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Leptin-mediated regulation of ICAM-1 is Rho/ROCK dependent and enhances gastric cancer cell migration

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Background: Our previous study indicates that leptin enhances gastric cancer (GC) invasion. However, the exact effect of leptin on GC metastasis and its underlying mechanism remain unclear. Intercellular adhesion molecule-1 (ICAM-1), a major molecule in stabilising cell–cell and cell–extracellular matrix interactions, is overexpressed and has crucial roles in tumour metastasis.

Methods: Here, we investigated leptin and ICAM-1 expression in GC tissues. Furthermore, we characterised the influence of leptin on ICAM-1 expression in GC cells and elucidated the underlying mechanism.

Results: Leptin and ICAM-1 were overexpressed in GC tissues, and a strong positive correlation was observed. They were also related with clinical stage or lymph node metastasis. Furthermore, leptin induced GC cell (AGS and MKN-45) migration by upregulating ICAM-1, and knockdown of ICAM-1 by small interference RNA (siRNA) blocked this process. Cell surface ICAM-1, as well as soluble ICAM-1 (sICAM-1), was also enhanced by leptin. Moreover, leptin increased ICAM-1 expression through Rho/ROCK pathway, which was attenuated by pharmacological inhibition of Rho (C3 transferase) or its downstream effector kinase Rho-associated protein kinase (ROCK) (Y-27632).

Conclusions: Our findings indicate that leptin enhances GC cell migration by increasing ICAM-1 through Rho/ROCK pathway, which might provide new insight into the significance of leptin in GC.

Gastric cancer (GC) ranks as the second leading cause of cancer-related death in the world (Parkin *et al*, 2005). The high aggressiveness and rapid metastasis of GC contribute to its high mortality rate (Wang and Chen, 2011). The metastasis involves multiple factors, such as obesity, an important risk factor in GC (Calle *et al*, 2003; Howard *et al*, 2010). Adipocytes provide fatty acids for rapid tumour growth, and the dysfunction of lipid metabolism can lead to the pathogenesis of human GC (Song *et al*, 2012). Leptin is an adipokine of the obesity (*ob*) gene, and has been known to have roles in the homeostasis of body weight by

regulating food intake and energy metabolism (Chan *et al*, 2006). Recent studies have revealed that leptin is implicated in the tumorigenesis of cancer, such as ovarian cancer, endometrial cancer, GC, and so on (Zhao *et al*, 2007; Wu *et al*, 2012; Xu *et al*, 2013). Leptin exerts its function through leptin receptor, and the use of leptin receptor antagonists has been recognised as a potential therapeutic approach for cancer (Otvos *et al*, 2011). Our previous study indicates that leptin enhances GC cell invasion by upregulating the expression of membrane type 1-matrix metalloproteinase (MT1-MMP) (Dong *et al*, 2013). Converging studies

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reveal that leptin expression is elevated in GC patients, and is also significantly correlated with GC metastasis (Shida *et al*, 2005; Ishikawa *et al*, 2006; Zhao *et al*, 2007). However, the exact effect of leptin on GC metastasis and its underlying mechanism remain poorly understood.

Recent findings of upregulation of intercellular adhesion molecule-1 (ICAM-1, also known as CD54) by leptin in allergic inflammation may shed light on how leptin modulates cell migration (Wong *et al*, 2007). This suggests a link between leptin and ICAM-1. ICAM-1 is a transmembrane glycoprotein of the immunoglobulin superfamily. It binds to two major ligands, lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) and macrophage-1 antigen (Mac-1, CD11b/CD18). LFA-1 and Mac-1 expressed on NK cell and macrophages share a common β chain (Diamond *et al*, 1990; Zimmerman and Blanco, 2008). ICAM-1 has an essential role in cell–cell and cell–extracellular matrix interaction. Binding to LFA-1 and Mac-1, ICAM-1 can facilitate the metastasis of cancer cells by escaping the recognition and attack of immunocytes (Makrilia *et al*, 2009). Previous studies show that ICAM-1 is associated with more aggressive lesions in prostate cancer (Chen *et al*, 2012), lung cancer (Grothey *et al*, 1998), and GC (Maruo *et al*, 2002). Laboratory models have demonstrated that the upregulation of ICAM-1 can promote tumour cell invasion and metastasis. In addition, overexpressed ICAM-1 may be released from the local cancer cell nests and enter the serum as sICAM-1 (Essick *et al*, 2008). Elevated serum ICAM-1 level is correlated significantly with serum CA19-9 and CEA in GC (Polychronidis *et al*, 2003); consistently, suppression of ICAM-1 expression is related to attenuated tumour progression and metastasis (Kaliora *et al*, 2008; Chen *et al*, 2012; Yu *et al*, 2012). Interestingly, both leptin and ICAM-1 are increased after *Helicobacter pylori* infection (Fan *et al*, 1995; Azuma *et al*, 2001). To our knowledge, there is no published data about the effect of leptin on ICAM-1 and its role in GC metastasis.

The expression of ICAM-1 may be regulated by a variety of signalling pathways, such as Rho (the small G protein Ras homologue gene family) and its immediate downstream effector Rho-associated coiled-coil-forming protein kinase (ROCK) (Jung *et al*, 2012). ROCK is ubiquitously expressed serine–threonine protein kinases that are involved in diverse cellular functions, including actin cytoskeleton organisation, cell adhesion, and motility (Burrige and Wennerberg, 2004). Hyperactivation of the Rho/ROCK pathway is known to be linked with more aggressive tumour properties such as metastasis (Sahai and Marshall, 2002). Rho/ROCK may be regulated by some factors in tumours. For example, lysophosphatidic acid triggers focal adhesion assembly through Rho/ROCK pathway in human ovarian cancer cells (Sawada *et al*, 2002). A previous study reveals that leptin may induce Rho/ROCK pathway (Schram *et al*, 2011). On the basis of these reports, we hypothesise that Rho/ROCK pathway may be involved in the leptin-mediated expression of ICAM-1. This study has demonstrated that leptin promotes the migration of GC cells by upregulating ICAM-1 expression via the involvement of Rho/ROCK pathway. Targeting ICAM-1 and its regulation pathway might provide preliminary experimental clues for the development of therapies against the metastasis of GC.

MATERIALS AND METHODS

Patients and specimens. Archived paraffin-embedded GC tissues and matched adjacent normal gastric tissues were collected from 84 patients who underwent surgery for primary gastric carcinoma in the Department of General Surgery, Qilu Hospital, Shandong University (Shandong, China). The clinical parameters and pathologic classifications are presented and summarised in

Table 1. There were 34 males (age, 31–77 years; median, 57 years) and 50 females (age, 32–75 years; median, 53 years); all patients had not received medical therapy before surgery. The diagnosis of each case was confirmed by two pathologists. The research was approved by the Ethics Committee of Qilu Hospital and we obtained written informed consent from all patients.

Reagents and Antibodies. Rabbit anti-human ICAM-1 polyclonal antibody for immunohistochemistry and western blot (WB) and anti- β -actin mAb were obtained from Cell Signaling Technology (Boston, MA, USA). Rabbit anti-human leptin polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Recombinant human leptin (rhleptin), mouse anti-human ICAM-1 mAb for flow cytometry, sICAM-1 commercial ELISA (enzyme-linked immunosorbent assay) kit were obtained from R&D Systems (Minneapolis, MN, USA). Rabbit anti-human phospho-ROCK polyclonal antibody was purchased from AnaSpec Inc. (Fremont, CA, USA). Rat anti-human ROCK was obtained from BD Biosciences (San Jose, CA, USA). PE-goat anti-mouse secondary mAb was purchased from Invitrogen (Carlsbad, CA, USA). All isotype controls were purchased from corresponding vendors.

GC cell lines and cell culture. The GC cell lines AGS and MKN-45 from the Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and the Riken Cell Bank (Tsukuba, Japan) were grown in DMEM/F12 medium containing 10% fetal calf serum (FCS) (Hyclone-Pierce, Rockford, IL, USA). Cells were seeded upon 80% confluence. In the following experiments, cells were serum-deprived for 12 h prior to the administration of exogenous stimulation, and they were then incubated with serum-free DMEM/F12 medium except for special indications.

Immunohistochemical analysis. Paraffin-embedded tissues were deparaffinised and rehydrated. After antigen retrieval, all tissues were exposed to respective primary antibodies (leptin, 1:400; ICAM-1, 1:1000) overnight at 4 °C, and then incubated with biotin-conjugated secondary antibody for 30 min at 37 °C, followed by peroxidase-conjugated avidin. Staining was observed with DAB (3,3'-diaminobenzidine tetrahydrochloride), and evaluated by two pathologists. An immunohistochemical score was generated by incorporating both the percentage of positive tumour cells and the intensity of staining as previously described (Yang *et al*, 2011).

Transient transfection in AGS cells. The vector pcDNA 3.1/myc-His A was kindly provided by Dr Yinghong Xiao (Raul Andino lab, Department of Microbiology and Immunology, University of California, San Francisco, CA, USA). The human full-length ICAM-1 cDNA (Genebank: NM_000201) was amplified and subcloned between the EcoR I and BamH I sites of the vector to construct eukaryotic expression vector pcDNA3.1-ICAM-1 in Central Research Laboratory (Bethune Second Hospital of Jilin University, Changchun, China). About 2×10^5 cells per well were seeded onto six-well plates, and then transfected with pcDNA3.1-ICAM-1 or an empty vector (MOCK) as control, using Lipofectamine2000 following the manufacturer's protocol. After 24 h, the transfection efficacy was evaluated.

Cell migration assay. The migration assay was performed using transwell inserts (8 μ m pores, BD Biosciences, Bedford, MA, USA) in 24-well dishes as previously described (Chen *et al*, 2012). Two hundred microliters of serum-free medium containing 1×10^4 cells were seeded in the upper chamber. The lower chamber was filled with 600 μ l medium containing 10% FCS. Cells were incubated for 1 h for adherence, and leptin was then added in the upper chamber for another 24 h at 37 °C in 5% CO₂. After 24 h, the non-migrated cells were gently scraped off by cotton swab. The migrated cells were fixed by 10% formalin for 5 min, stained with eosin, and counted. Three fields of each well were photographed, and the cell

Table 1. Relationship between leptin, ICAM-1 expression, and clinicopathological variables.

Parameters	No. of patients	Leptin			ICAM-1		
		-/+	++/+++	P-value	-/+	++/+++	P-value
Gender							
Male	34	13	21	0.541	12	22	0.764
Female	50	23	27		18	32	
Age							
> 50 years	52	24	28	0.135	22	30	0.687
≤ 50 years	32	12	20		8	24	
BMI							
	84	36	48	0.541	30	54	0.554
Tumour size							
≤ 5 cm	41	17	24	0.212	16	25	0.147
> 5 cm	43	19	24		14	29	
Differentiation							
Well	25	5	20	0.395	8	17	0.786
Moderate	28	18	10		11	17	
Poor	31	13	18		11	20	
Borrmann-type							
I	21	12	9	0.143	8	13	0.278
II	19	9	10		4	15	
III	28	7	21		13	15	
IV	16	8	8		5	11	
Invasion depth							
T1	9	2	7	0.589	3	6	0.232
T2	15	5	10		8	7	
T3	26	16	10		10	16	
T4	34	13	21		9	25	
Lymph nodes metastasis							
N0	11	4	7	0.021*	7	4	0.001**
N1	21	10	11		13	8	
N2	23	11	12		7	16	
N3	29	11	18		3	26	
Invaded adjacent organs							
Yes	26	11	15	0.243	8	18	0.089
No	58	25	33		22	36	
Clinical stage							
I	14	9	5	0.011**	7	7	0.028*
II	16	8	8		5	11	
III	21	11	10		8	13	
IV	33	8	25		10	23	

*P<0.05; **P<0.01.

numbers were determined by Kodak MI software. Each experiment was performed in double and repeated three times. The number of migrating cells in each experiment was corrected for proliferation effects using a cell viability assay (corrected migrating cell number = counted migrating cell number/percentage of viable cells).

RT-PCR and real-time RT-PCR. Total RNA was extracted from human GC cells using TRIzol reagent (Invitrogen), and 1 µg of

total RNA was reverse transcribed using M-MuLV reverse transcriptase (Fermentas, St Leon-Rot, Germany) for 60 min at 42 °C in the presence of the oligo-dT primer. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described (Xu *et al.*, 2012). The products were separated on 1.8% agarose gel and visualised by ethidium bromide. For real-time RT-PCR, cDNA was amplified in triplicate with the LightCycler FastStart DNA Master SYBR Green I Reagent

Kit (Roche Molecular Biochemicals, Mannheim, Germany) in the LightCycler 2.0 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instruction. The amplification condition was at 95 °C for 15 min, followed by 40 cycles (15 s at 95 °C; 1 min at 60 °C). Melting curve analysis was used to confirm amplification specificity. The quantification data were analysed with LightCycler analysis software version 4.0 (Roche Applied Science). The average C_t of triplicate wells was calculated as the amount of gene product present in the sample. The relative gene expression level was determined by the ratio between C_t value for ICAM-1 and β -actin. The primer sequences used in this study were as follows: ICAM-1 primer for real-time RT-PCR: sense 5'-GCCACTTCTCTGTAAAGTCTGTGGG-3', anti-sense 5'-CTACCGGCCCTGGGACG-3'. ICAM-1 primer for RT-PCR: sense 5'-CTGCTGGGAATTTTCTGGCCAA-3', anti-sense 5'-CTATGGCAACGACTCCTTCTCG-3'. β -actin: sense 5'-AGCGAGCATCCCCAAAGTT-3', anti-sense 5'-GGGCACGAAGGCTCATATT-3'.

WB analysis. The cultured cells were lysed on ice as described previously (Xu *et al*, 2012). Equal amounts of proteins from each sample were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes were incubated with respective primary antibodies (ICAM-1 (1:500), ROCK (1:1000), P-ROCK (1:1000), and β -actin (1:2000)) overnight at 4 °C. The specific horseradish peroxidase-conjugated goat anti-rabbit or goat anti-rat secondary antibody was used to blot the target proteins, and the immunoreactivity of the target proteins was assessed using an enhanced chemiluminescence detection system.

Flow cytometric analysis. Cells were collected, washed three times with PBS containing 0.1% BSA. Cells were incubated with anti-ICAM-1 mAb for 30 min at room temperature and then incubated with PE-goat anti-mouse secondary mAb for 30 min. After another three washes, cells were suspended in PBS containing 0.1% BSA and then immediately analysed by FACSCalibur (BD Biosciences). Data were analysed by the software WinMDI 2.8.

Enzyme-linked immunosorbent assay. GC cells (AGS, MKN-45) (2×10^5 cells per well) were seeded onto six-well plate. Cells were serum-deprived for 12 h when confluence attained 80%. Then the medium was removed, and cells were treated in the absence (control) or presence of leptin (100 ng ml^{-1}) after 1 h pretreatment with C3 transferase ($0.25 \mu\text{g ml}^{-1}$) or Y-27632 ($3.3 \mu\text{M}$). The inhibitors were present for the duration of leptin treatment. After 24 h at 37 °C in 5% CO_2 , cell culture supernatants were collected and centrifuged at $300 \times g$ for 5 min. The levels of sICAM-1 in the cell-free supernatants were detected using a commercial ELISA kit according to the manufacturer's instruction.

RNA interference. Small interference RNA constructs targeting ICAM-1 and stable negative control (NC) were designed and purchased from GenePharma Co., Ltd (Shanghai, China). For transient silencing, $2 \times 10^5 \text{ ml}^{-1}$ cells were seeded onto six-well plates, and transfected with ICAM-1-siRNA (80 nmol l^{-1}) using Lipofectamine RNAiMAX reagent (Invitrogen), following the manufacturer's protocol. The interference efficiency was evaluated via WB analysis. The sequence for ICAM-1-siRNA was: sense strand: 5'-GCCUCAGCACGUACCUCUATT-3', antisense strand: 5'-UAGAGGUACG UGCUGAGGCTT-3'. The sequence for control siRNA was: sense strand: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense strand: 5'-ACGUGACACGUUCGGAG AATT-3'.

RhoA GTPase activity assay. RhoA GTPase activity was detected using the G-LISA RhoA activation assay kit (Cytoskeleton Inc., Denver, CO, USA) according to the manufacturer's instructions. Briefly, cells were lysed, and equal amount of protein from each sample was added to pre-coated Rho-GTP binding plates. After 30 min of shaking at 4 °C, 200 μl of antigen-presenting buffer were

added to each well and incubated for 2 min at room temperature. Following the conjugation of the anti-actin and secondary antibodies, RhoA activity was quantified using the absorbance at 490 nm.

Statistical analysis. Data were primarily presented as the mean \pm s.d. The SPSS software package (version 13.0; SPSS, Chicago, IL, USA) was used for all statistical analysis. The distribution of the samples was determined via Kolmogorov-Smirnov test. Correlations of leptin and ICAM-1 expression with clinicopathological factors were analysed by Kruskal-Wallis test or Mann-Whitney *U*-test, as appropriate. Chi-squared test was applied to analyse the correlation of leptin and ICAM-1, respectively. Other data from experiments were analysed by paired Student's *t*-test or one-way ANOVA wherever appropriate. Tukey *post hoc* comparison or SNK test was performed when statistical significance ($P < 0.05$) was found between observations. Each variable was tested twice, and the experiment was repeated three times.

RESULTS

Expression of leptin and ICAM-1 in primary GC lesions was related with tumour progression. To investigate the correlation of leptin and ICAM-1 in GC and whether these proteins were involved in GC progression, the expression of leptin and ICAM-1 was immunohistochemically detected in 84 carcinoma tissues and matched with adjacent normal tissues. Moderate to strong (+ + / + + +) immunoreactivity for leptin and ICAM-1 was identified in 57.1% (48/84) and 64.2% (54/84) of carcinomas, respectively. In contrast, only moderate immunoreactivity (+ +) for these markers were detected in 44.3% and 17.8% of normal tissues, respectively (Figure 1A and B, Table 1). Immunostains revealed that leptin and ICAM-1 were expressed both in the cytoplasm and on the cell surface, but not in the nucleus (Figure 1A). Moreover, leptin and ICAM-1 were positively correlated with lymph nodes metastasis and clinical stage (Table 1), and the levels of both proteins were significantly correlated with each other (Figure 1C).

Leptin induced the migration of human GC cells. Our previous study showed that leptin could promote GC cell invasion (Dong *et al*, 2013). Some studies indicated that leptin induced migration of several tumours such as endometrial cancer and glioma (Yeh *et al*, 2009). To investigate the effects of leptin on GC cell migration, we first examined the levels of leptin and leptin receptor in two GC cell lines (AGS and MKN-45). RT-PCR and WB analysis revealed that leptin and all leptin receptors were expressed in both cell lines, though the level of leptin was far lower than the physiological level (Dong *et al*, 2013). Our data showed that leptin ($10\text{--}500 \text{ ng ml}^{-1}$) treatment significantly enhanced cell migration, with a maximal response at the dose of 500 ng ml^{-1} ($P < 0.001$ for both cell lines) compared with controls (Figure 2A and B). Moreover, a maximal response to leptin (100 ng ml^{-1}) was observed at 36 h (Figure 2C).

ICAM-1 was involved in leptin-induced GC cell migration. Previous studies reveal that ICAM-1 has an essential role in cancer cell migration (Maruo *et al*, 2002). To investigate whether ICAM-1 is regulated by leptin (100 ng ml^{-1} , a dose that could induce a significant effect), we examined ICAM-1 expression after being treated with leptin. The results of RT-PCR demonstrated that leptin significantly increased ICAM-1 expression in AGS and MKN-45 cells (Figure 3A; 4.06 ± 0.54 -fold for AGS, $P < 0.001$; 2.56 ± 0.33 -fold for MKN-45, $P = 0.005$). The similar trend was also observed in the results of real-time RT-PCR (Figure 3B) and the protein level (Figure 3C; 3.07 ± 0.25 -fold for AGS, $P < 0.001$; 2.9 ± 0.26 -fold for MKN-45, $P = 0.003$). As shown in Figure 3D,

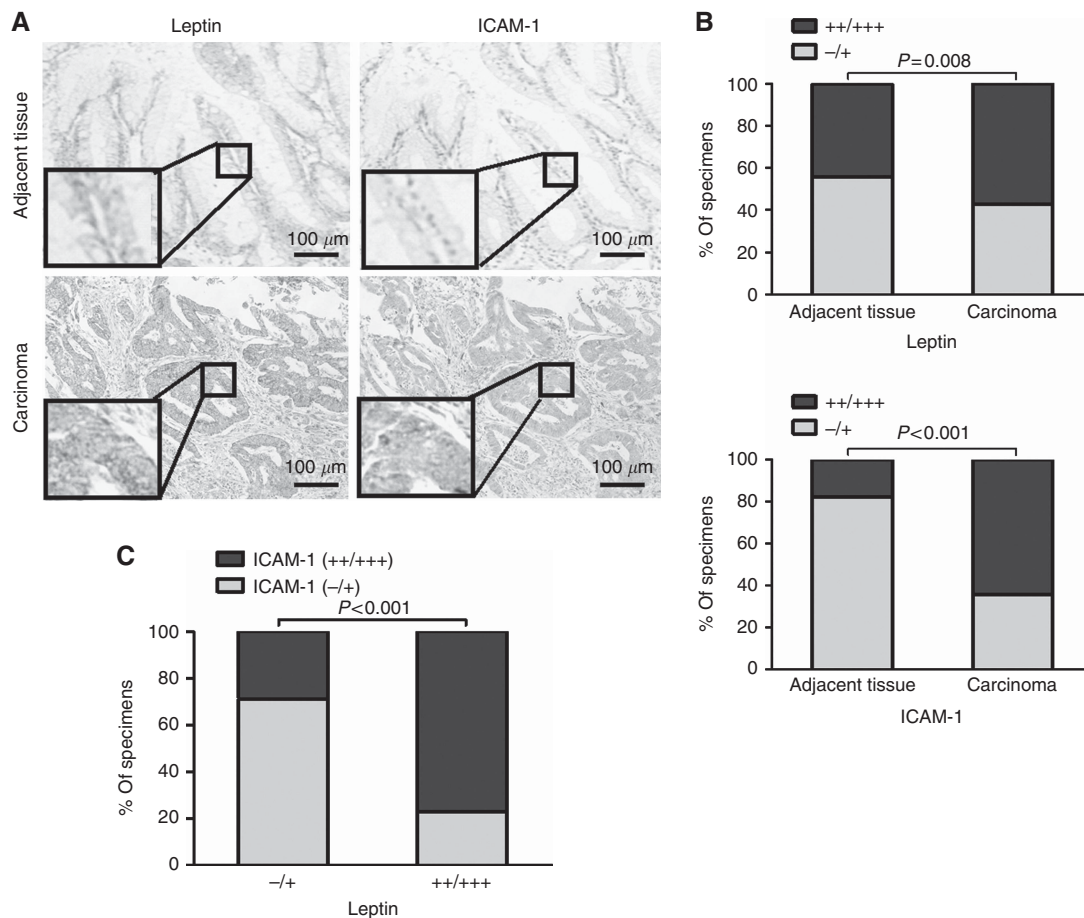


Figure 1. Expression levels of leptin and ICAM-1 in human gastric carcinoma and adjacent tissues. **(A)** Immunohistochemical staining of leptin and ICAM-1 in human gastric carcinoma and adjacent tissues. Two representative specimens are shown. Scale bar, 100 μm . **(B)** The corresponding percentages of specimens with low (-/+) or high (+ +/+ + +) were summarised. **(C)** Percentages of specimens with low (-/+) or high (+ +/+ + +) leptin expression in which ICAM-1 low (-/+) or high (+ +/+ + +) observed.

the cell surface ICAM-1 was also induced by leptin (100 ng ml⁻¹), as evidenced by the increased percentage of positive cells (AGS: from 43.65% \pm 2.42% to 78.96% \pm 2.09%, $P < 0.001$; MKN-45: from 35.35% \pm 1.61% to 59.64% \pm 3.51%, $P < 0.01$). Furthermore, sICAM-1 level in the supernatant of cultured cells (AGS and MKN-45) was also elevated after leptin treatment ($P < 0.05$, Figure 3E).

To test whether leptin-mediated ICAM-1 upregulation was involved in GC cell migration, we first transduced GC cells to overexpress ICAM-1 (pcDNA3.1-ICAM-1). The transfection efficacy was analysed by WB (Figure 4A). Our findings showed that overexpressed ICAM-1 could promote GC cell migration (Figure 4B). Moreover, we performed knockdown of ICAM-1 by siRNA. The knockdown efficiency was evaluated by WB (Figure 4C). As shown in Figure 4D, leptin-induced migration of GC cells was significantly reduced by transient transfection of ICAM-1-siRNA (AGS: 53.9% \pm 3.6%, $P = 0.020$; MKN-45: 42.79% \pm 3.78%, $P = 0.005$). The NC group was transfected with control siRNA.

Leptin increased the expression of ICAM-1 through Rho/ROCK pathway. Rho GTPases (e.g., RhoA, Rac1, and Cdc42) is the binary switch between an active GTP-bound form and an inactive GDP-bound form. A previous study described that Rho/ROCK signalling was related with GC (Matsuoka *et al*, 2011). Therefore, we investigated whether leptin modulated RhoA activity in GC cells. As shown in Figure 5A, the RhoA activity was significantly increased after leptin stimulation at 15 min (1.2 \pm 0.2-fold for AGS; 1.1 \pm 0.1-fold for MKN-45), 30 min (1.6 \pm 0.1-fold for AGS;

1.36 \pm 0.06-fold for MKN-45), and 60 min (1.67 \pm 0.12-fold for AGS; 1.5 \pm 0.1-fold for MKN-45). Furthermore, the phosphorylation of ROCK was also induced by leptin in AGS and MKN-45 cells at 15, 30, and 60 min, though the total ROCK expression remained unaltered over the time course of leptin treatment in these studies (Figure 5B).

To further explore whether leptin regulated the expression of ICAM-1 through the Rho/ROCK pathway, cells were pretreated with the specific pharmacological inhibitor C3 transferase (Rho inhibitor; Cytoskeleton) at 0.25 $\mu\text{g ml}^{-1}$ or Y-27632 (ROCK inhibitor; Calbiochem, Nottingham, UK) at 3.3 μM (Schram *et al*, 2011), followed by leptin treatment. The expression of ICAM-1 was analysed by WB, flow cytometry, and ELISA (for sICAM-1). As shown in Figure 5C, pretreatment with either Rho (C3 transferase) or ROCK (Y-27632) inhibitor prevented the leptin-induced upregulation of ICAM-1 in AGS and MKN-45 cells, and there was no difference between C3 transferase and Y-27632. Although treatment with inhibitors alone resulted in a subtle decrease in the expression of ICAM-1 compared with control, the inhibitors alone did not influence the expression of ICAM-1. A similar trend was also observed with the cell surface ICAM-1 (Figure 5D) and sICAM-1 (Figure 5E).

DISCUSSION

Metastasis, such as lymph node metastasis, is a common cause of death from GC (Wang and Chen, 2011). Leptin has been

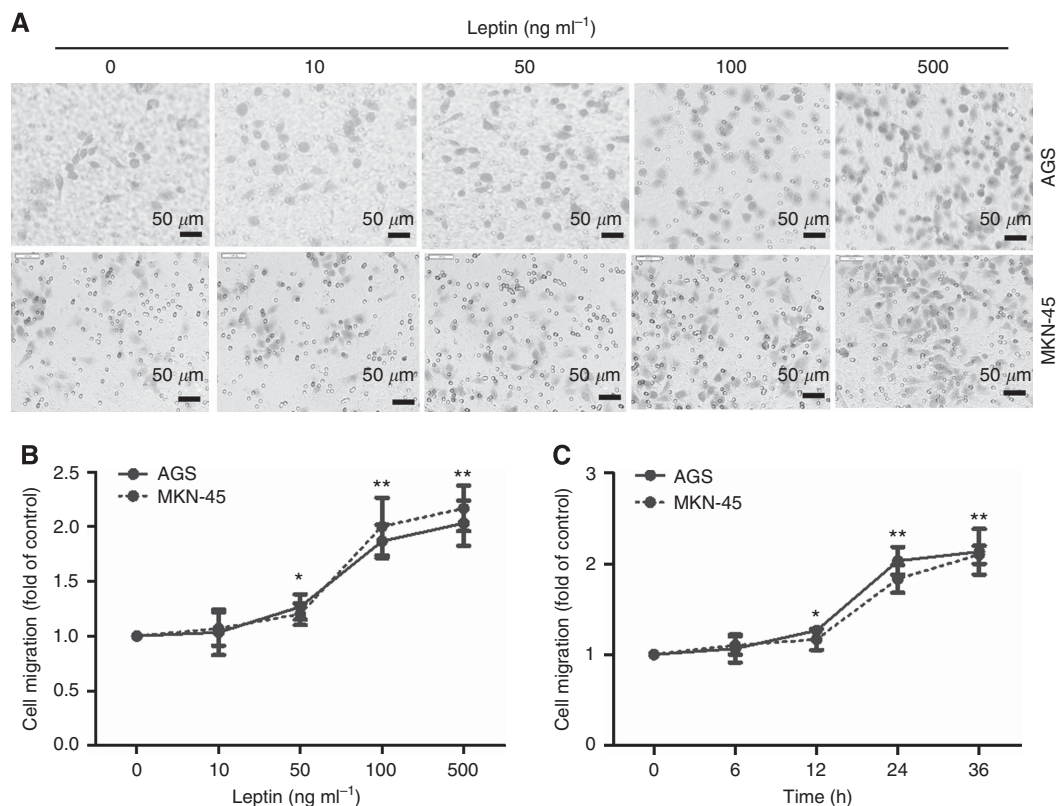


Figure 2. Effect of leptin on the migration of GC cells. **(A)** AGS and MKN-45 cells (1×10^4 cells/well) were treated in the absence (control) or presence of various doses of leptin (10, 50, 100, and 500 ng ml⁻¹) for 24 h. The migration was measured using transwell assay. Scale bar, 50 μ m. The migration numbers were expressed relatively to control **(B)**. **(C)** In a separate experiment, AGS and MKN-45 cells were treated with leptin (100 ng ml⁻¹), and the migration was detected at 0, 6, 12, 24, and 36 h. All data shown were expressed as mean \pm s.d. of three independent experiments. The images were from a representative experiment. * $P < 0.05$, ** $P < 0.01$.

associated with an increased risk of GC and has critical roles in tumour metastasis (Zhao *et al*, 2007). However, little is known about the exact effect of leptin on GC cell migration and its underlying mechanism. ICAM-1 is overexpressed in several cancers and implicated in tumour migration (Maruo *et al*, 2002; Chen *et al*, 2012). In this study, leptin and ICAM-1 levels were strongly correlated with each other in human GC tissues, as well as with clinical stage and lymph node metastasis, suggesting a causal connection between leptin pathway and ICAM-1. Our results also demonstrated that leptin may enhance the migration of GC cells (AGS and MKN-45) by upregulating ICAM-1 expression *in vitro*. Moreover, leptin induces the activation of Rho/ROCK pathway, which contributes to the subsequent upregulation of both surface ICAM-1 and sICAM-1. Taken together, our findings indicate that leptin enhances GC migration by increasing ICAM-1 expression through Rho/ROCK pathway.

Metastasis is a multi-step process during which cancer cells detach from the primary tumour, migrate, adhere, and invade into the blood or lymphatic vessels. Finally cancer cells extravasate out of the vessel and interact with the target tissue. Although multiple factors are involved in this process, adipocyte provides fatty acids for rapid tumour growth, and the dysfunction of lipid metabolism can lead to the pathogenesis of GC (Song *et al*, 2012). Leptin, secreted predominantly from adipocytes, may promote GC cell proliferation, suggesting an essential role of leptin in the progression of GC (Pai *et al*, 2005; Shida *et al*, 2005). This study has indicated that leptin induces the migration of GC cells (AGS and MKN-45) in a dose- and time-dependent manner, which keeps in line with previous studies that leptin induces migration of endometrial cancer and glioma cells (Yeh *et al*, 2009; Wu *et al*, 2012). Further clarifying the mechanisms responsible

for leptin-induced GC metastasis is important for better understanding and control of tumour metastasis in patients with GC.

Our results revealed that leptin and ICAM-1 were overexpressed in GC tissue and correlated with lymph node metastasis, which is consistent with the previous studies (Maruo *et al*, 2002; Zhao *et al*, 2007). Further analysis indicated that leptin was positively correlated with ICAM-1, suggesting a possible link between leptin and ICAM-1 in the progression of GC. This is also supported by the findings that leptin and ICAM-1 are elevated after stimulation with inflammatory cytokines, for example, in patients with *H. pylori* infection (Fan *et al*, 1995; Azuma *et al*, 2001). Previous studies show that ICAM-1 undergoes cleavage at its extracellular stalk domain, and release a soluble form of the protein (sICAM-1), and this process may be mediated by MT1-MMP (MMP14) (Essick *et al*, 2008). In this study, we found that leptin enhanced the expression of ICAM-1, as well as cell surface ICAM-1 and sICAM-1 in GC cells. Furthermore, we also observed an enhanced expression of MT1-MMP by leptin in GC cells (Dong *et al*, 2013). Therefore, we speculate that leptin promotes ICAM-1 expression and subsequently enhances the level of sICAM-1 via upregulating MT1-MMP. Increased ICAM-1 may promote the adhesion of cancer cells, leading to tumour growth and mediating cell-extracellular matrix interaction. When metastasis occurs, cancer cells detach from the primary lesion, penetrate through the tissue basement membrane, and invade blood vessels and lymphatic vessels. On the other hand, as the host killer cells (NK cells, LAK cells, and CTLs) and macrophages express LFA-1 and Mac-1, two major receptors of ICAM-1, partially via which they recognise and attack the tumour cells (Diamond *et al*, 1990), upregulated sICAM-1 may inhibit NK and macrophage activity by competitively binding to LFA-1 and Mac-1 (Koyama, 1994). Thus, it is

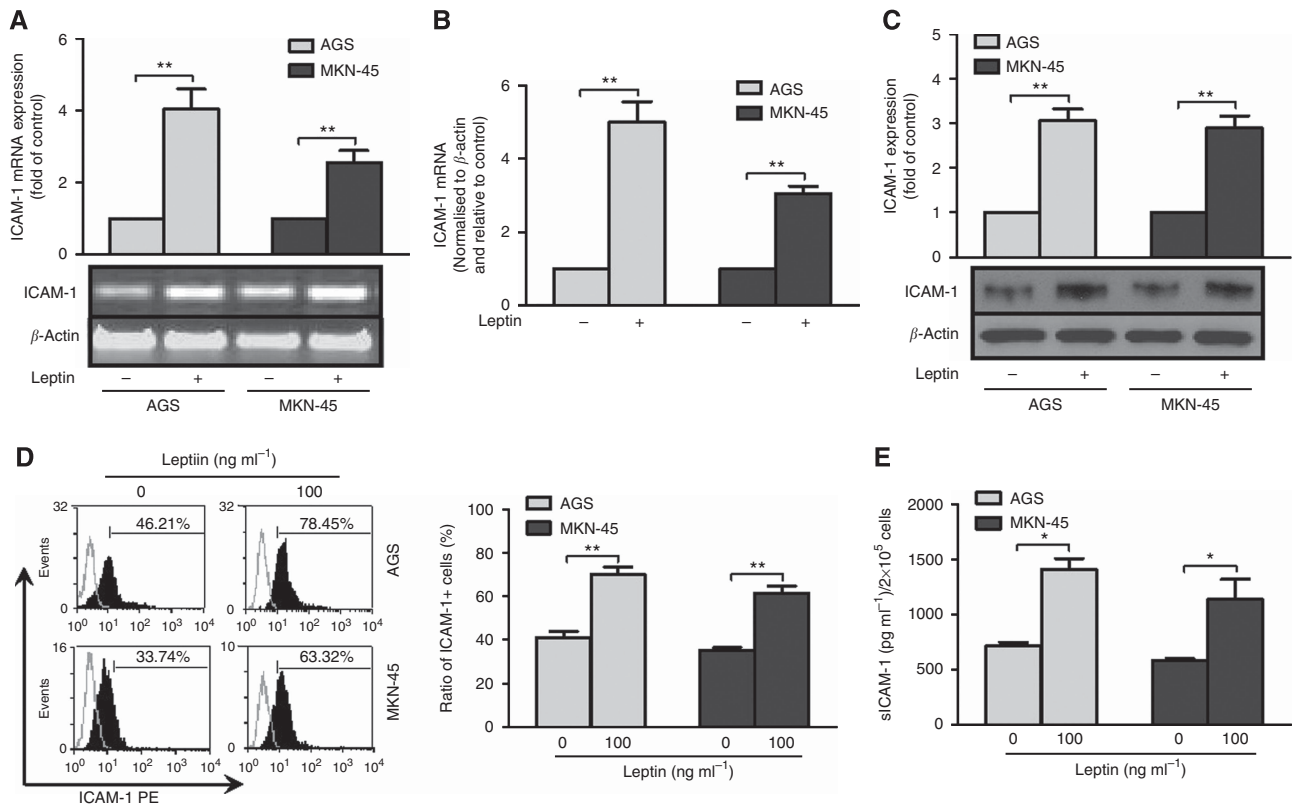


Figure 3. Leptin enhanced the expression of ICAM-1 in GC cells. AGS and MKN-45 cells were treated with leptin (100 ng ml⁻¹) for 24 h. The expression of ICAM-1 was measured by RT-PCR (A), real time RT-PCR (B) and WB (C). The cell surface expression of ICAM-1 was measured by flow cytometry (D), and the level of sICAM-1 in supernatant was detected by ELISA (E). All data shown are expressed as mean \pm s.d. of three independent experiments. **P*<0.05, ***P*<0.01.

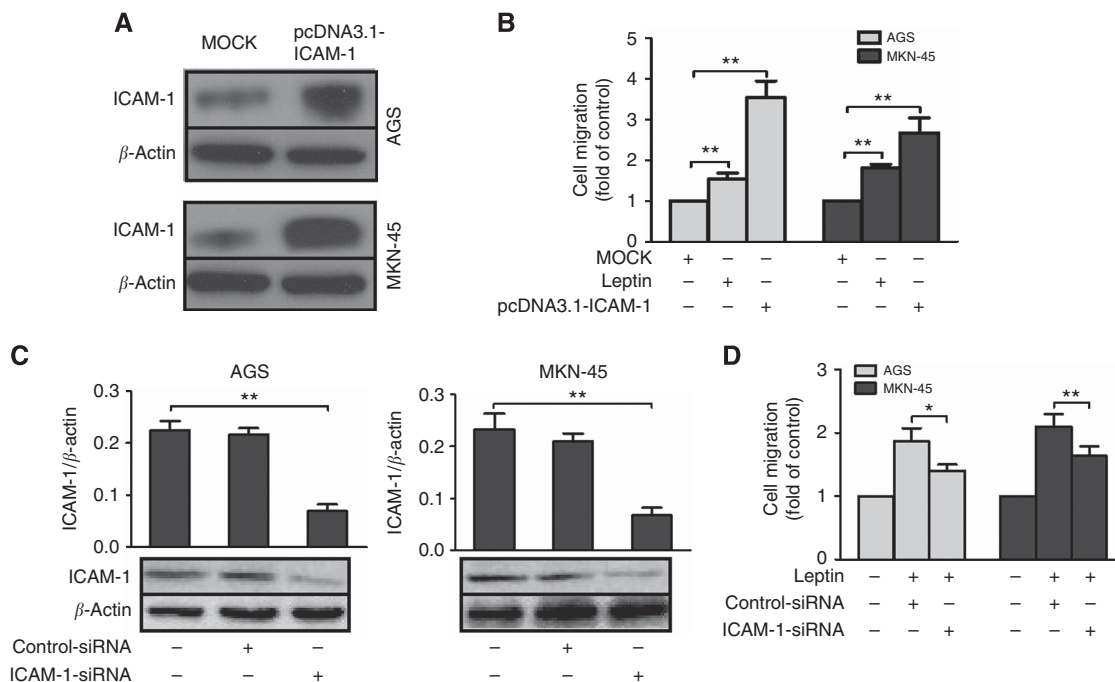


Figure 4. Leptin enhanced GC cell migration via upregulating ICAM-1. The expression level of ICAM-1 in GC cells was measured by WB after transfection with pcDNA3.1-ICAM-1 (A) or ICAM-1-siRNA (B). One representative result out of three independent experiments is shown. Graphs represent quantitation with β -actin as the internal standard. Each bar represents the mean \pm s.d. (*n*=3, ***P*<0.01). In addition, AGS and MKN-45 cells were transfected with pcDNA3.1-ICAM-1 (C) or ICAM-1-siRNA (D) for 24 h and then treated with leptin (100 ng ml⁻¹) for 24 h. The migration was measured by transwell assay. The migration numbers were expressed as relative to control. **P*<0.05, ***P*<0.01.

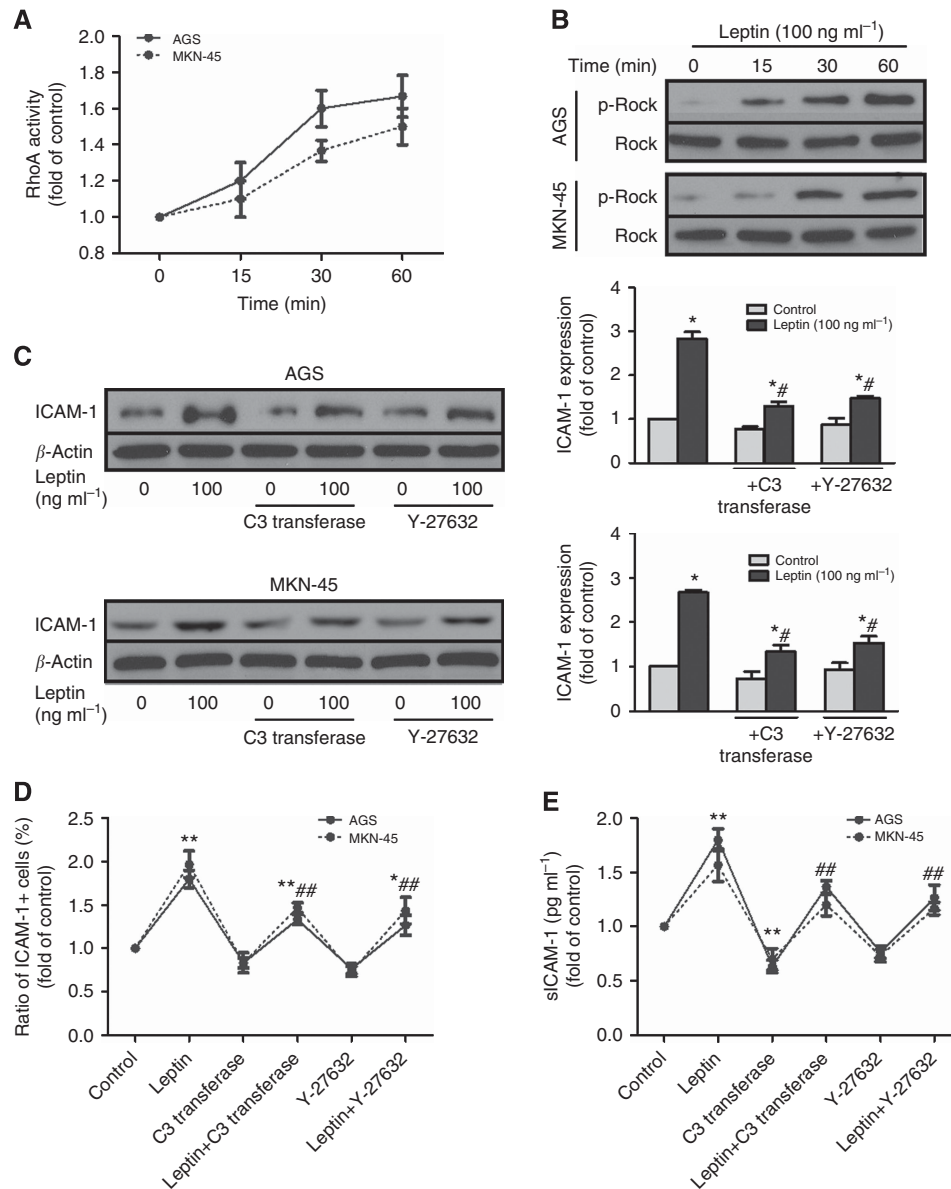


Figure 5. Rho/ROCK pathway was involved in the leptin-induced ICAM-1 expression in GC cells. AGS and MKN-45 cells were treated with leptin (100 ng ml^{-1}) for various times (0, 15, 30, and 60 min). The RhoA activity was detected by ELISA (**A**), and the phosphorylation levels of ROCK in cell lysates were determined by WB (**B**). In a separate experiment, AGS and MKN-45 cells were treated in the absence (control) or presence of leptin (100 ng ml^{-1}) after 1 h pretreatment with C3 transferase ($0.25 \mu\text{g ml}^{-1}$) or Y-27632 ($3.3 \mu\text{M}$). The inhibitors were present for the duration of leptin treatment. After 24 h, the levels of ICAM-1 (**C**), cell surface ICAM-1 (**D**), and sICAM-1 (**E**) in the presence or absence of C3 transferase or Y-27632 were determined. One representative result out of three independent experiments is shown. β -actin was used as an internal control. The results are shown as mean \pm s.d. * $P < 0.05$ vs control, ** $P < 0.01$ vs control, # $P < 0.05$ vs control, ## $P < 0.01$ vs the group of leptin.

possible that there is a balance between ICAM-1/sICAM-1 and immunocytes, which can also be explained by the fact that it takes a long time for detectable tumour to form a metastatic lesion. With the development of GC, leptin increased sICAM-1 level, breaking the balance and subsequently resulting in a failure of the host immunocytes to effectively kill the tumour cells, which may have, therefore, protected the tumour cells from elimination and facilitated tumour migration. The hypothesis is well supported by previous findings that serum leptin and sICAM-1 are elevated and positively correlated with the clinical stage and lymph node metastasis of tumour (Maruo *et al*, 2002; Capelle *et al*, 2009). Taken together, these results suggest that leptin/ICAM-1/sICAM-1 is implicated in GC metastasis and may thus provide a potential therapeutic target against GC, which needs to be further confirmed by more experiments with animal models.

Sunami *et al* (2000) have shown that lymph node metastasis reduced after successful transfection of ICAM-1 gene into the cancer cells. Because of the enhancement of immune surveillance mechanism, tumour cells might be easily captured and destroyed by the lymphocytes. However, their cell model is different from ours, in addition, these authors did not discuss the production of sICAM-1 in their ICAM-1-overexpressing cells, though previous study from the same group revealed that the survival rate in high sICAM-1 group significantly decreased compared with that in low sICAM-1 group (Nakata *et al*, 2000).

The present study has demonstrated the importance of leptin-induced upregulation of ICAM-1 in GC cell migration and further elucidated the underlying mechanism. It is well known that the activity of small GTPases (including RhoA) is important for the signalling derived from lipid metabolism (Bolick *et al*, 2005).

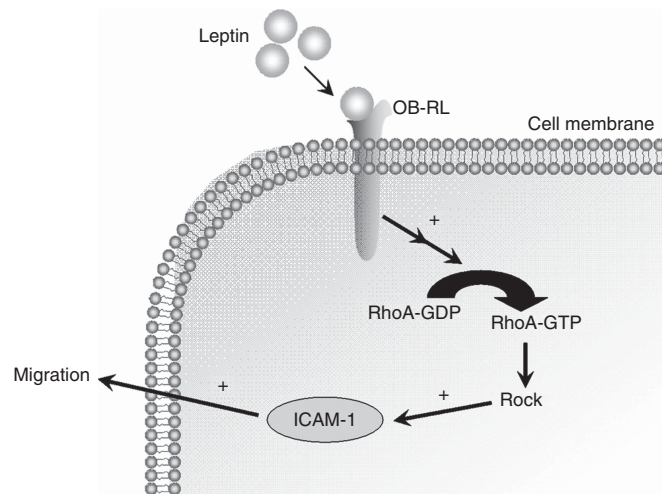


Figure 6. Schematic diagram summarising the leptin-RhoA/ROCK-ICAM-1 pathway on the migration of GC cells. By binding with OB-RL, leptin activates the RhoA/ROCK pathway, which subsequently induces the expression of ICAM-1. All of these finally promoted migration of GC. Abbreviation: OB-RL = leptin receptor.

This study has shown that leptin may activate Rho/ROCK pathway, which is consistent with the previous findings that leptin regulates myocardial matrix remodelling by activating Rho/ROCK (Schram *et al*, 2011). Further analysis indicates that the inhibitor of either Rho (C3 transferase) or ROCK (Y27632) attenuates leptin-induced ICAM-1 expression, illustrating that the use of ROCK inhibitors might be a potential therapeutic approach. This is also supported by the finding that Rho/ROCK pathway critically regulates the plasticity of metastatic GC (Matsuoka *et al*, 2011). Notably, the inhibitor treatment in this study failed to completely block the leptin-induced upregulation of ICAM-1, implying a possible contribution from other unidentified leptin-activated pathway(s). Several signalling pathways activated by leptin have been identified in GC, that is, Janus kinase/signal transducers and activators of transcription (JAK/STAT), extracellular signal-regulated kinase 1/2 (ERK1/2), and mitogen-activated protein kinase (MAPK) pathways, as well as phosphatidylinositol 3-kinase (PI3K)/AKT pathways (Pai *et al*, 2005; Shida *et al*, 2005). Previous study reveals that leptin upregulates ICAM-1 expression in eosinophils by the combined activation of MAPK and JAK pathways under allergic inflammation (Wong *et al*, 2007). However, their changes were not examined in this study. Although we cannot exclude the possible effect of these signalling pathways, the upregulation of ICAM-1 induced by leptin in the cells of this study might primarily depend on the RhoA/ROCK pathway. Further experiments will be conducted to test these possibilities. In addition, Rho GTPases are the binary switch between an active GTP-bound form and an inactive GDP-bound form. This process might be mediated by several molecules, such as Rho guanine nucleotide exchange factors (Rossman *et al*, 2005), NF- κ B (Jung *et al*, 2012), and reactive oxygen species (Jin *et al*, 2004). Although these mechanisms remain an interesting research topic, they are beyond the scope of the current study.

In conclusion, this study has shown that leptin enhances GC cell migration by increasing ICAM-1 expression, which is demonstrated to be largely dependent on the activation of Rho/ROCK signalling pathway. A summarised schematic diagram is shown in Figure 6. Our results may provide preliminary experimental clues for future development of novel therapies against the metastasis of GC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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