SFRP5 inhibits gastric epithelial cell migration induced by macrophage-derived Wnt5a

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Secreted frizzled-related protein 5 (SFRP5) is frequently found downregulated in gastric cancer due to SFRP5 gene hypermethylation, and there is a great necessity to elucidate the role of its downregulation in gastric cancer. By binding Wnt molecules, SFRP5 is generally supposed to exert negative effects on Wnt signal pathways widely linked to human cancers. This study found that macrophages over-produced Wnt5a under the stimulation of Lipopolysaccharide (LPS) or Helicobacter pylori, the most common infectious agent in human stomach. Wnt5a-conditioned medium from macrophages enhanced cell migration and CXCR4 expression in either SFRP5-negative gastric epithelial cells (GEC) harboring SFRP5 methylation or SFRP5-positive cells treated with SFRP5 small interfering RNA (siRNA). However, such induced effect was remarkably eliminated by either Wnt5a siRNA in macrophages or treatment with recombinant SFRP5. We also found that Wnt5a-conditioned and medium stimulated phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun, and JNK inhibitor SP600125 blocked Wnt5a-induced CXCR4 expression and cell migration in SFRP5-negative cells. Taken together, these findings suggest that epithelium-derived SFRP5 may play a probable defensive role in impeding gastric cancer progression, characteristically by inhibiting GEC migration induced by macrophage-derived Wnt5a via JNK signaling activation.

Introduction

Secreted frizzled-related proteins (SFRPs) resemble Wnt receptor frizzled proteins in cysteine-rich domains, which enable SFRPs to bind Wnt proteins, and interfere the binding of Wnt proteins to their frizzled receptors, attenuating Wnt signaling. According to our previous report, SFRP5 was found downregulated in gastric cancer due to SFRP5 gene hypermethylation (1). This finding is consistent with the results from the studies on some other cancers (2–6). However, further investigations are needed to elucidate the roles of reduced SFRP5 expression in cancers (especially in gastric cancer), though it is generally hypothesized that downregulation of SFRP5 may lead to excessive activation of Wnt signal pathways.

It is established that Wnt5a plays complicated roles in cancer initiation as well as progression. On one hand, it has an inhibitory effect on the proliferation of some types of cells, acting as a tumor suppressor; and exhibit signs of downregulation in some malignant diseases (7,8). On the other hand, Wnt5a is overexpressed in a series of cancers, including gastric cancer (9). It is probably that Wnt5a upregulation may not be involved in cancer initiation, but in cancer progression. Such a concept is justified by the association of Wnt5a with cell invasion and migration in melanoma (10), osteosarcoma (11), pancreatic cancer (12) and prostate cancer (13).

Despite the difficulties in determining the intrinsic mechanisms about Wnt5a overexpression in gastric cancer, some relevant studies have helped to elucidate them by identifying tumor-associated macrophage as an important source of Wnt5a (14,15). In fact, macrophage infiltration has been recognized as a hallmark of many human cancers, including gastric cancer (16,17). In addition, other recent studies have shown that inflammatory stimuli are able to induce Wnt5a expression in certain cells (18,19). This study attempted firstly to investigate whether inflammatory stimulation, especially, Helicobacter pylori infection, could stimulate macrophage to express Wnt5a, and then proceeded to explore whether macrophage-derived Wnt5a would play a positive role in gastric cancer invasion and migration. We also made a further speculation that gastric epithelium-derived SFRP5 might antagonize Wnt5a and thereby could play a defensive role in gastric cancer progression.

Materials and methods

Gastric epithelial cells

Human gastric cancer cells MKN45, SGC-7901, HGC-27, BGC-803 and non-cancerous gastric epithelial cell (GEC) GES-1 were provided by Keygen Biotech Co. (Nanjing, China). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, at 37°C in a humid incubator with 5% CO₂.

Macrophages and Wnt5a-conditioned medium

Mononuclear cells were isolated from human peripheral blood by Ficoll-Hypaque density gradient centrifugation and purified by CD14 microbeads. Monocytes were cultivated in RPMI 1640 supplemented with 10% fetal calf serum for 7–10 days until differentiation into macrophages. Wnt5a-conditioned medium was generated from cultured macrophages treated with LPS (100 ng/ml) for 8h. Then, cells were washed to remove LPS and maintained in culture for an additional 24h. The medium was collected, centrifuged and filtered. All experiments were carried out with the approval of the ethical committee of China Medical University.

H pylori strain

H pylori strain 26695 (ATCC 700392) was grown on sheep blood agar plates at 37°C under microaerophilic conditions. Bacteria were transferred after 48h into Brucella broth containing 5% fetal bovine serum for 24h. A multiplicity of infection of 100 was used in all studies.

Real-time reverse-transcription PCR

Total RNA was isolated from cells by Trizol (Takara, Dalian, China) according to the protocol supplied by the manufacturer. Complementary DNA was synthesized from 1 µg RNA using random 9mers and Avian Myeloblastosis Virus (AMV) reverse transcriptase. Real-time PCR was performed using the LightCycler system together with the LightCycler DNA Master SYBR Green I Kit (LightCycler, Roche Diagnostics). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Gene expression was quantified by the comparative computerized tomography method, normalizing computerized tomography values to glyceraldehyde-3-phosphate dehydrogenase and calculating relative expression values. Primer sequences for Wnt5a were described in (9), and for CXCR4 and glyceraldehyde-3-phosphate dehydrogenase in (20).

Western blot

A total of 10 µg protein of each sample was run on a 12% sodium dodecyl sulfate/acylamide gel. The proteins on acrylamide gel were transferred to a nylon membrane, which was blocked overnight (4°C in phosphate-buffered saline with 0.1% Tween and 10% milk powder). Polyclonal antibodies for SFRP5 (Abcam), Wnt5a, CXCR4, phosphorylated c-jun N-terminal kinase (JNK), phosphorylated c-Jun (Santa Cruz), de-phosphorylated β-catenin (dp-β-catenin) (Mybiosource) and the corresponding secondary antibodies (Santa Cruz) were applied before immunoblotting. The human gene β-actin (Santa Cruz) was employed as an internal control.

Methylation-specific PCR and DNA demethylation

Genomic DNA was extracted from cells by a standard phenol/chloroform extraction and ethanol precipitation procedure. Methylation of SFRP5 was detected by Genmed MSP Kit (Genmed, Shanghai, China). The procedure was performed according to the manufacturer’s instructions. Primers for SFRP5 methylated sequence and unmethylated sequence were described...
in (1). Cells with SFRP5 methylation were demethylated by 5-aza-2’-deoxycytidine (DAC) (2 μmol/l; Sigma). Cells were seeded at a density of 3 x 10⁵ cells/cm² in a 24-well plate on day 0 and exposed to DAC on day 1, 2, and 3. After each treatment, the cells were cultured in fresh medium. Control cells were incubated without the addition of DAC. Cells were harvested on day 4 for experiment.

**RNA interference**
Wnt5a small interfering RNA (siRNA) plasmid, SFRP5 siRNA plasmid and non-silencing control siRNA plasmid were purchased from Takala (Dalian, China). Cells were seeded into a 24-well plate at a density of 2 x 10⁵. On the following day, cells were transfected with siRNA plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Migration assays**
Migration of cultured cells was assayed using Matrigel invasion chamber (24-well format, 8 μm pore; BD pharmingen). Cells (5 x 10⁵) were added to the upper chamber and medium supplemented with CXCL12 (100 ng/ml; Sigma) was added to the lower chamber. Migration assays were incubated for 18 h at 37°C and 5% CO₂. Migrated cells on the lower surface were stained using 1% toluidine blue after fixation with 100% methanol. For each transwell, the number of migrated cells in 10 medium power fields (x 20) was counted.

**Statistical analysis**
Expression of messenger RNA (mRNA) in gastric cells was compared using Student’s t-test or one-way analysis of variance. Statistical analysis was carried out using SPSS version 11.0 (SPSS, Chicago, IL). Difference was considered significant when P < 0.05.

**Results**
Wnt5a is upregulated in macrophages by inflammatory stimulation
To explore the effect on Wnt5a expression under inflammatory environment, we treated macrophages with LPS (10 ng/ml, 100 ng/ml and 1 µg/ml, respectively; Sigma) for 8h. Real-time-PCR and western blot detection revealed that LPS upregulated Wnt5a expression significantly in mRNA and protein level (Figure 1A and 1B). Next, we evaluated the effect of H. pylori, the most common infectious agent to stomach, on Wnt5a expression in macrophages. After infection with H. pylori 26695 for 24h, macrophages expressed more Wnt5a mRNA and protein. However, this induction was significantly inhibited by neutralizing tumor necrosis factor (TNF)-α antibody infliximab (4 µg/ml; Sigma), suggesting TNF-α may be involved in the regulation of Wnt5a by H. pylori infection (Figure 1C and 1D).

Wnt5a expression was also measured in gastric cancer cells MKN45, SGC-7901, HGC-27 and BGC-803 and non-cancerous GEC GES-1. Of these five GECs, only MKN45 was found to slightly express Wnt5a (Figure 1E). In addition, we investigated the effect of inflammatory stimulation on Wnt5a expression in these gastric cells. As expected, induction of Wnt5a expression was also present exclusively in MKN45 (Figure 1E) after treatment with LPS (100ng/ml).

SFRP5 inhibits gastric epithelial cell migration by Wnt5a
Western blot was used to determine SFRP5 expression in GECs. The detection showed that SFRP5 was strongly expressed in...
BGC-803; S, SGC-7901; G, GES-1.

SFRP5 was observed in MKN45, HGC-27 and BGC-803 after
of
re-expression
SFRP5 was found in MKN45, HGC-27 and BGC-803, not in GES-1
(B
MKN45, HGC-27 and BGC-803 by western blot.
Expression of SFRP5 was detected in GES-1 and SGC-7901, not in
non-cancerous cell GES-1; of four malignant cells, SFRP5 was
exclusively expressed in SGC-7901 (Figure 2A). Next, methylation-
specific PCR was used to investigate SFRP5 methylation status in these
cells, and the examination indicated that SFRP5 gene was
methylated in MKN45, HGC-27 and BGC-803, but not in
SFRP5-negative GECs. After incubation with Wnt5a-conditioned
medium or rWnt5a (0.1 µg/ml), we found no significant change for
expression of SFRP5 in these cells (Supplementary Figure 1A and
1C, available at Carcinogenesis Online; Figure 4A and 4B). Induction of
CXCR4 expression was observed when these cells were
incubated with Wnt5a-conditioned medium. However, CXCR4 expression in
these cells was upregulated remarkably by SFRP5 siRNA in the presence
of Wnt5a-conditioned medium (Supplementary Figure 1B and
1C, available at Carcinogenesis Online; Figure 4E and 4F).

We also examined CXCR4 mRNA expression in SFRP5-positive
SGC-7901 and GES-1 by real-time PCR. No significant induction of
CXCR4 expression was observed when these cells were incubated
with Wnt5a-conditioned medium. Moreover, CXCR4 expression in these
cells was upregulated remarkably by SFRP5 siRNA in the presence
of Wnt5a-conditioned medium (Supplementary Figure 1B and
1C, available at Carcinogenesis Online; Figure 4A and 4B). Similar results were obtained in another SFRP5-negative gastric cancer cell
BGC-803 (Figure 4C and 4D).

SFRP5 inhibits CXCR4 expression in GECs induced by Wnt5a

To determine the molecular mechanisms underlying the increased
migration induced by Wnt5a, expression of CXCR4 was investigated
in SFRP5-negative GECs. After treatment with Wnt5a-conditioned
medium from macrophages, the expression was upregulated conside-
ably in mRNA and protein level in HGC-27 (Figure 4A and 4B). However,
the upregulation was abolished when macrophages were treated with
Wnt5a siRNA (Supplementary Figure 1A, available at Carcinogenesis
Online; Figure 4A and 4B). Induction of CXCR4 expression in HGC-
27 by Wnt5a-conditioned medium was also suppressed by rSFRP5
(Figure 4A and 4B). Similar results were obtained in another SFRP5-
negative gastric cancer cell BGC-803 (Figure 4C and 4D).

JNK is involved in migration induction by Wnt5a

To investigate the downstream signals affected by SFRP5 deficiency in
the presence of Wnt5a, firstly we determined the expression levels of dp-
β-catenin, a key molecule in canonical Wnt signal pathway, and its target
cyclinD1 in SFRP5-negative cell BGC-803. After incubation with Wnt5a-conditioned medium or rWnt5a (0.1 µg/ml), we found no significant changes for the expression levels of dp-
β-catenin and cyclinD1 in BGC-803, suggesting canonical Wnt signal pathway is not involved in Wnt5a-induced migration of SFRP5-negative cells (Figure 5A).

We next assessed whether non-canonical Wnt/JNK signal pathway
was responsible for Wnt5a-induced cell migration. Western blot revealed
expression of phosphorylated JNK and c-Jun, a downstream substrate of JNK, was stimulated in BGC-803 after treatment with Wnt5a-conditioned medium. However, the increased expression was
antagonized by rSFRP5 (0.5 µg/ml) (Figure 5B). Furthermore, CXCR4 expression and cell migration induced by Wnt5a were blocked by JNK
inhibitor SP600125 (10 µg/ml; Santa Cruz) in BGC-803 (Figure 5C
and 5D). Collectively, these data indicate JNK plays important roles in Wnt5a-induced migration in the absence of epithelium-derived SFRP5.

Discussion

This study demonstrates that *H. pylori*, the most powerful carcinogen
to stomach, upregulates Wnt5a expression in macrophages. Consistently, a recent study has shown that Wnt5a levels are significantly lower in gastric cancer tissue than that in the normal stomach, upregulates Wnt5a expression in macrophages (21). These studies collectively suggest that *H. pylori* may be a
Wnt5a inducer in gastric cancers. The signaling involved in Wnt5a
upregulation by *H. pylori* may be associated with toll-like receptor and nuclear factor-kappaB pathway. A conserved nuclear factor-
 kappaB-binding site has been found within the Wnt5a promoter
region (22); and toll-like receptor signaling and nuclear factor-
 kappaB activation are indispensable for Wnt5a expression in human
mononuclear cells after stimulation with mycobacterial species (18).
Other than inflammatory stimulation, cancer cells were also found
to induce macrophages to express Wnt5a (14), indicating complex
interactions between macrophages and tumor cells.

Given the close relation between chronic inflammation and cancer,
it is quite probable that under inflammatory environment, macrophage-
derived Wnt5a, a putative linker between inflammation and cancer,
would play positive roles in human cancers. This study showed that
*H. pylori* induced Wnt5a expression in macrophages through TNF-α,
and Wnt5a elicited a strong stimulation in CXCR4 expression in SFRP5-negative GECs. These results suggest that there exists a particular pathway, where macrophage-derived TNF-α, under H. pylori infection, induces macrophages to produce and secrete Wnt5a in an autocrinal manner; and then, Wnt5a stimulates CXCR4 expression by GECs in a paracrinal manner.

As to the exact membrane receptor mediating the intracellular signal pathway initiated by Wnt5a, it deserves further explorations and is still a subject of ongoing investigation in our laboratory. Some frizzled proteins, as we speculate, may have mediated Wnt5a signaling in GECs in a paracrinal manner.

In this study, Wnt5a-conditioned medium or recombinant Wnt5a exerted no effects on the expression levels of dp-β-catenin and cyclinD1 in gastric cancer cells, and this largely excluded the possibility of the involvement of canonical Wnt signal pathway in Wnt5a-induced migration of gastric cancer cells. On the contrary, phosphorylation of JNK and c-Jun, a downstream substrate of JNK, was stimulated in gastric cancer cells by treatment with Wnt5a-conditioned medium or recombinant Wnt5a. Furthermore, the upregulation of CXCR4 expression induced by Wnt5a was effectively blocked by JNK inhibitor SP600125. Collectively, all these data indicate that JNK plays important roles in Wnt5a-induced cell migration.

Generally, cell migration is recognized as a hallmark of cancer metastases. Studies have shown both canonical Wnt pathways

In most cases, intracellular Wnt5a signal pathways are β-catenin non-dependent, failing to induce translocation and accumulation of cytosolic β-catenin in nucleus (10,13,26). There are generally two major intracellular pathways, CaMKII–PKC and JNK, mediating Wnt5a signals involved in cancer and inflammation (10,13,14,19,26–28). In this study, Wnt5a-conditioned medium or recombinant Wnt5a exerted no effects on the expression levels of dp-β-catenin and cyclinD1 in gastric cancer cells, and this largely excluded the possibility of the involvement of canonical Wnt signal pathway in Wnt5a-induced migration of gastric cancer cells. On the contrary, phosphorylation of JNK and c-Jun, a downstream substrate of JNK, was stimulated in gastric cancer cells by treatment with Wnt5a-conditioned medium or recombinant Wnt5a. Furthermore, the upregulation of CXCR4 expression induced by Wnt5a was effectively blocked by JNK inhibitor SP600125. Collectively, all these data indicate that JNK plays important roles in Wnt5a-induced cell migration.
and non-canonical Wnt pathways are involved in cell migration by upregulating chemokines/chemokine receptors, especially CXCL12/CXCR4. For instance, canonical Wnt/β-catenin signaling was found to upregulate CXCL12 gene expression in bone marrow stromal cells (29) and CXCR4 expression in a zebrafish model (30). Besides canonical Wnt pathway, non-canonical Wnt5a pathway is also implicated in regulation of CXCL12/CXCR4. One recent study has shown that Wnt5a/PKC signaling is required for CXCL12-mediated T-cell migration and the sustained expression of CXCR4 (31). This study reveals for the first time that macrophage-derived Wnt5a induces CXCR4 expression in either normal or malignant GECs, contributing to the increased migration of these cells.

This study also indicates that SFRP5 deficiency promotes GEC migration, as a result of excessive activation of Wnt5a signaling and subsequent CXCR4 upregulation. Generally, SFRP5 gene is inactivated by promoter methylation in a series of human malignant tumors, including gastric cancer (1), mesothelioma (2), bladder cancer (3), breast cancer (4), malignant astrocytic gliomas (5) and pancreatic cancer (6). It was reported that methylation levels of SFRP5 were higher in H.pylori-positive samples than those in H.pylori-negative ones, suggesting H.pylori infection might be involved in SFRP5
methylation process (32). Epigenetic inactivation of SFRP5 gene was found to be associated with unfavorable prognosis in breast cancer as well as cisplatin resistance in ovarian cancer (33,34). Thus, SFRP5 methylation may be a novel biomarker potentially useful in clinical cancer management.

Furthermore, SFRP5 has been found to co-ordinate foregut specification and morphogenesis by antagonizing both canonical and non-canonical Wnt signaling (35). Likewise, in adult tissues, SFRP5 also seem to inhibit both canonical and non-canonical Wnt pathways and plays complex roles in cell growth, apoptosis, invasion and migration (33,34). As shown in this study, SFRP5 suppressed Wnt5a activation and inhibited JNK signals, which pointed to its probable role in attenuating non-canonical Wnt5a pathways in gastric cell migration.

Studies have shown that H.pylori can directly stimulate invasion, migration and angiogenesis of gastric cancer cells (36–38), indicating that the stomach carcinogen is involved not only in gastric cancer initiation but also in gastric cancer progression. Consistent with these studies, our previous study has shown that CXCR4 level is significantly higher in H.pylori-positive gastric cancers compared with H.pylori-negative ones (20). In addition, in stomach, H.pylori has been shown to recruit and activate macrophages (16,17) which has been confirmed to be implicated in tumor metastasis and progression (39,40).

In summary, this study presents evidence that H.pylori is a Wnt5a inducer to macrophages; and macrophage-derived Wnt5a enhances gastric cancer cell migration through upregulating CXCR4 expression in a paracrine manner. We also find that, due to SFRP5 gene hypermethylation, Wnt5a exerts this effect on gastric cancer cells in the absence of gastric epithelium-derived SFRP5. Moreover, rSFRP5 has a suppressive effect on Wnt5a-induced CXCR4 expression and cell migration. These findings collectively suggest that SFRP5 plays a defensive role in obstructing gastric cancer progression via antagonizing Wnt5a/JNK signaling; and therefore, its

Fig. 5. Involvement of JNK in migration induction by Wnt5a. (A) Expression of dp-β-catenin and cyclinD1 did not change significantly in BGC-803 in the presence of Wnt5a-conditioned medium or rWnt5a. (B) Expression of phosphorylated JNK and c-Jun was increased by Wnt5a-conditioned medium in BGC-803; however, the increase was suppressed by rSFRP5. *P < 0.01, versus control; **P < 0.01, versus mWnt5a + rSFRP5. (C) and (D) JNK inhibitor SP600125 blocked CXCR4 expression and cell migration induced by Wnt5a in BGC-803. *P < 0.01, versus control; **P < 0.01, versus mWnt5a + SP. mWnt5a, Wnt5a-conditioned medium; SP, SP600125. Data are expressed as mean ± SD, n = 3.

Fig. 6. Schema: epithelium-derived SFRP5 plays a defensive role in gastric cancer progression. Inflammatory stimuli induce macrophages to overexpress Wnt5a, which enhances CXCR4 expression and cell migration in SFRP5-negative (SFRP5−) GECs. However, SFRP5 derived from SFRP5-positive (SFRP5+) GECs blocks the induced CXCR4 expression and cell migration via binding macrophage-derived Wnt5a.
deficiency in gastric epithelium, together with Wnt5a overproduction by macrophages, may substantially promote gastric cancer cell migration (Figure 6).

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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References


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