki et al., 2003; Tuynman et al., 2008).

Here we report a previously unrecognized mechanism by which COX-2 inhibition impinges on the Wnt/β-catenin signaling pathway, namely through upregulation of MAGI1, a multi-domain scaffolding molecule present at adherens junctions in complex with E-cadherin/β-catenin. CRC cells treated in vitro with the COXIBs celecoxib and NS398, or the pan-COX-1/2 inhibitor ibuprofen, expressed more of the MAGI1 mRNA and protein. The induction of MAGI1 upon COX-2 silencing shows that this activity was not because of the off-target effects of COXIBs (Abassi et al., 2009). MAGI1 overexpression induced an epithelial-like morphology; stabilized E-cadherin and β-catenin localization at cell–cell junctions; increased paxillin-positive focal adhesions, actin stress fibers and cell adhesion to matrix proteins; and suppressed TCF/LEF1 transcriptional activity, anchorage-independent growth, migration and invasion in vitro. MAGI1 silencing decreased E-cadherin localization at cell–cell junctions, disrupted stress fibers and focal adhesions, and enhanced anchorage-independent growth, migration, invasion and TCF/LEF activity in vitro. MAGI1 overexpression in tumor cells in vivo suppressed primary colorectal tumor growth and lung metastasis formation in an intestinal orthotopic transplantation CRC model and an experimental metastasis model. These observations extend the published data on the activities of MAGI1 in non-transformed cells and uncover a previously unrecognized tumor-suppressive role of MAGI1 in CRC. MAGI1, however, is not the
only mediator of the antitumor activity of celecoxib. Additional mechanisms are involved, as MAGI1 silencing only partially blocked celecoxib from inhibiting tumor growth. Inhibition of tumor-associated angiogenesis is likely to contribute to this effect. In view of the observed phenotypic and functional changes, we considered the possibility that MAGI1 might modulate epithelial-to-mesenchymal transition. We monitored the expression levels of E-cadherin, N-cadherin, vimentin, Zeb1 and high mobility group AT-hook2 (HMGA2) by immunostaining and reverse transcription (RT)-PCR in MAGI1-overexpressing and -silenced SW480 cells but did not observe any differences (data not shown). These results suggest that MAGI1 modulates morphological and functional (that is, invasion and migration) features independently of epithelial-to-mesenchymal transition.

**E-cadherin/β-catenin complexes are forming a signalosome** that recruits multiple scaffolding molecules such as

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**Figure 6** MAGI1 overexpression suppresses metastases formation. (a) Spontaneous lung metastasis formation was monitored *in vivo* by luciferase activity over the thorax region in mice bearing orthotopic SW480 tumors. The results represent the mean photon values within the tumor region of interest. MAGI1 overexpression suppresses metastases formation. *P < 0.05 compared with the WT (n = 3–4). (b) Representative histological images (hematoxylin–eosin staining) confirming the presence of metastases in lung and mesenteric tissue. (c) Experimental lung metastasis assay. WT and MAGI1-overexpressing cells were injected into the tail vein and mice were killed 4 weeks later. Lung metastases were quantified by counting the number of nodules per lung, and measuring the surface (as surrogate of size) or individual nodules. MAGI1 overexpression suppresses both the number and the size of the metastatic nodules. *P < 0.01, **P < 0.001 compared with the WT (n = 7). (d) Representative examples of lung metastases of WT (left) and MAGI1-overexpressing (right) SW480 cells. MAGI1, MAGI1-overexpressing; MAGI1, MAGUK with Inverted domain structure-1; MAGUK, Membrane-Associated Guanylate Kinase; shMAGI1, MAGI1-silenced; WT, wild type.
MAG1, PTEN (Kotelevets et al., 2005), Rap1 (Mino et al., 2000) and TRIP6 (Chastre et al., 2009). The stabilization of E-cadherin/β-catenin at the cell-cell borders by MAG1 overexpression and its loss from cell-cell borders following MAG1 silencing is consistent with the model that MAG1 suppresses Wnt/β-catenin signaling by decreasing the pool of free β-catenin. This model is also supported by the published evidence that the COX-2 main product, PGE₂, leads to the inactivation of glycogen synthase kinase-3β, release of β-catenin from the axin/APC complex and its translocation to the nucleus, resulting in the activation of TCF/LEF1-dependent transcription (Castellone et al., 2005; Shao et al., 2005).

As tumor cell exposure to PGE₂ results in MAG1 reduction, it is conceivable that inflammatory and cancer conditions associated PGE₂ production, as it occurs in cancer, may cause a reduction of MAG1 expression resulting in facilitated Wnt/β-catenin signaling. It will be important to unravel the signalling pathways linking PGE₂/E-prostanoid receptors to suppressed MAG1 expression, and identify molecules representing potential therapeutic targets, to maintain high MAG1 levels under conditions of elevated PGE₂ production as an alternative to COXIBs-based treatments. This could create new opportunities to replace COXIB in pharmacological approaches to prevent CRC in high-risk patients or to treat manifest CRC, thereby potentially avoiding COXIB cardiovascular side effects (Cha and DuBois, 2007). As COX-2 activity suppresses E-cadherin expression in intestinal epithelial cells (Tsujii and DuBois, 1995) and COX-2 inhibition upregulates the same (Noda et al., 1998; Wolff and Rubin, 1998). In epithelial cells, COX-2 inhibition upregulates Wnt/β-catenin signaling, and anti-p-FAK, anti-human FAK antibody, anti-phospho-S473-FAK, anti-Akt, anti-ERK1/2 and anti-phospho-T202/Y204, ERK1/2 (from Cell Signaling Technology, Danvers, MA, USA); anti-integrin function-blocking mAbs—mAbs L1a/2 (anti-β1), and mAb G19 (anti-α5) and GoH3 (anti-αv) (from Beckman Coulter, Nyon, Switzerland); Sam-1 (anti-α6) and LM609 (anti-αvβ3) (from Millipore, Zug, Switzerland). The biotinylated rat anti-CD31 mAb was from Pharmingen (San Diego, CA, USA). Matrigel was purchased from BD Biosciences, and NS-398 (Futaki et al., 1997) and ibuprofen (Meade et al., 1993) were from Biomol (Enzo Life Sciences, Lausanne, Switzerland). Celecoxib was provided by Pfizer AG (Dübendorf, Switzerland). 16,16 dimethyl PGE₂ was purchased from Cayman (Ann Arbor, MI, USA). D-luciferin was obtained from Caliper Life Sciences (Oftringen, Switzerland).

Cell lines and cell culture

The human colorectal carcinoma cell lines SW480, SW620, HCT116, DLD1, HT29 and T84 were from American Type Culture Collection (LGC Standards, Molsheim, France). For all experiments, cells were grown in high-glucose Dulbecco’s modified Eagle’s medium (HCT116, HT29 and T84) or RPMI (SW480, SW620 and DLD1) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, and maintained in a humidified incubator at 37 °C under 5% CO₂. All cell culture reagents were purchased from Invitrogen. Cells were treated with 50 μM Celebrex, 100 μM NS-398 and 50 μM ibuprofen for 4 days (medium with the appropriate drug was changed daily).

In vitro migration and invasion assay

Assays were performed as described previously by Monnier et al. (2008).

Cell adhesion and proliferation assays

Assays were performed as described previously by Zarin and Ruegg (2005) and Bieler et al. (2007).

Soft agar assay

For soft agar assay, 5 × 10³ cells were seeded in triplicate in 1 ml of 0.3% (w/v) agar (Difco; BD Biosciences) in complete Dulbecco’s modified Eagle’s medium (HCT116, HT29 and T84) or RPMI (SW480, SW620 and DLD1) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin, and maintained in a humidified incubator at 37 °C under 5% CO₂. All cell culture reagents were purchased from Invitrogen. Cells were treated with 50 μM Celebrex, 100 μM NS-398 and 50 μM ibuprofen for 4 days (medium with the appropriate drug was changed daily).

Luciferase assay

For dual luciferase reporter assays, SW480 and HCT116 cells were transfected with the firefly luciferase reporter constructs (TOPFlash or FOPFlash; Millipore-Upstate, Upstate, MA, USA) and the control Renilla luciferase reporter pRL-TK purchased from Sigma Chemie (Buchs, Switzerland). The antibodies used are as follows: anti-MAG1 polyclonal antibody (M5691) and anti-actin monoclonal antibody (mAb), phallolidin-fluorescein isothiocyanate (from Sigma Chemie); anti-panaxillin (clone 349) (from Transduction Laboratories, Lexington, KY, USA); anti-c-Myc mAb (sc-40) (from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-COX-2 mAb (from Cayman Chemical; Chemie Brunschwig, Basel, Switzerland); anti-β-catenin mAb (from Transduction Laboratories; BD Biosciences, Basel, Switzerland); anti-phospho-Y425-FAK antibody (from Invitrogen, Basel, Switzerland); anti-pY925-FAK, anti-human FAK antibody, anti-phospho-S473-FAK, anti-Akt, anti-ERK1/2 and anti-phospho-T202/Y204, ERK1/2 (from Cell Signaling Technology, Danvers, MA, USA). Assays were performed as described previously by Monnier et al. (2008).

Materials and methods

Antibodies and chemicals

Bovine serum albumin, paraformaldehyde, poly l-lysine (PLL), fibronectin, laminin, gelatin and collagen-I were

In conclusion, we have identified MAG1 as a COXIB-induced inhibitor of Wnt/β-catenin signaling with tumor- and metastasis-suppressive activity in colon cancer cells.

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Assays were performed as described previously by Zarin and Ruegg (2005) and Bieler et al. (2007).

Soft agar assay

For soft agar assay, 5 × 10³ cells were seeded in triplicate in 1 ml of 0.3% (w/v) agar (Difco; BD Biosciences) in complete Dulbecco’s modified Eagle’s medium (for HCT116) or RPMI (for SW480) medium in six-well plates on 1 ml of a 0.6% bottom agar layer. The cells were fed twice a week and the number of colonies per well was scored 4 weeks later: colonies were counted in four random fields per well under a microscope (n = 3). The results are presented as colonies per field and represent the mean values ± s.d.
The human MAG1 cDNA was provided by Dr Y Hata (Department of Medical Biochemistry, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan) and sub-cloned into the pRRLSIN.cPPT.PKG.GFP.WPRE lentiviral vector under the control of the phospho glyceral kinase (PGK) promoter (=pSD44 plasmid). For gene silencing experiments, the pCMV-GIN-ZEO lentiviral shRNAmir-expression system was purchased from Open Biosystems (Huntsville, AL, USA). For the shRNAmir sequences directed against the human MAG1, three different clones were obtained (clone IDs: V2LHS-36239 (= MAG1shRNAmir#1)/V2LHS-36236 (= MAG1shRNAmir#2)/V2LHS-36238 (= MAG1shRNAmir#3)) that consisted of the shRNAmir sequences cloned into the pSHAG-MAGIC2 (pSM2c) retrovector. The three shRNAmir sequences, or a non-silencing control shRNAmir sequence that contains no homology to known mammalian genes, were sub-cloned into the pCMV-GIN-ZEO lentiviral system. For silencing of human COX-2 (PTGS2), three clones were used, with the following clone IDs: V2LHS-6305 (= COX-2shRNAmir#1)/V2LHS-131533 (= COX-2shRNAmir#2)/V2LHS-131532 (= COX-2shRNAmir#3). The luciferase-expressing lentiviral vector for in vivo cell tracking, pLV-CAG-Luci-IRESPuroR, was a gift from Dr M Aguet (ISREC-EPFL, Lausanne, Switzerland). Lentiviruses were produced in 293T cells by following the calcium phosphate transfection method or co-transfecting the pCMV-GIN-ZEO constructs or the pSD44-MAG1 cDNA with the pMD2G plasmid (VSV-G viral envelope construct) and the pMDLgpRRE plasmid. Cell cultures were transduced by overnight incubation at 37°C in virus-containing media in the presence of 8 µg/ml polybrene. Selection was started on bulk cultures 48 h after transduction using 2 µg/ml puromycin (Sigma Chemie) for the pSD44-MAG1-cDNA construct or 800 µg/ml G418 (Calbiochem, San Diego, CA, USA) for the pCMV-GIN-ZEO constructs.

In vivo studies

Subcutaneous implantation model. SW480 cells (1 × 10^6) and derived lines were injected subcutaneously into Swiss nu/nu female mice (6–8 weeks) and HCT116 cells (1 × 10^6) were injected subcutaneously into NSG mice. Tumor volume was calculated using the following formula: length × (width)^2. For celecoxib treatment, mice were randomized at day 7 and assigned to a treated (that is, celecoxib mixed with a powdered rodent chow diet (2018 Global Rodent diet; Harlan Teklad Europe, Lyon, France; 1000 p.p.m. (=1 g/kg chow) and provided continuously during the course of the experiment) or an untreated group (chow without celecoxib). This protocol allowed attaining celecoxib serum concentrations that are clinically relevant as shown previously by Jacoby et al. (2000) and Gupta et al. (2007). Orthotopic transplantation model. SW480 cells were tagged with firefly luciferase using lentiviral transduction. The carcass was exteriorized by laparotomy and 1 × 10^6 cells were injected into each single spot in the wall using a Hamilton syringe under binocular guidance. The injected carcass was returned to the abdominal cavity and the wound was sutured. The development of primary tumors and metastatic spread was monitored weekly using the IVIS Xenogen Imaging System (Caliper Life Sciences) after intraperitoneal injection of 150 mg/kg 0-luciferin. The results are expressed as photon emission within the ‘region of interest’. Experimental metastasis model. SW480 cells (1 × 10^6) were injected into the tail vein of 10-week-old NSG females. The
mice were killed 4 weeks later and serial sections of paraffin-embedded lungs were hematoxylin-eosin-stained. The number of metastases was quantified by counting nodules manually and quantifying the surface (using the ImageJ software; NIH) of six sections per lung per animal. The results are presented as the average of the number of metastasis per lung ± s.d. and surface of individual metastasis ± s.d. (in arbitrary values).

**Histopathological and immunohistochemical analyses**

Standard hematoxylin-eosin staining procedures were performed on paraffin-embedded tissues. For immunohistochemical detection of micro-vessels, tissue was stained with biotinylated rat anti-mouse CD31 as described earlier by Monnier et al. (2008).

**Statistical analysis**

Statistical significance was determined by Student's t-test. P-value < 0.05 was considered significant.

**Conflict of interest**

The authors declare no conflict of interest.

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**References**


