Interplay between Trx-1 and S100P promotes colorectal cancer cell epithelial–mesenchymal transition by up-regulating S100A4 through AKT activation

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Abstract
We previously reported a novel positive feedback loop between thioredoxin-1 (Trx-1) and S100P, which promotes the invasion and metastasis of colorectal cancer (CRC). However, the underlying molecular mechanisms remain poorly understood. In this study, we examined the roles of Trx-1 and S100P in CRC epithelial-to-mesenchymal transition (EMT) and their underlying mechanisms. We observed that knockdown of Trx-1 or S100P in SW620 cells inhibited EMT, whereas overexpression of Trx-1 or S100P in SW480 cells promoted EMT. Importantly, S100A4 and the phosphorylation of AKT were identified as potential downstream targets of Trx-1 and S100P in CRC cells. Silencing S100A4 or inhibition of AKT phosphorylation eliminated S100P- or Trx-1-mediated CRC cell EMT, migration and invasion. Moreover, inhibition of AKT activity reversed S100P- or Trx-1-induced S100A4 expression. The expression of S100A4 was higher in human CRC tissues compared with their normal counterpart tissues and was significantly correlated with lymph node metastasis and poor survival. The overexpression of S100A4 protein was also positively correlated with S100P or Trx-1 protein overexpression in our cohort of CRC tissues. In addition, overexpression of S100P reversed the Trx-1 knockdown-induced inhibition of S100A4 expression, EMT and migration in SW620 cells. The data suggest that interplay between Trx-1 and S100P promoted CRC EMT as well as migration and invasion by up-regulating S100A4 through AKT activation, thus providing further potential therapeutic targets for suppressing the EMT in metastatic CRC.

KEYWORDS
colorectal cancer, epithelial–mesenchymal transition, S100A4, S100P, thioredoxin-1

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide.1 Despite the recent advances in diagnosis and treatment, CRC-related mortality remains high.2 Metastasis is one of the major causes of death for CRC patients. Epithelial-to mesenchymal transition (EMT), a key developmental regulatory programme, has been shown to play an important role in the invasion and metastasis of CRC.3 Despite an increased understanding in CRC EMT and...
metastasis, the underlying molecular mechanisms remain poorly understood.

Thioredoxin-1 (Trx-1), a 12-kDa cellular redox protein, is ubiquitously expressed in mammalian cells and has numerous, diverse functions including maintaining cellular redox homeostasis and cell survival. Trx-1 expression is increased in many tumours where its expression is associated with increased cancer cell growth, inhibited apoptosis and decreased patient survival. Trx-1 has been shown to regulate cell growth and survival through the modulation of transcription and cell signalling. We have previously reported a novel positive feedback loop between Trx-1 and S100P, which promotes CRC invasion and metastasis. Trx-1 increases S100P gene transcription through interaction with transcription factor SP1, and S100P in turn promotes the expression and nuclear localization of Trx-1 through up-regulation of p-ERK1/2 and down-regulation of TXNIP expression. S100P is a member of the S100 calcium-binding protein family involved in the regulation of a number of cellular processes. S100P expression is up-regulated in various cancers and has an important role in tumour growth, invasion and metastasis. In our previous studies, we found that overexpression of Trx-1 or S100P enhances CRC cell invasion and metastasis, and conversely, suppression of Trx-1 or S100P expression inhibits CRC cell invasion and metastasis. However, the molecular mechanisms of Trx-1 and S100P in regulating the invasion and metastasis of CRC need further exploration.

Here, for the first time, we demonstrate that Trx-1 and S100P promote CRC EMT, migration and invasion by up-regulating S100A4. This up-regulation of S100A4 by Trx-1 and S100P in CRC cells is partly dependent upon AKT activation. Our study reveals a novel mechanism in the Trx-1/S100P axis that regulates CRC EMT as well as migration and invasion, which may be a potential therapeutic target for the management of CRC.

2 MATERIALS AND METHODS

2.1 Cell culture and chemicals

Cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The human CRC cell lines SW480 and SW620 are derived from primary (SW480) and metastatic lesions (SW620) of a single colon cancer patient. Cells were maintained in RPMI 1640 (for SW480 cells) or Dulbecco’s modified Eagle’s medium (DMEM, for SW620 and HEK293T cells) supplemented with 10% foetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA). MK2206 (Cat.# S1078), an AKT inhibitor, was purchased from Selleck Chemicals (Houston, TX, USA). shRNA sequences were targeted to the Trx-1 mRNA (shTrx-1: 5’-GAC TGT CAG GAT GTT CTC GAG TGT GA-3’) or S100P mRNA (shS100P: 5’-AAC TCA CTG AAG TCC ACC TGG GCA TCT CC-3’). Lentiviral vectors carrying Enhanced Green Fluorescent Protein (EGFP) gene or shRNA targeting firefly luciferase (shLuc: 5’-TGCTC GGT GGT GCC AAC CCT ATT CT-3’) were used as controls. Cells were then transduced with the lentivirus as previously described.

2.3 Western blotting

For Western blot analysis, cells were lysed on ice using a lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein (30 μg) was separated by gel electrophoresis and transferred to PVDF membranes. The immunoreaction was carried out using primary antibodies against Trx-1 (ab133524), S100P (ab133554), S100A4 (ab27957) (Abcam, ITK Diagnostics BV, The Netherlands); AKT (9272), phospho-AKT (P-AKT, 4060), β-Actin (4970) (Cell Signaling Technology, Bioke, The Netherlands); E-cadherin (610181), vimentin (550513) (BD Biosciences, San Jose, CA, USA); and fibronectin (sc18825) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4 Migration and invasion assay

Cell migration and invasion assays were performed using 24-well Transwell cell culture chamber inserts with 8 μm pores (Corning Costar Corp., Cambridge, MA, USA) according to the manufacturer’s instructions. For the migration assay, 3 × 10⁵ cells per 200 μL in each well were seeded into Transwell inserts and cultured for 24 hours. The bottom chamber of each well was filled with 600 μL of medium containing 10% FBS. Migrated cells were fixed with 4% paraformaldehyde and subsequently stained with 0.1% crystal violet. The invasion assays were carried out using the same methods as the migration assays except that chambers were coated with 1:3 diluted matrigel (BD Biosciences). The migration or invasion cells were counted in 5 random selected fields at a 100 magnification using a microscope. Cell migration and invasion were expressed as relative cell count relative to the each control group. The value from each control group was arbitrarily set as 1. All experiments were performed in triplicates.

2.5 siRNA transfection

Cells were transfected with S100A4-siRNA or control siRNA (negative control, NC) at a final concentration of 50 nM using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocol. The sequence of siRNA targeting S100A4 was 5’-AAC GAG GAC GTG GTC AAC CCT ATT CT-3’. Cells were then transduced with the lentivirus as previously described.

2.6 Patient recruitment and immunohistochemistry

Immunohistochemistry (IHC) analysis was performed to evaluate the expression of protein in human CRC tissues as described
A total of 112 CRC cases from 1997 to 2003 at the First Affiliated Hospital of Wenzhou Medical University were included. Immunostainings for S100A4, Trx-1 and S100P were performed on 4 μm sections from paraffin-embedded tissue samples. The sections were incubated with primary antibodies against S100A4, Trx-1 or S100P overnight at 4°C. These slides were independently reviewed by 2 trained pathologists and a "H Score" was used. The H score was obtained by adding the scores of percentage positivity and staining intensity. A cut-off score was generated by the Receiver Operating Characteristic (ROC) curve analysis, which determined the overexpression of S100A4, Trx-1 and S100P. The score with the closest distance from the curve to the point (0.0, 1.0) in the ROC curve which maximizes the sensitivity and specificity as the cut-off score. According to ROC curve analysis, the cut-off scores for Trx-1, S100P and S100A4 were 118.3, 123.7 and 103.5, respectively. Recurrence was defined as CRC detected after surgical resection of the patient’s primary CRC. Recurrence may be local, lymph nodes or in a distant area. This study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

### 2.7 Immunofluorescence staining

Cells grown on coverslips were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde at 4°C for 20 minutes. Cells were then permeabilized with 0.3% Triton X-100 and blocked with 10% normal serum. Cells were incubated with primary antibodies to E-cadherin or vimentin and then incubated with Alexa-Fluor 594 goat anti-rabbit antibody IgG (Life Technologies). Nuclei were counterstained with DAPI (4',6-Diamidino-2-Phenylindole; Life Technologies). Samples were examined and photographed using a fluorescence microscope (Olympus, Lake Success, NY, USA).

### 2.8 Statistical analysis

All data are presented as mean ± standard deviation. The correlation between protein expression and clinicopathological features was analysed by the chi-squared test. Comparisons of the continuous variables between 2 groups were performed with an independent samples t-test. One-way ANOVA followed by post hoc Tukey’s or Dunnet’s analysis was used for multiple comparisons. A Kaplan-Meier analysis was used to obtain the survival curve, and the log-rank test was used for comparing survival curves. A P value of less than .05 was considered statistically significant.

### 3 RESULTS

#### 3.1 The expression levels of Trx-1 and S100P influence the EMT phenotype of CRC cells

In this study, the CRC cell lines SW480 and SW620 that are derived from primary (SW480) and metastatic lesions (SW620) of the same patient were chosen as model systems for studying EMT. Protein expression levels were determined by Western-blot assays, and protein levels relative to β-actin protein levels were assessed by densitometric analysis. Figure 1A shows that protein levels of S100P, Trx-1, S100A4, vimentin and fibronectin in the SW620 are higher than that seen in SW480 cells, while the level of epithelial marker E-cadherin is lower in SW620 than in SW480 cells. As SW480 cells exhibited lower expressions of Trx-1 and S100P than SW620 cells do, we overexpressed Trx-1 or

**FIGURE 1** The expression levels of S100P, Trx-1, S100A4 and EMT-associated proteins in SW480 and SW620 cells. A, S100P, Trx-1, S100A4 and EMT-associated proteins (E-cadherin, vimentin and fibronectin) were examined by Western blotting. β-actin was used as the loading control. B, EMT morphological changes induced by S100P or Trx-1. Representative microscopic views of SW480 and SW620 cells were shown. Scale bar, 50 μm
S100P in SW480 cells by lentiviral-mediated gene transfer. Overexpression of S100P or Trx-1 showed an elongated, mesenchymal morphology as compared to the parental SW480 cells (Figure 1B). In contrast, SW620 cells with S100P or Trx-1 knockdown showed a reversed EMT morphology: the cells were more epithelial-like as compared to the control cells (Figure 1B). In addition, ectopic overexpression of Trx-1 or S100P in SW480 cells resulted in down-regulation of E-cadherin, whereas the expressions of the 2 mesenchymal markers vimentin and fibronectin were up-regulated (Figures 2A and B). On the other hand, knockdown of Trx-1 or S100P in SW620 by shRNA resulted in an increased expression of E-cadherin and decreased expressions of vimentin and fibronectin. In addition, overexpression of Trx-1 or S100P up-regulated the levels of S100A4 and P-AKT in SW480 cells, whereas knockdown of Trx-1 or S100P down-regulated the levels of S100A4 and P-AKT in SW620 cells (Figure 2A,B). Moreover, the expression of the mesenchymal marker, vimentin, and the epithelial marker, E-cadherin, were examined by immunofluorescence. Immunofluorescent staining showed that E-cadherin expression decreased while vimentin expression increased after the overexpression of Trx-1 or S100P in SW480 cells (Figure 2C,D). Conversely, knockdown of Trx-1 or S100P in SW620 cells caused an increase in E-cadherin expression and a decrease in vimentin expression (Figure 2E,F).

These results suggested that S100P or Trx-1 could induce EMT in CRC cells.

### 3.2 Inhibition of AKT activity caused a mesenchymal-to-epithelial transition, and decreased S100A4 expression, and migration and invasion abilities in CRC cells

The previous experiments showed that AKT activation and S100A4 expression up-regulation were observed with Trx-1- or S100P-induced EMT in CRC cells (Figure 2). These observations lead to the question of whether AKT activation or S100A4 is involved with EMT process and migration and invasion of CRC cells. Figure 3A shows that blocking AKT activity with MK-2206 in SW620 cells increased the level of E-cadherin and decreased the levels of the vimentin and fibronectin. In particular, inhibition of AKT activity decreased the expression of S100A4 (Figure 3A) and suppressed SW620 cell migration and invasion (Figure 3B). These results indicate that the AKT activation is associated with the onset of EMT in CRC cells, and S100A4 might be a downstream target of AKT signalling.

### 3.3 Silencing S100A4 by siRNA induces mesenchymal-to-epithelial transition and inhibits migration and invasion of SW620 cells

To study the role of S100A4 in the process of EMT as well as migration and invasion in CRC cells, we used siRNA targeting S100A4 to down-regulate S100A4 expression. As Figure 4A shows, silencing S100A4 by siRNA in SW620 cells up-regulated the expression of E-cadherin but down-regulated the expressions of vimentin and fibronectin. However, the AKT and P-AKT protein expressions were not significantly altered (Figure 4A). Transwell assays showed that silencing S100A4 by siRNA inhibited the migration and invasion rate of SW620 cells by up to 41.2 and 70.5%, respectively (Figure 4B).

### 3.4 Clinical and pathological significance of S100A4 expression in CRC

To further explore the role of S100A4 in CRC development and progression, S100A4 expression was visualized using IHC for the 112 CRC samples (Figures 5A-C). We observed that the expression of S100A4 in CRC tissues was significantly higher than that of the matched adjacent normal tissues (Figure 5D, $P < .01$). Significant up-regulation of S100A4 protein expression was also seen in CRC with lymph node metastasis, relative to CRC without lymph node metastasis (Figure 5E, $P < .05$). The elevated expression of S100A4 was significantly correlated with lymph node metastasis ($P = .045$) in CRC (Table 1). Kaplan-Meier survival analysis showed that an overall survival rate was associated with the S100A4 overexpression patients in comparison with patients with a normal level of S100A4 expression (Figure 5F, $P = .042$). In addition, a significant positive correlation between the overexpression of S100P and S100A4 (Table 2, $P < .05$) or between the overexpression of Trx-1 and S100A4 (Table 3, $P < .01$) was observed in our cohort of CRC tissues.

### 3.5 Inhibition of AKT activity or silencing S100A4 reverses Trx-1-mediated EMT, migration and invasion in CRC cells

We further examined whether Trx-1 and S100P promote CRC cell EMT, migration and invasion via up-regulating S100A4 expression and activating AKT. We then silenced S100A4 using siRNA targeting S100A4 or blocked the phosphorylation of AKT with an AKT inhibitor MK-2206 in SW480-Trx-1 and SW480-S100P cells. Following siRNA-S100A4 or MK-2206 treatment, Trx-1-induced EMT was reversed, as evidenced by the increased expression of the epithelial marker, E-cadherin and the decreased expression of the mesenchymal marker, vimentin, in SW480-Trx-1 cells (Figure 6A,B). Moreover, blocking AKT phosphorylation with MK-2206 reversed Trx-1-induced S100A4 expression. In addition, Transwell assays showed that silencing S100A4 or blocking the phosphorylation of AKT significantly impaired Trx-1-induced migration and invasion of SW480 cells (Figure 6C,D).

### 3.6 Inhibition of AKT activity or silencing S100A4 reverses S100P-mediated EMT, migration and invasion in CRC cells

After siRNA-S100A4 or MK-2206 treatment, S100P-induced EMT was also reversed in SW480-S100P cells (Figure 7A,B). Moreover,
FIGURE 2  Effects of Trx-1 and S100P on epithelial–mesenchymal transition of colorectal carcinoma cells. (A) Western blotting revealed that overexpression of Trx-1 resulted in a decreased expression of epithelial marker E-cadherin and increased expressions of mesenchymal markers (vimentin and fibronectin), S100A4 and phosphorylated AKT (P-AKT) in SW480 cells, whereas knockdown of Trx-1 by shRNA resulted in an increased expression of E-cadherin and decreased expressions of vimentin, fibronectin, S100A4 and P-AKT in SW620 cells. (B) Western blotting showed that overexpression of S100P resulted in a decreased expression of E-cadherin and increased expressions of vimentin, fibronectin, S100A4 and P-AKT in SW480 cells, whereas knockdown of S100P by shRNA resulted in an increased expression of E-cadherin and decreased expressions of vimentin, fibronectin, S100A4 and P-AKT in SW620 cells. \( \beta \)-Actin was used as the loading control. (C) Immunofluorescence staining of Trx-1 overexpression down-regulated E-cadherin expression while up-regulating vimentin expression in SW480 cells. (D) Knockdown of Trx-1 by shRNA up-regulated E-cadherin expression and down-regulated vimentin expression in SW620 cells. (E) S100P overexpression caused a decrease in E-cadherin expression and an increase in the expression of vimentin in SW480 cells. (F) Knockdown of S100P by shRNA increased the expression of E-cadherin and decreased vimentin expression in SW620 cells. Nuclei were counterstained using DAPI (blue). Merged figures show the co-localization of E-cadherin or vimentin (red) with DAPI. Scale bar = 20 \( \mu \)m.
inhibition of AKT activity reversed S100P-induced S100A4 expression and migration and invasion of SW620 cells. Transwell assays showed that silencing S100A4 or inhibition of AKT activity significantly suppressed the S100P-induced migration and invasion of SW620 cells (Figure 7C,D).

3.7 | Ectopic expression of S100P partially reverses the inhibition of EMT, migration and invasion caused by Trx-1 knockdown

When S100P was introduced into Trx-1-silenced SW620 cells, the inhibited EMT of CRC cells and S100A4 expression was substantially re-enhanced (Figure 8A,B). Also, ectopic expression of S100P partially reversed Trx-1 knockdown-induced inhibition of migration and invasion in SW620 cells (Figure 8C,D).

4 | DISCUSSION

Epithelial-to-mesenchymal transition, an intricate process by which epithelial cells lose epithelial characteristics and gain a migratory mesenchymal phenotype, plays a key role in cancer invasion and metastasis. EMT is characterized by a decreased expression of epithelial markers, for example E-cadherin, and increased expressions of mesenchymal markers, for example vimentin and fibronectin.
Preventing or reversing EMT processes might be a promising therapeutic strategy for metastatic CRC treatment.3 Trx-1 is an essential component of the Trx system which plays crucial roles in the

**FIGURE 5** Expression of S100A4 in colorectal carcinoma tissues and its prognostic significance in colorectal cancer patients. (A) Strong immunopositive staining of cancerous tissue, H score = 200; (B) moderate immunopositive staining of cancerous tissue, H score = 120; (C) negative staining of cancerous tissue, H score = 0; D, S100A4 expression of colorectal carcinoma tissue was significantly higher than that of the matched adjacent normal tissues as indicated by IHC. **P < .01. Scale bar = 20 µm.** E, Significant up-regulation of S100A4 protein expression by IHC was shown in colorectal carcinoma with lymph node metastases, relative to colorectal carcinoma without lymph node metastasis. *P < .05. F, Kaplan-Meier survival analysis according to CRC patients with S100A4 overexpression (log-rank test, *P = .042)

| TABLE 1 | S100A4 expression and clinicopathological parameters in colorectal cancer specimens |
|---------|------------------------------------------------------------------------------------------------------------------|
|         | S100A4 protein | All cases | Normal expression | Overexpression | P value |
| Sex     |               |           |                  |              |        |
| Male    | 49            | 33        | 16               | .551         |
| Female  | 63            | 39        | 24               |              |
| Histologic grade (WHO) | | | | |
| Low     | 96            | 60        | 36               | .334         |
| High    | 16            | 12        | 4                |              |
| Clinical stage | | | | |
| I-II    | 73            | 49        | 24               | .391         |
| III-IV  | 39            | 23        | 16               |              |
| pN status | | | | |
| N0      | 59            | 43        | 16               | .045*        |
| N1-N2   | 53            | 29        | 24               |              |
| Recurrence | | | | |
| No      | 58            | 40        | 18               | .284         |
| Yes     | 54            | 32        | 22               |              |

pN, pathological node.
Statistical analyses were performed by χ² test.
*P < .05.

| TABLE 2 | Correlation between S100P and S100A4 expressions in colorectal cancer tissues |
|---------|--------------------------------------------------------------------------------|
|         | S100A4 | Cases | Normal expression | Overexpression | P value |
|          |        |       |                  |              |        |
| S100P    | Normal expression | 57    | 41 (70.7%)        | 16 (48.5%)   | <.05   |
|          | Overexpression  | 34    | 17 (29.3%)        | 17 (51.5%)   |        |

| TABLE 3 | Correlation between Trx-1 and S100A4 expressions in colorectal cancer tissues |
|---------|--------------------------------------------------------------------------------|
|         | S100A4 | Cases | Normal expression | Overexpression | P value |
|          |        |       |                  |              |        |
| Trx-1    | Normal expression | 33    | 28 (40.0%)        | 5 (12.5%)    | <.01   |
|          | Overexpression  | 77    | 42 (60.0%)        | 35 (87.5%)   |        |

Preventing or reversing EMT processes might be a promising therapeutic strategy for metastatic CRC treatment.3 Trx-1 is an essential component of the Trx system which plays crucial roles in the
regulation of cellular redox signalling pathways. Trx1 is mainly located in cytosol, and it is involved in maintaining redox homeostasis and interacts with several proteins related to oxidative stress, cell proliferation and apoptosis, such as thioredoxin-interacting protein (TXNIP), apoptosis signal-regulated kinase (ASK-1), SENP1 and PTEN thereby regulating their functions. In response to oxidative stress, Trx1 is translocated into the nucleus and activates several transcription factors, such as NF-κB, p53, AP-1, glucocorticoid receptor and SP1, by promoting their DNA binding activity.

Our previous report showed a positive feedback mechanism between Trx-1 and S100P, which promotes CRC invasion and metastasis. Ectopic expression of Trx-1 promotes CRC cell migration and invasions in vitro and liver metastasis in vivo. Conversely, Trx-1 knockdown inhibits CRC cell migration and invasion abilities in vitro and liver metastasis in vivo. Similar results were also obtained in CRC cells with S100P overexpression or knockdown. Trx-1 promotes CRC invasion and metastasis through crosstalk with S100P. These results suggested that interplay between Trx-1 and S100P plays important roles in CRC metastasis. It has been reported that S100P acts as a ligand for the receptor for advanced glycation endproducts (RAGE) and activates ERK1/2, NF-kB and the JAK/STAT pathway. The S100P/RAGE signalling pathway contributes to cancer progression by triggering the oncogenic miR-155 and miR-214 in colon cancer. S100P has also been reported to interact with integrin α7, myosin II or EZRIN, leading to increase cell migration. Here, we report yet another novel mechanism wherein Trx-1 and S100P promote CRC EMT as well as migration and invasion by up-regulating S100A4 through the activation of AKT.

SW480 and SW620 colon carcinoma cell lines are derived from primary and secondary tumours resected from the same patient. SW480 cells are mainly epithelial, whereas SW620 cells are mesenchymal. The increase in the expression levels of Trx-1, S100P and S100A4 were observed in SW620 cells compared with SW480 cells. Therefore, we overexpressed Trx-1 or S100P in SW480 cells and silenced Trx-1 or S100P in SW620 cells. Our results showed that down-regulation of Trx-1 or S100P in SW620 cells inhibited EMT, whereas overexpression of Trx-1 or S100P in SW480 cells promoted EMT. S100P was reported to promote EMT and the invasion and metastasis of CRC by activating RAGE/ERK signalling. Moreover, Jiang et al. demonstrated that Trx-1 is a critical mediator of TGF-beta induced EMT in salivary adenoid cystic carcinoma.
S100P could promote cancer invasion and metastasis by promoting the EMT in CRC\textsuperscript{21,50} and lung cancer.\textsuperscript{21}

In this study, we saw that the down-regulation of S100P or Trx-1 inhibited S100A4 expression, whereas overexpression of S100P or Trx-1 promoted S100A4 expression. Down-regulation of S100A4 expression by siRNA reversed S100P- and Trx-1-induced EMT, migration and invasion of CRC cells. S100A4 is also a ubiquitous small, calcium-binding protein that is directly involved in tumour metastasis via increased cell motility and invasion.\textsuperscript{51} S100A4 is regarded as an important regulator of metastasis and EMT.\textsuperscript{51-53} Overexpression of S100A4 promotes metastasis in several experimental animal models, in contrast, down-regulation of S100A4 expression reduces the metastatic capacity of cancer cells.\textsuperscript{24,51,54-58} In CRC, S100A4 is overexpressed and its expression is correlated with patient outcome.\textsuperscript{59,60} Consistent with that, our results showed that the expression of S100A4 was higher in human CRC tissues compared with their normal counterpart tissues, and higher expression of S100A4 was significantly correlated with lymph node metastasis and poor survival. Furthermore, the overexpression of S100A4 protein was positively correlated with S100P or Trx-1 protein overexpression in our cohort of CRC tissues. Dahlmann M et al. have reported that stable knockdown of S100A4 in CRC HCT116 cells by transfection with S100A4-shRNA expression plasmids resulted in reduced the liver metastatic potential of CRC cells when intrasplenically transplanted in mice.\textsuperscript{61} Moreover, hydrodynamics-based systemic treatment with plasmids DNA for S100A4-specific shRNA, via repeated tail vein injection, inhibited the formation of liver metastases.\textsuperscript{61} These results suggest that S100A4 may be a critical downstream target of S100P and Trx-1 and is responsible for the S100P- or Trx-1-induced EMT and invasiveness of CRC cells.

Next, we tried to elucidate the underlying mechanism by which Trx-1 and S100P regulates S100A4. Our results further showed that S100P or Trx-1 could regulate AKT phosphorylation, and blocking AKT signalling with MK-2206 down-regulated S100A4 expression and inhibited EMT as well as migration and invasion of SW620 cells. Liu S et al. reported that HBXIP induces S100A4 expression through PI3K/AKT signalling pathway in breast cancer cells.\textsuperscript{52} S100P could also activate AKT signalling in lung cancer cells.\textsuperscript{21} Indeed, we observed that blocking AKT signalling partially reversed S100P- or Trx-1-induced S100A4 expression, EMT, and migration and invasion of CRC cells. Therefore, we speculate that AKT signalling may be involved in S100A4 up-regulation and EMT mediated by S100P or Trx-1. Further, we demonstrated that overexpression of S100P could reverse the Trx-
1 knockdown-induced inhibition of S100A4 expression, EMT, and migration and invasion of CRC cells.

In summary, our findings demonstrate for the first time a novel molecular mechanism involving Trx-1 and S100P in the regulation of the invasion and metastasis capabilities of CRC cells. EMT has been shown to play a critical role in tumour metastasis, and inhibition of EMT is considered to be a promising approach to prevent metastasis.63 The identification of functionally relevant regulators of EMT may offer new appropriate targets and therapeutic opportunities for controlling cancer progression and metastasis.53 Our results demonstrate that the interplay between S100P and Trx-1 promotes CRC cell EMT, migration and invasion by up-regulating S100A4 through AKT activation (Figure 8E), suggesting that the inhibition of AKT or S100A4 may serve as important therapeutic strategies in suppressing the EMT in metastatic CRC with S100P or Trx-1 overexpression.

**FIGURE 8** S100P partially reverses the inhibition of EMT, migration and invasion caused by Trx-1 knockdown. A representative Western blot (A) and the summarized data (B) showed that overexpression of S100P partially reversed Trx-1 silencing-induced mesenchymal–epithelial transition in SW620 cells. SW620-shTrx-1 cells were transfected with Lenti-S100P; the expression levels of E-cadherin, S100A4 and vimentin were assessed by Western blotting. β-Actin was used as the loading control. (C, D) Overexpression of S100P partly reversed Trx-1 silencing-induced inhibition of migration and invasion in SW620 cells. (E) Model for crosstalk between S100P and Trx-1 that ultimately promotes colorectal cancer cell EMT, migration and invasion by up-regulating S100A4 through AKT activation.
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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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