MTA1 Is Up-regulated in Colorectal Cancer and Is Inversely Correlated with Lymphatic Metastasis

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Abstract. *Background: Metastasis-associated protein 1 (MTA1) plays an important role in tumourigenesis and progression of certain cancer types. In the current study, we analyzed the relationship between MTA1 expression and disease progression of colorectal cancer (CRC). Materials and Methods: CRC tissues (n=93) and adjacent normal colorectal tissues (n=70) were analyzed by quantitative real-time polymerase chain reaction. MTA1 knockdown was established in RKO and HT115 cells using MTA1 siRNA. Results: The expression of MTA1 was significantly increased in CRC tissues compared to paired normal colorectal tissues, but decreased expression of MTA1 was correlated with poor prognosis (higher lymph node involvement stage, TNM stage, local invasion and recurrence) that was associated with increased expression of VEGFC and -D and the receptor VEGFR3. Conclusion: MTA1 is up-regulated in CRC. MTA1 expression is inversely associated with lymphatic metastases and the expression of VEGFC, VEGFD and VEGFR3.*

Worldwide, colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.3 million new cancer cases and 693,900 deaths estimated to have occurred in 2012 (1). The highest incidence rates are found in Australia and New Zealand, Europe, and North America, whereas the lowest rates are found in Africa and South-Central Asia (1). Rates are substantially higher in males than in females (1). Colorectal cancer is the second most common cause of cancer-related death in the UK. Around 16,200 people died of CRC in 2012 in the UK (2). In China, information from the National Central Cancer Registry indicates that colorectal cancer is the fifth most common cause of cancer-related death in men and the fourth in women (3).

The metastasis-associated protein (MTA) family consists of three well-known members, MTA1, MTA2, and MTA3. *MTA1* gene was first identified by differential cDNA library screening using the rat mammary adenocarcinoma metastatic system and was found to be positively associated with breast cancer invasion and metastasis (4). MTA1 is an essential part of the nucleosome remodelling and histone deacetylation complex and is considered to act as a negative transcriptional regulator (5). In the past two decades, MTA1 overexpression has been demonstrated in a variety of human malignancies and may be associated with vascular endothelial growth factor (VEGF) (6). Both nuclear expression of MTA1 protein and increased frequency of tumour microvessels were seen in higher-grade human ductal breast carcinoma sections using immunohistochemistry (IHC) analysis (7). Furthermore an *in vitro* study has shown that MTA1 up-regulates expression of VEGF and its receptor FLT1 *via* extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathway in HEK-293 and MCF-7 cells (7). The protein level of MTA1 and VEGFA was consistently lower in invasive ductal carcinomas with high maspin expression compared to tumours that had lost maspin expression (7). Furthermore, decreased MTA1 protein expression accompanied by reduced angiogenesis has been observed in prostate cancer tumours in castrated and interleukin 17 (IL17) receptor C-deficient mice, suggesting that *MTA1* might be a target gene of the IL17 signaling pathway (8).

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Table I. *Primers used for polymerase chain reaction (PCR) and quantitative real time PCR (QPCR).*

Gene	Forward primer	Reverse primer
MTA1 (QPCR)	GAGGAACAGCTCCCGATG	ACTGAACCTCACCGTACAGAAGGGGAAATAGAAGAGGA
VEGFC (PCR)	TTTGCCAATCACACTTCCTG	CAGGCACATTTTCCAGGATT
VEGFC (QPCR)	GGAAAGAAGTTCCACCACCA	ACTGAACCTGACCGTACAGAAAATCCTGGCTCACAAGC
VEGFD (PCR)	TGGAACGATCTGAACAGCAG	TTCTTCAGGGATCTGGATGG
VEGFR2 (QPCR)	CTTGCTCAAGACAGGAAGAC	ACTGAACCTGACCGTACACCCAATACTTGTCGTCTGAT
VEGFR3 (QPCR)	AGCATCGTGTGGTACAAAG	ACTGAACCTGACCGTACACTCAGCTTCTGGTTGGAGT
GAPDH (PCR)	GGCTGCTTTTAACTCTGGTA	GACTGTGGTCATGAGTCCTT
β-Actin (QPCR)	CATTAAGGAGAAGCTGTGCT	ACTGAACCTGACCGTACAGCTCGTAGCTCTTCTCCAG

MTA1: Metastasis-associated protein 1; *VEGF*: vascular endothelial growth factor; *VEGFR*: VEGF receptor; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase.

VEGFC was initially purified and cloned from human prostatic carcinoma cells in 1996 (9). VEGFC and its receptor VEGFR3 play an important role in angiogenesis and lymphangiogenesis (9). The VEGFC–VEGFR3 axis enhances tumour growth and metastases in certain types of solid cancers (10).

Aberrant expression of MTA1 has also been observed in CRC. *MTA1* mRNA was highly expressed in 14 out of 36 (38.9%) CRCs and was associated with deeper invasion through the intestine wall and a higher rate of lymph node metastasis (11). Higher *MTA1* mRNA levels were seen in CRC tumours compared to normal colorectal mucosa in a cohort of 40 CRC samples (12). Positive IHC staining of MTA1 was shown in 38 out of 74 (51.4%) CRCs. MTA1 expression was correlated with the depth of tumor invasion and vascular invasion (13). Furthermore, the 5-year survival rate in the MTA1-positive group (50.5%) was significantly lower compared to the MTA1-negative group (73.1%) (13). MTA1 expression analysis by IHC indicated that MTA1 expression was significantly higher in moderately and poorly differentiated tumours and live-metastatic tumours compared to normal colonic tissues in a cohort which comprised of 18 normal colon tissues and 91 tumor samples (14). MTA1 overexpression in HCT-116 cells enhanced proliferation, adhesion, migration and invasion, while silencing of *MTA1* inversely affected these cellular functions (14). In an examination of 81 CRC samples using IHC, MTA1 and VEGFC expression levels were correlated with lymph node metastasis and Dukes' stages (15). Furthermore, knockdown of *MTA1* in HCT116 cells resulted in decreased VEGFC expression, whilst the opposite effect was seen in LoVo MTA1-overexpressing cells (15).

In the current study, we aimed to determine the expression of MTA1 and VEGFC in CRC. *MTA1* and *VEGFC* expression in in a clinical cohort of 93 CRC tissues and 70 normal colorectal tissues was determined using quantitative real-time polymerase chain reaction (QPCR) and the association with the clinical and pathological features was further analysed.

MTA1 knockdown was established in RKO cells using anti-*MTA1* small interfering RNA (siRNA) and *VEGFC* transcript level was also determined. The effect of *MTA1* knockdown on cell growth, migration and invasion were also assessed.

Materials and Methods

Cell lines and culture conditions. Human colon cancer cell lines RKO, Caco-2, HRT-18 and HT115 were purchased from the European Collection of Cell Cultures (Salisbury, UK) and were cultured at 37˚C, with 5% CO_2 and 95% humidity. The wild-type cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (PAA Laboratories Ltd., Somerset, UK), amphotericin B, penicillin and streptomycin.

Fresh human colorectal cancer tissues. Colorectal cancer tissues (n=93) and normal background tissues (n=70) were collected immediately after surgery and stored at −80˚C until use. All specimens were verified using haematoxylin and eosin (H&E)-stained frozen sections by consultant pathologists. Histological information was obtained from pathology reports. The study was approved by the South East Wales Local Research Ethics Committee (05/WSE03/92) and consent was obtained from the patients.

RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and QPCR. RNA was extracted from confluent cells in a 25 cm² flask using total RNA isolation (TRI) reagent and following the protocol provided (Sigma-Aldrich, Dorset, UK). Fresh frozen tissues were first homogenised in the TRI reagent by a handheld homogenizer. cDNA was synthesised from 1 μg RNA by using a first-strand DNA systhesis kit (BioRad, Hemel Hempstead, UK). Quantitative analysis of *MTA1* mRNA expression in CRC tissues was determined by QPCR using Amplifor™-based technologies, in which a 6-carboxy-fluorescine-tagged Uniprimer™ (Biosearch Technologies, Inc., Petaluma, CA, USA) was used as a probe together with a pair of target-specific primers and reverse primer with an additional Z-sequence (actgaacctgaccgtaca). The quality of cDNA samples was verified using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene. All the primers are listed in Table I.

MTA1 knockdown using siRNA. MTA1 siRNA and control siRNA were bought from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Transfection of *MTA1* siRNA was conducted following the manufacturer's protocol. Briefly, 7×10^5 cells were seeded to each well of a 6-well plate in antibiotic-free DMEM supplemented with 10% foetal calf serum and incubated overnight. Lyophilized *MTA1* siRNA was dissolved in 330 μl of the RNAse-free water, and 6 μl of *MTA1* siRNA was added to 94 μl siRNA transfection medium to make solution A. Meanwhile, 3 μl of siRNA transfection reagent was added to 97 μl siRNA transfection medium to make solution B. Solution A and solution B were mixed and incubated for 30 min at room temperature and this was subsequently used to transfect cells. After incubation for 72 h, cells were harvested for expression analysis.

Western blot analysis. The protein concentrations in the cell lysates were determined using the DC protein assay kit (Bio-Rad) and an ELx800 spectrophotometer (Biosearch Technologies, Inc.). After separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis, proteins were blotted and probed with the antibody to MTA1 (1:500; Santa Cruz Biotechnology, Inc.), antibody to VEGFC (1:500; Santa Cruz Biotechnology, Inc.), and antibody to GAPDH (1:1,000; Santa Cruz Biotechnology, Inc.) as a housekeeping gene control, followed by a peroxidase-conjugated secondary antibody (1:2,000; Sigma-Aldrich Ltd., Dorset, UK). Protein bands were visualised using a chemiluminescence detection kit (Luminata, Millipore Ltd., Feltham, UK) and photographed using Syngene imager (Syngene International Ltd., Cambridge, UK).

In vitro cell growth assay. CRC cells were seeded into a 96-well plate at a density of 3,000 cells/200 μl per well. Cells were incubated for 1, 3 and 4 days, and were then fixed with 4% formaldehyde. Following staining with 0.5% crystal violet, absorbance was determined at a wavelength of 540 nm using an ELx800 spectrophotometer.

In vitro invasion assay. The procedure for the *in vitro* invasion assay has previously been described (16). Culture inserts with 8-μm pores were pre-coated with 50 μg of Matrigel Matrix Basement Membrane (BD Bioscience, Oxford, UK) and air-dried. Following a rehydration, 3×10^5 cells were seeded into each insert. After incubation for 72 hours, cells that had migrated through the matrix to the other side of the insert were fixed, stained and counted.

Wound-healing assay. The assay was performed as previously described (16). A total of 3×10^5 cells were seeded into 96-well plates and incubated overnight. The monolayer of cells was scraped with a 10 μl pipette tip to ensure a linear wound. The migration of cells was photographed using a time-lapse video recorder and analysed using Optimas 6.0 motion analysis (Meyer Instruments, Inc., Houston, TX, USA).

Statistical analysis. Student *t*-test and one-way ANOVA analysis were performed using SPSS statistical software (SPSS version 22.0; IBM Inc., Armonk, NY, USA). Data are shown as mean±SE. Differences were considered to be statistically significant at *p*<0.05.

Results

The correlation of mRNA of MTA1 and clinical parameters in CRC. The transcript level of *MTA1* in a cohort of 93 CRC

Table II. *Correlation of mRNA expression of metastasis-associated protein 1 (MTA1) (copies) and clinical parameters.*

Category	No.	MTA1, mean±SE	*p*-Value
T/N			
Normal	70	7.1±3.31	
Tumour	91	2983±1178	0.013*
Location			
Left colon	22	1823±1511	
Right colon	28	5325±2526	0.24
Transverse colon	2	962±9626	0.64
Rectum	21	3611±3501	0.64
Dukes' stage			
A	7	13658±7539	
B	32	5174±27691	0.33
C	32	164±113	0.12
Tumour stage			
T1	2	12457±12036	
T2	10	14183±6545	0.92
T3	40	2407±1872	0.56
T4	17	192±121	0.49
Lymph node involvement stage			
N0	38	6873±2715	
N1	16	290±224	0.021*
N2	15	31.9±19.7	0.016*
N12	31	165±116	0.018*
TNM stage			
I	9	18526±6850	
II	29	3256±25746	0.063
III	26	179±139	0.028*
IV	6	96.0±42.26	0.027*
Clinical outcome			
No invasion	49	5334±2139	
Invasion	26	216±141	0.021*
Disease-free	34	2891±1700	
Incidence	23	3979±3188	0.77
No recurrence	57	4555±1854	
Local recurrence	7	282±274	0.026*
No metastasis	49	3683±1602	
Metastasis	19	4299±3863	0.88
Alive	35	1487±1034	
Death	22	4162±3331	0.45
Non therapy	42	4309±1855	
Neoadjuvant chemoradiotherapy	13	427±424	0.047*

*Significant at *p*<0.05.

tissues and 70 adjacent normal colorectal tissues was determined using QPCR which was normalised against corresponding β-actin. The association of expression with the clinical and pathological features was analyzed using one-way ANOVA (Table II). The median follow-up period was 21.7 months (range=0.7-88 months) for the current cohort. Higher levels of *MTA1* transcript were observed in the CRC samples compared to normal colorectal tissue (*p*=0.013). However, the level of *MTA1* transcripts were decreased in tumours with lymph node involvement (N1 and N2) in comparison no-lymph node involvement (N0) (*p*=0.021 and 0.016, respectively). In

Table III. *Correlation of metastasis-associated protein 1 (MTA1) expression with expression of vascular endothelial growth factor (VEGF), and its receptor.*

	VEGFA	VEGFB	VEGFC	VEGFD	VEGFR1	VEGFR2	VEGFR3
Correlation coefficient with MTA1	0.0911	−0.173	−0.208	−0.321	0.00518	0.413	−0.266
p-Value	0.473	0.114	0.0451*	0.00886*	0.961	0.000107*	0.0312*
Number of samples	64	84	93	66	93	84	66

All the values are normalized to those for glyceraldehyde 3-phosphate dehydrogenase. *Significant at $p<0.05$.

line with this finding, decreased levels of *MTA1* transcript were also seen in more advanced tumours (T3 and T4) according to the TNM staging in comparison with that of tumours at an early stage (T1) ($p=0.028$ and 0.027, respectively). Similarly, decreased *MTA1* expression was also seen in tumours of Dukes' stage B and C compared to Dukes' stage A tumours. Reduced levels of *MTA1* transcript were seen in locally invasive tumours compared non-invasive tumours ($p=0.021$). In terms of clinical outcomes, patients with tumours with local recurrence exhibited lower *MTA1* expression compared to those that remained disease-free ($p=0.026$). Lower *MTA1* expression was seen in the group of patients who received neoadjuvant chemoradiotherapy compared to the non-treated group ($p=0.047$). No correlation with survival of patients was observed for MTA1 expression in the current cohort.

The correlation between MTA1 and VEGF, VEGFR in CRC. The transcript levels of VEGF family members and their receptors (VEGFA-D and VEGFR1-3) in the CRC cohort were also determined using QPCR. Their correlation with *MTA1* expression was analyzed using Spearman correlation analysis (Table III). A significant correlation was found between increased expression of *MTA1* and reduced expression of *VEGFC*. Similarly, the expression of *MTA1* was also significantly inversely correlated with that of *VEGFD* and VEGFR3. Conversely, the expression of *MTA1* was significantly positive correlated with that of *VEGFR2*. No correlation was found between the *MTA1* expression and other VEGF and VEGFRs.

MTA1, VEGF and VEGFR expression in CRC cell lines. The expression of *MTA1*, *VEGFs* and *VEGFRs* by CRC cell lines was also examined using PCR. Similar transcript levels of *MTA1* were observed in RKO, Caco-2, HRT-18 and HT115 cell lines (Figure 1A). *VEGFC* expression was higher in RKO cells compared to Caco-2, HRT-18 and HT115 cells. Expression of *VEGFD*, *VEGFR2* and *VEGFR3* was found to be very low or absent from these CRC cell lines.

MTA1 knockdown in RKO and HT115 cells. Reduced MTA1 expression was seen in the RKO and HT115 cells transfected with *MTA1* siRNA. The altered MTA1 expression was verified using RT-PCR (Figure 1B and 1E). Reduced *MTA1* transcripts in RKO *MTA1* knockdown cells were also confirmed using real-time PCR (Figure 1C). Due to lower expression or absence of VEGFs and VEGFRs in other cell lines, only expression of VEGFC was determined in RKO cells. Following knockdown of *MTA1*, an increased expression of *VEGFC* mRNA was evident both in the PCR (Figure 1B) and also real-time PCR analyses (Figure 1C). However, subsequent determination of protein levels in these cells did not exhibit any difference in VEGFC expression at the protein level. In line with the PCR and real-time PCR results, reduced protein expression of *MTA1* was seen in the RKO MTA1 knockdown cells.

The effect of MTA1 knockdown on growth, migration and invasion of RKO cells. Cellular functions, including *in vitro* growth, migration and invasion, were determined using *in vitro* functional assays. Knockdown of *MTA1* in RKO cells resulted in a decreased growth compared with control cells (Figure 2). In subsequent analyses of cell migration, reduced migration was seen in the RKO MTA1 knockdown cells compared with the control cells over a period up to 16 h (Figure 3). Similarly to the observation in the functional tests of migration, a decrease was also seen in the invasion of RKO *MTA1* knockdown cells in comparison to control cells ($p<0.001$; Figure 4).

Discussion

Several genes, including activation of *KRAS* and inactivation of adenomatous polyposis coli (*APC*) and p53, play profound roles in tumourigenesis and progression in CRC (17, 18). The expression of many genes in CRC is changed compared to normal tissues, as assessed using cDNA microarrays (19, 20). This suggests that aberrations in the expression of multiple genes or their protein functions are required during tumourigenesis and progression of CRC. *MTA1* is one of the genes that has been found to be overexpressed in CRC tissues and cell lines. Overexpression of *MTA1* is positively correlated with cell invasion, higher metastasis rate, poorer
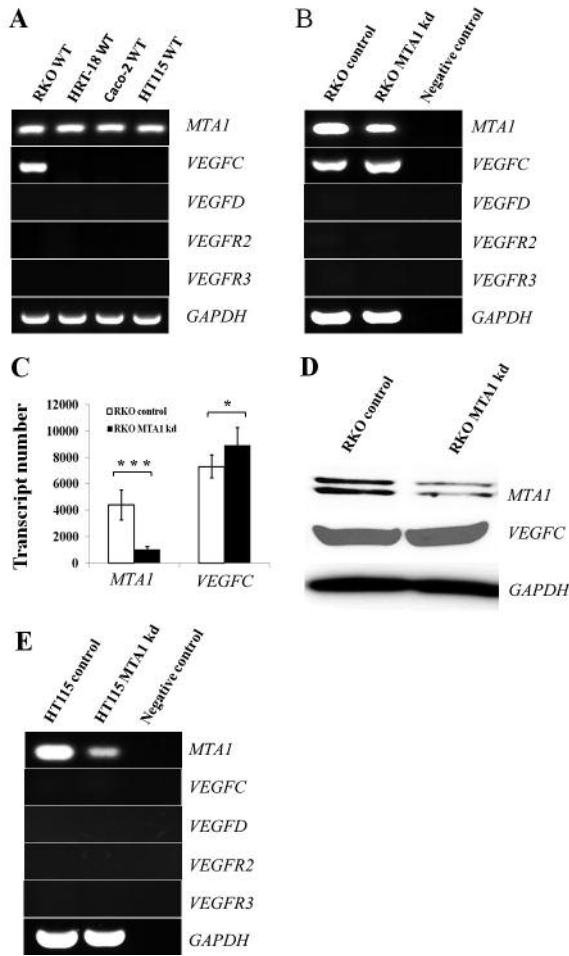
Figure 1. *Expression of metastasis-associated protein 1 (MTA1), vascular endothelial growth factors (VEGFs) and VEGF receptors (VEGFRs) in CRC cells. A: MTA1, VEGFC, VEGFD, VEGFR2 and VEGFR3 expression in RKO, Caco-2, HRT-18 and HT115 cell lines were determined using reverse transcription-polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. B: The expression of MTA1, VEGFC, VEGFD, VEGFR2 and VEGFR3 in RKO cells transfected with MTA1 siRNA (kd) and control siRNA was determined using RT-PCR. C: The expression of MTA1 and VEGFC in RKO cells was also quantified using real-time quantitative PCR. D: Knockdown of MTA1 and consequent effects on the expression of VEGFC at their protein levels were also examined using western blot analysis. E: MTA1, VEGFC, VEGFD, VEGFR2 and VEGFR3 expression in HT115 cells was also assessed using RT-PCR. Significantly different at: *p<0.05, and ***p<0.001. WT: Wild-type cells.*

Figure 2. *Influence of metastasis-associated protein 1 (MTA1) knockdown on in vitro cell growth. The cell growth at day 3 and day 4 in RKO control and RKO MTA1 knockdown (kd) cells. Three experiments were performed. The mean and standard error of mean (error bars) are shown. Significantly different at: *p<0.05, and **p<0.01.*

Figure 3. *Effect of metastasis-associated protein 1 (MTA1) knockdown on cell migration. A: The wound-healing assay at 0, 8 and 16 h in RKO control and RKO MTA1 knockdown (kd) cells (×100). B: Quantification of wound closure. Each cell line was tested in triplicate for an individual experiment. Three experiments were performed. Representative images (A) and average migration (mean and standard error of mean, B) are shown. Significantly different at: **p<0.01, and ***p<0.001.*

prognosis, and VEGFC expression (11-15). The present study shows that the expression of *MTA1* is significantly elevated in CRC tissues compared to paired normal colorectal tissues. This is in line with findings by other researchers, suggesting that *MTA1* plays a role in tumourigenesis and development of CRC (12, 13). However,
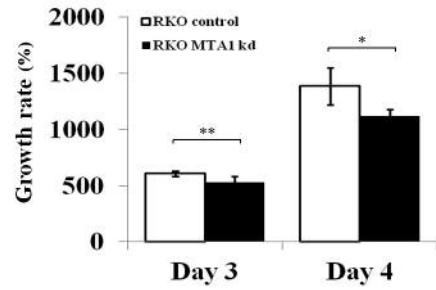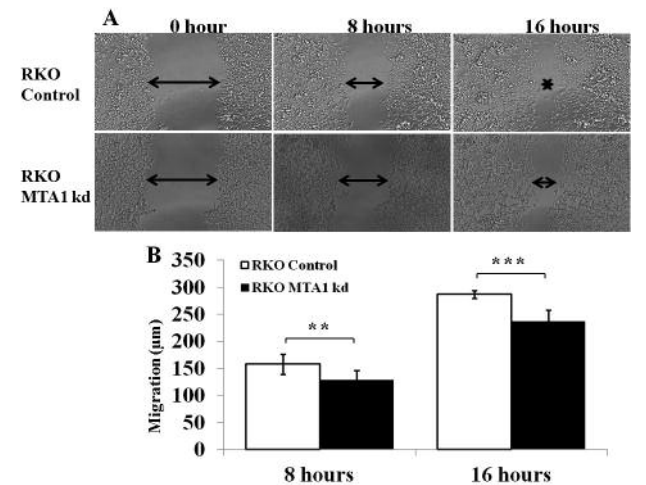
further analysis of *MTA1* expression, comparing its expression in tumours at different stages of disease, showed a reduced expression of *MTA1* transcripts in locally advanced tumours with invasion, raising the question of whether *MTA1* plays the same role during the whole process of CRC development and progression. A different role may be played by *MTA1* during early stages of disease progression, for example, when CRC cells are dissociating from the primary tumour and begin to invade through surrounding tissue. This is also reflected in the analyses of *MTA1* expression levels
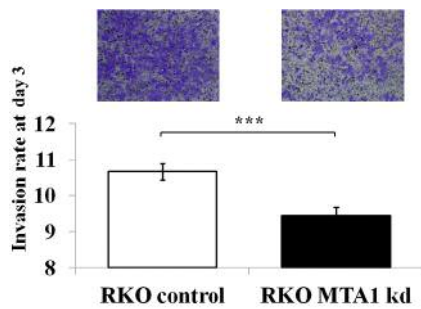
Figure 4. *Metastasis-associated protein 1 (MTA1) and its effect on invasion of CRC cells. Cell invasion assays were carried out to assess the effect of MTA1 knockdown on invasiveness of RKO cells. Three experiments were performed. The mean and standard error of the mean (error bars) are shown. ***Significantly different at p<0.001.*

in tumours according to TNM staging, and also in patients who received neoadjuvant chemoradiotherapy.

We further investigated the influence of *MTA1* on functions of CRC cells by interfering with its expression using siRNA. Knockdown of *MTA1* in RKO cells resulted in a reduction of *in vitro* growth, migration and invasion. This appears to provide supportive evidence for its positive role in CRC cell function that has been evident in other studies (12, 13) and also in the current study, by comparing its expression in tumours with normal colonic tissues. Moreover, an even more complicated mechanism may underlie these observations. For instance, *MTA1* may play a different role when cancer cells interact with surrounding tissues and cells of other types within or surrounding the tumours. Our current study suggests a potential role for this molecule in the lymphatic metastasis of CRC.

Lymph node metastases are commonly seen in CRC and have been utilised as a prognostic factor and also a criterion for the selection of adjuvant therapies (21). In the current study, reduced expression of *MTA1* was observed in tumours with lymph node metastases. Decreased expression was also seen in more advanced tumours in comparison to tumours at an early stage according to their TNM staging. We then analysed the link between *MTA1* expression and VEGFs and VEGFRs in the current cohort of CRC. In line with its reduced expression in tumours with lymphatic metastases, an inverse correlation was seen between MTA1 expression and that of VEGFC, VEGFD and VEGFR3. This suggests that reduced MTA1 expression in the tumours facilitates lymphangiogenesis and dissemination of CRC cells *via* lymphatic vessels. *MTA1* may play a negative role in tumour-associated lymphangiogenesis and consequent lymphatic metastasis.

VEGFC can regulate both angiogenesis and lymphangio-genesis through VEGFR3-mediated signaling (9, 10). Du *et*

al. reported that MTA1 regulates lymphangiogenesis by inducing *VEGFC* expression in CRC (15). However, a negative correlation between *MTA1* and *VEGFC* was revealed in the current cohort of CRC by quantitative analysis of their transcripts. Further investigation of VEG-C in the RKO CRC cell line, which expresses both MTA1 and VEGFC, confirmed such a correlation existing in this CRC cell line. This suggests that at least in some CRC cells, MTA1 can negatively regulate expression of *VEGFC*. On the other hand, we also observed a positive correlation between *MTA1* and *VEGFR2* in the current CRC cohort. Dual roles may, therefore, be played by *MTA1* in coordinating angiogenesis and lymphangiogenesis.

In conclusion, *MTA1* expression is elevated in CRC. Reduced *MTA1* expression in CRC is associated with local invasion and lymphatic metastasis. It has been suggested that differential roles may be played by *MTA1* at different stages of the disease, *i.e.* tumourigenesis and progression, lymphangiogenesis and angiogenesis. The exact mechanisms underlying such controversial roles require further investigation employing different techniques (*i.e.* robust epigenomics and proteomics analysis), co-culture, 3-D models and also *in vivo* experiments.

Acknowledgements

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