EUROPEAN JOURNAL OF CANCER 47 (2011) 121–130

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Four and a half LIM protein 2 (FHL2) negatively regulates the transcription of E-cadherin through interaction with Snail1

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ARTICLE INFO

Article history:
Received 25 June 2010
Received in revised form 28 July 2010
Accepted 29 July 2010

Keywords:
FHL2
Snail1
E-cadherin
Epithelial–mesenchymal transition
Colon cancer

ABSTRACT

E-cadherin is a hallmark of epithelial–mesenchymal transition (EMT), which plays a crucial role in cancer metastasis. We previously demonstrated that four and a half LIM protein 2 (FHL2) inhibited E-cadherin expression and promoted invasive potential and EMT in colon cancer. Here, we aim to further define the mechanism underlying the inhibition of E-cadherin by FHL2 in colon cancer. The expression profiles of FHL2 and Snail1 were first observed by Western blot, immunofluorescence and immunohistochemistry. We found that both the protein level and the cellular localisation of Snail1 were quite similar to FHL2 in colon cancer; reciprocal co-immunoprecipitation assay showed that FHL2 was able to bind Snail1 and its intact structure was required. The expression of FHL2 was positively correlated to Snail1 while negatively to E-cadherin and phospho-Snail1. FHL2 over-expression induced the accumulation of Snail1 in the nucleus. Moreover, dual luciferase assay revealed that FHL2 over-expression decreased while FHL2 siRNA increased the transcriptional activities of two E-cadherin promoter constructs which contained E-box sites (Snail1-binding elements). Mutation of E-boxes increased the transcriptional activities and FHL2 expression was involved in the function of mutation. These results suggested that FHL2 negatively regulated E-cadherin transcriptional activity through interaction with Snail1. Our study established a novel regulatory function of FHL2 and revealed a potential mechanism on promoting the process of EMT.

1. Introduction

E-cadherin is an important cell–cell adhesion molecule and a hallmark of epithelial–mesenchymal transition (EMT), which plays a crucial role in development, inflammation and tumour progression.[1–3] Abolishing E-cadherin function In Vitro confers invasive properties to non-invasive cells.[4,5] Down regulation of E-cadherin may result from genetic mutations, promoter hypermethylation or dysfunction of transcriptional factors.[6–8] In particular, transcriptional

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doi:10.1016/j.ejca.2010.07.045

repression is regarded as a fundamental mechanism of E-cadherin silencing during tumour progression. Several transcriptional repressors have been found, such as members of the Snail, zinc finger E-box binding (ZEB) and basic helix–loop–helix (bHLH) families. Among them, Snail1 is a strong repressor of E-cadherin and its role has been highlighted due to its over-expression in many tumours and the lack of E-cadherin during gastrulation of Snail1-deficient murine embryos.[9,10] In E-cadherin-positive carcinoma cells, transfection of Snail1 induces a full EMT occurrence with E-cadherin downregulation and mesenchymal molecule, vimentin and matrix metalloproteinase 2 (MMP2) upregulation.[11] Snail1 is over-expressed in lung, breast, pancreas and colon cancers.[12–15] Its function is regulated by glycogen synthase kinase 3 beta (GSK-3β) through phosphorylation. Zhou and colleagues found that there were two GSK-3β phosphorylation motifs in Snail1. GSK-3β binds to and phosphorylates the first motif to regulate the β-Trcp-mediated ubiquitination of Snail1 resulting in its degradation, whereas phosphorylation of the second motif induces its nuclear export.[16]

FHL2 (four and a half LIM-only protein 2) is a LIM-only protein and participates in cell transcription and signal transduction.[17] It is highly expressed in cancers including giloma, colon, prostate and breast cancers.[18–22] Over-expression of FHL2 stimulates cell proliferation and inhibits apoptosis in melanocyte or prostate cancer cells. As a trans-activator, FHL2 is able to physically interact with a large number of transcription factors such as AP-1 (c-jun and c-fos), ERK2, β-catenin and α3β1 integrin receptor.[19,23,24]

In our previous study, we found that over-expression of FHL2 in gastrointestinal cancers was essential to maintain their malignant phenotype and suppression of FHL2 increased the expression of E-cadherin.[19] Furthermore, FHL2 induced the progression of EMT in colon cancer and was involved in the formation of E-cadherin/β-catenin complex.[25] In this study, we aim to further define the mechanism underlying the inhibition of E-cadherin expression by FHL2 in colon cancer.

2. Materials and methods

2.1. Reagents, cell lines and tissue specimens

Rabbit anti-human Snail1 used for Western blot, immunofluorescence and immunohistochemistry (IHC), rabbit anti-p-Snail1 (phosphor S246), rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rabbit anti-human FHL2 antibody used for IHC were purchased from Abcam (Cambridge, UK). Mouse anti-human FHL2 antibody used for immunofluorescence and Western blot was a product of MBL international incorporation (Woburn, Japan). Rabbit anti-human Snail1 (H-130) used for co-immunoprecipitation, rabbit anti-human E-cadherin, bovine anti-mouse immunoglobulin G-TR (IgG-TR) and goat anti-rabbit IgG-FITC were purchased from Santa Cruz (Santa-Cruz, CA, USA). Mouse anti-human Topoisomerase I (TOPO I) and flag were purchased from Sigma (St. Louis, MO, USA). Human colon cancer cell lines SW480, HCT116, HCT15, HT29, DLD1 and SW1116 were maintained in our laboratory and cultured as previously described.[26] Seventeen pairs of surgically resected colon cancer tissues and their matched metastatic cancer sites from the same patient were obtained from the Department of Pathology, Nanfang Hospital, The First Affiliated Hospital of Southern Medical University, China. All tissue slices were subjected to histopathological review before further investigation. The Ethics Committee of Southern Medical University of China approved our experimental protocols and the consent was obtained from all patients.

2.2. Constructs, transient transfection, and establishment of stable transfectants

Flag-tagged FHL2 (PCI 3X flag) plasmids, named as 76, 77, 78 and 79 expressed the full length, the last three, two and one LIM domain of FHL2 protein, respectively. Transient and stable transfections were performed as described before.[27] The over-expressions of FHL2 or tagged proteins were confirmed by Western blot. DLD1-FHL2-pcDNA was generated to express full-length FHL2 protein with DLD1-pcDNA as the vector control. Transfectants of SW480-76, SW480-77, SW480-78 and SW480-79 were generated to express full-length or truncated FHL2 proteins with SW480-PCI as the vector control.

2.3. Immunohistochemistry (IHC)

Paraffin-embedded tissue blocks were cut into 5 μm sections and transferred to glass slides. The slides were deparaffinised with xylene, rehydrated with ethanol, washed and subjected to microwave retrieval. Sections were then immersed in 3% hydrogen peroxide to block endogenous peroxidase activity and incubated with first antibodies (1:50) followed by incubation with peroxidase-conjugated anti-rabbit secondary antibody (Dako, 1:100). The expressions of FHL2 and Snail1 were then visualised using 1 mg/ml 3,3′-diaminobenzidine and counterstained with haematoxylin. The tissue in which more than 10% of cancer cells being positively stained was considered positive. For quantitative analysis, the ratio of positively stained cells to all tumour cells in five random areas at 200-fold magnification was recorded. Scoring of tissue slides was performed independently by two investigators; the percentage of positive cells and the intensity of staining were scored from 0 to 3.0, less than 10% of cells stained: (1) 10–50% of cells stained; (2) 50–75% of cells stained; and (3) more than 75% of cells stained.

2.4. siRNA transfection

The sequences of FHL2 siRNA, Snail1 siRNA and control siRNA were as follows: CGAAUCUCUCUUUGGCAAGdTdT, UGCACAUCCGAAGCCACACdTdT and CGUACGCGGAAUACUUCGAdTdT, respectively. Cells seeded in tissue culture plate were grown to 30–50% confluence and transfected by lipofectamin™ RNAiMAX as per the instructions of the manufacturer. The effect of gene knockdown was evaluated by Western blot 24–48 h later.

2.5. Preparation of cytoplasmic and nuclear extract and Western blot

Whole cell lysates, cytosolic and nuclear extract were prepared routinely.[28] The cell lysates were electrophoresed on

denaturing SDS–PAGE gel (5% stacking gel and 12% separating gel). The proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The blots were probed with primary antibody followed by the HRP-conjugated secondary antibody. Antigen-antibody complexes were visualised by the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Little Chalfont Buckinghamshire, UK).

2.6. Generation of E-cadherin promoter-luciferase constructs

A 232 bp (–94 to –325 nt) and a 601 bp (–94 to –694 nt) of E-cadherin promoter fragments upstream of the ATG translation starting codon were cloned into pGL3 basic vector to generate two reporter constructs, named as pLuc232 and pLuc601. The sequences with KpnI and XhoI sites were added into the reverse and forward primers, respectively. The details were as follows: E-cadherin forward 1: 5′-TAACCCACCTAGACCCTAGCAA-3′; forward 2: 5′-GGGCAATACAGGGAGACACAG-3′; E-cadherin reverse: 5′-CTCACAGGTGCTTTGCAGTTC-3′. Lengths of the two constructs were 232 bp and 601 bp. Promoter segments were obtained by PCR amplification with genomic DNA extracted from cancer cells as the template. Hotstart PCR was performed for 32 cycles with 95 °C denaturation for 30 min (first cycle), 94 °C denaturation for 45 s, 55 °C annealing for 45 s, and 72 °C elongation for 1 min and 7 min (final cycle). The cloned fragments were verified by gene sequencing.

2.7. Promoter-luciferase reporter expression

Dual luciferase assay was performed to evaluate the transcriptional activities of promoter-reporter constructs using the Dual-Luciferase reporter assay system (Promega) as we reported previously.[28] Firefly luciferase activity value was normalised to renilla activity value. Promoter activity was presented as the fold induction of relative luciferase unit (RLU) compared with the basic vector control. RLU = value of firefly luciferase unit/value of renilla luciferase unit. All treatments were triplicated for each single experiment.

2.8. Immunofluorescence

Cells grown in cover glass were fixed with 4% paraformaldehyde and the non-specific bindings were blocked by incubation with 1% bovine serum albumin (BSA). The glasses were probed with the first antibodies (1:50) followed by TR- (Texas red) or FITC-conjugated second antibodies (1:50). Nuclei were counterstained with 1 μg/ml Hoechst 33258. After mounting, the slips were visualised under Zeiss Axioscop fluorescence microscope.

2.9. Co-immunoprecipitation (Co-IP)

The lysates of cells without or with stable transfection of tagged constructs were incubated with 3 μg first antibody for 3 h at 4 °C followed by incubation with the precleared protein A-agarose bead (Roche, Mannheim, Germany) slurry. After extensive washing, samples were subjected to Western blot for detection of the potential-interacting proteins.

2.10. Site-directed mutagenesis analysis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jalla, CA, USA) was used to generate constructs with mutation of Snail1-binding E-box element (E-box 1: –98 to –103 nt; E-box 4: –584 to –590 nt, the nucleotide adjacent to ATG starting codon were defined as –1) as previously reported.[26] Briefly, pLuc232 and pLuc601 constructs were PCR-amplified in the elongation process using Pfu DNA polymerase with the double strand mutation primers. PCR was performed for 16 cycles with 95 °C denaturation for 30 s (first cycle), 95 °C denaturation for 30 s, 55 °C annealing for 1 min, and 68 °C elongation for 10 min. The product was then treated with DpnI endonuclease to digest the methylated parental DNA template. The nicked vector DNA carrying the desired mutations was proliferated in Epicurian coli XL1-Blue supercompetent cells. Successful mutation was verified by gene sequencing.

2.11. Statistical analysis

Results obtained from triplicated luciferase experiments were expressed as the mean ± SD. RLU with different treatments was compared using a two-tailed Student's *t*-test. Correlation analyses for quantification of FHL2 and Snail1-positive staining were performed using Spearman correlation. Significant difference was considered if *p*-value was less than 0.05.

3. Results

3.1. Positive correlation between Snail1 and FHL2 expressions in colon cancer

We first detected FHL2 and Snail1 expressions in six colon cancer cell lines and found that the expression profiles of the two proteins were similar in five out six cell lines except HT29. As shown in Fig. 1A, FHL2 was expressed at a relative high level in SW480, HCT15 and SW1116 cell lines while at a relative low level in HCT116, DLD1 and HT29. Similarly, Snail1 was also expressed in SW480, HCT15 and SW1116 at a relative high level while at a low level or was undetectable in HCT116, DLD1 and HT29. Then the cellular localisation was observed by double-staining immunofluorescence analysis. As shown in Fig. 1B, most of the positive cellular signals of FHL2 and Snail1 showed a predominant nuclear staining. Thirdly, we detected Snail1 and FHL2 expressions by IHC in human colon cancer tissues taken from both the primary and metastatic sites. Similar to FHL2, Snail1 was expressed by all 17 primary and 16/17 of the metastatic cancer tissues. Nearly 90% of cancer cells and few of the adjacent normal epithelial cells expressed Snail1 (Fig. 1C). Semi-quantitatively, scoring of the two proteins showed that the expressions of both proteins in cancerous tissues were significantly higher than that of adjacent normal colon tissues ($p < 0.05$, Fig. 1D), Spearman correlation analysis showed a positive correlation between Snail1 and FHL2 expression (correlation coefficient $R = 0.756$, $p < 0.01$, Fig. 1E).

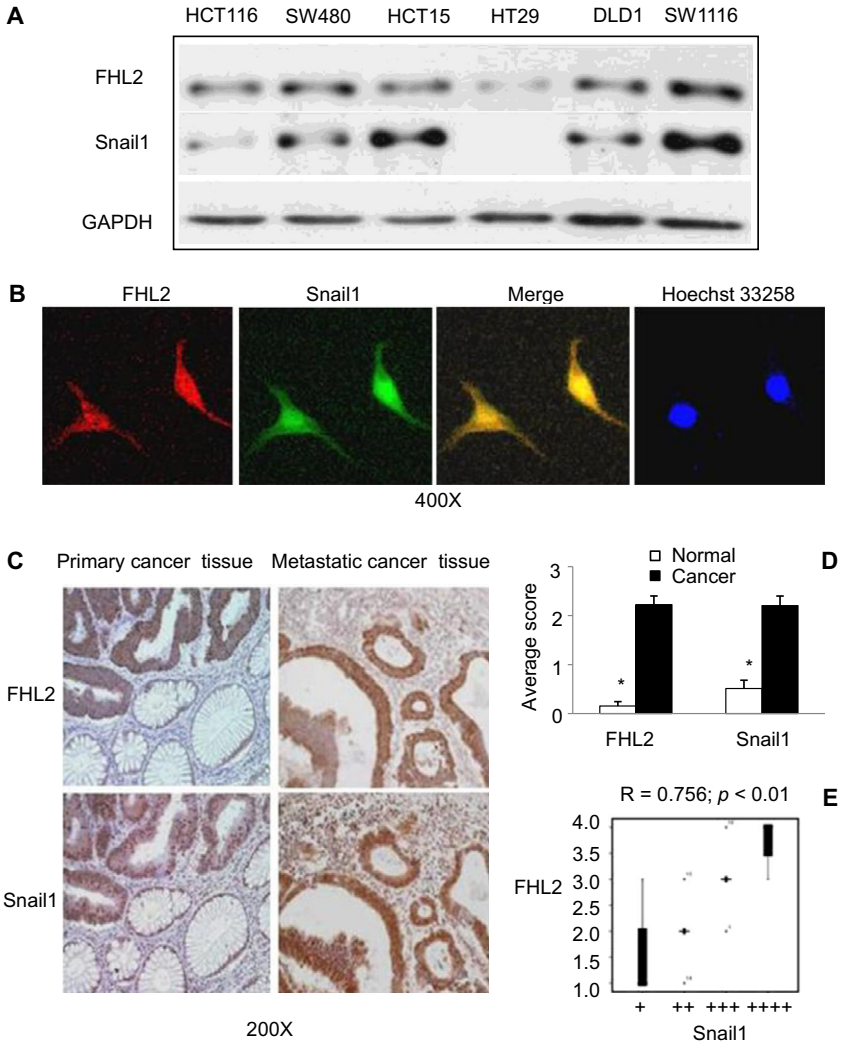

Fig. 1 – Expression profiles of FHL2 and Snail1 in colon cancer. (A) FHL2 and Snail1 expressions were detected in colon cancer cell lines by Western blot. GAPDH was used as the internal control. (B) Double staining of FHL2 and Snail1 in SW480 cells by indirect immunofluorescence assay with the nuclei being counterstained by Hoechst 33258 (original magnification = 400×). (C) The representative pictures of IHC staining for FHL2 and Snail1 in colon cancer tissues collected from the primary and the metastatic sites (original magnification = 200×). (D) Average scores of the two proteins in normal and cancerous colon tissues. *$p < 0.05$ between normal and cancer tissues. (E) FHL2 and Snail1 positive staining were quantified and their correlation was analysed using Spearman correlation method.

3.2. *FHL2 physically interacts with Snail1*

It is reported that FHL2 is capable of interacting with β-catenin to enforce β-catenin transactivational activity.[23] Interestingly, Snail1 can also bind to β-catenin and stimulates its transcriptional activity.[29] So a link between FHL2 and Snail1 might exist. Moreover, AJUBA, another LIM-containing protein, has been reported to interact with Snail1 in the nucleus and contribute to Snail1-dependent repression of E-cadherin.[30,31] Therefore, it is possible that FHL2 exerts its effect on E-cadherin by interaction with Snail1.

To verify such an interaction, we transfected flag-tagged full-length FHL2 into SW480 cells at first. Co-immunoprecipitation showed that Snail1 could be co-precipitated with flag-tagged-FHL2 in FHL2-over-expression cell line, SW480-76 (Fig. 2A). To further confirm that the FHL2–Snail1 interaction occurs with the endogenous FHL2, whole cell lysates from SW480 cells were prepared for immunoprecipitation. Indeed, the endogenous FHL2 was also capable of binding to Snail1 (Fig. 2B). Collectively, these findings suggest that FHL2 can physically interact with Snail1.

To define the binding domains that are responsible for these two protein interaction, plasmids encoding the flag-tagged, full-length FHL2 and the three truncated forms were transfected into SW480 cells, respectively. The results of immunoprecipitation experiments showed that the intact FHL2 protein could interact with Snail1. In contrast, the removal of the first one and a half, the two and a half or the three and a half LIM domains abrogated the interaction (Fig. 2C). These results indicate that all of the four and a half of LIM domains are essential during the interaction.

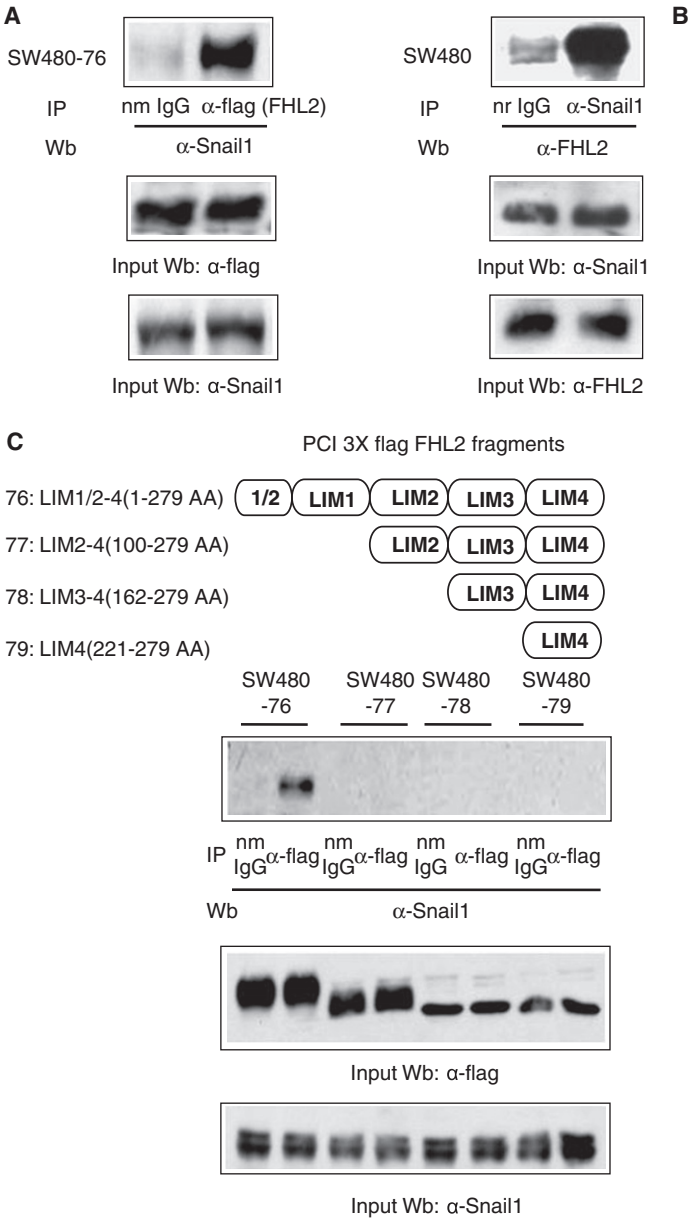

Fig. 2 – Interaction between FHL2 and Snail1 proteins. (A) Plasmid, PCI-76 encoding the full-length FHL2 protein, was transfected into SW480 cells. Immunoprecipitation was performed with anti-flag antibody, and preimmune normal mouse immunoglobulin G (nm IgG) was used as control. Western blotting was performed with anti-Snail1 antibody. The IP blot was probed with indicated antibodies to show the input of whole cell lysates. IP: immunoprecipitation and Wb: Western blot. (B) Cell lysates of SW480 cells was immmunoprecipitated by anti-Snail1 antibody or the control antibody, normal rabbit immunoglobulin G (nr IgG). Western blotting was carried out with anti-FHL2 antibody. The IP blot was probed with indicated antibodies to show the input. (C) Diagram showing the full-length FHL2 (76) and its truncations (77, 78, and 79). Immunoprecipitation was performed using the cell lysates of SW480 cells transfected with the above four constructs, respectively. Anti-flag or nm IgG were used as the bait to detect Snail1. The IP blot was probed with indicated antibodies to show the input. All of these pictures were the representatives of two or three independent experiments with identical results.

3.3. *FHL2 induces Snail1 expression and accumulation in the nucleus*

To evaluate the significance of FHL2–Sanil1 interaction, we checked the influence of FHL2 on the expression and metabolism of Snail1 protein in stable transfectant, DLD1-FHL2-pcDNA, with DLD1-pcDNA used as the internal control. As shown in Fig. 3A, we first confirmed our previous finding about the inverse correlation between FHL2 and E-cadherin expressions. Furthermore, a decrease of Snail1 phosphorylation at S246 was observed along with the increase of Snail1 protein level in DLD1-FHL2-pcDNA. Since there is no evidence for the association between the decrease of Snail1 S246 phosphorylation and the increase of Snail1 level, we further

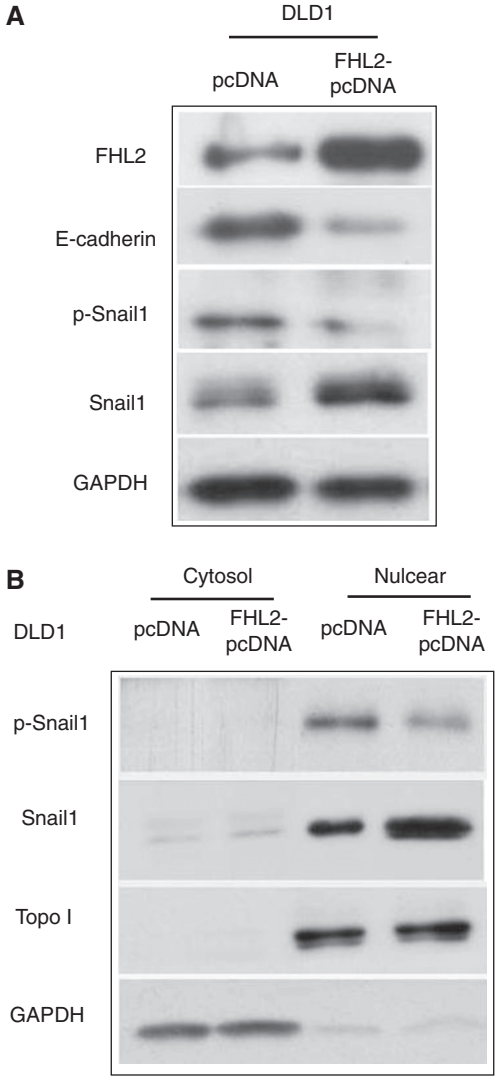

Fig. 3 – Effect of FHL2 on Snail1 expression and metabolism. (A) Expressions of FHL2, E-cadherin, phosphor-Snail1 (p-Snail1) and Snail1 in stable transfectants of FHL2-pcDNA or vector control (pcDNA) were detected by Western blotting. GAPDH was used as the internal control. (B) Expressions of Snail1 and p-Snail1 in the cytosolic and nuclear extracts of stable transfectants detected by Western blot. GAPDH and Topoisomerase I (Topo I) were detected as the internal control for cytosolic and nuclear proteins, respectively. All of these pictures were representatives of three independent experiments.

carried out cell fraction experiments to observe their cellular distribution and found that FHL2 decreased the level of phospho-Snail1 but increased the level of Snail1 in the nucleus in DLD1-FHL2-pcDNA cells (Fig. 3B). These findings suggest that FHL2 is able to induce the nuclear accumulation of Snail1.

3.4. *FHL2 over-expression inhibits wildtype E-cadherin promoter activity*

Subsequently, we detected the effect of FHL2 on the transcriptional activity of E-cadherin. There are four Snail1-binding E-boxes within E-cadherin promoter with only E-box 1 and 4 being functional in mediating the effect of Snail1.[9] Therefore, we generated four luciferase reporter constructs that contained 232 bp or 601 bp of E-cadherin promoter fragments with wildtype (wt) and mutant (mt) E-box 1 or E-box 4 element. Fig. 4A listed the primer sequences for site-directed mutation while Fig. 4B depicted the compositions of reporter constructs. We first assessed the effect of FHL2 on E-cadherin promoter activity. We found the RLU of pLuc232-wt and pLuc601-wt in DLD1-pcDNA cells were 176.04 ± 26.15 and 102.66 ± 10.27, respectively. The transcriptional activity of

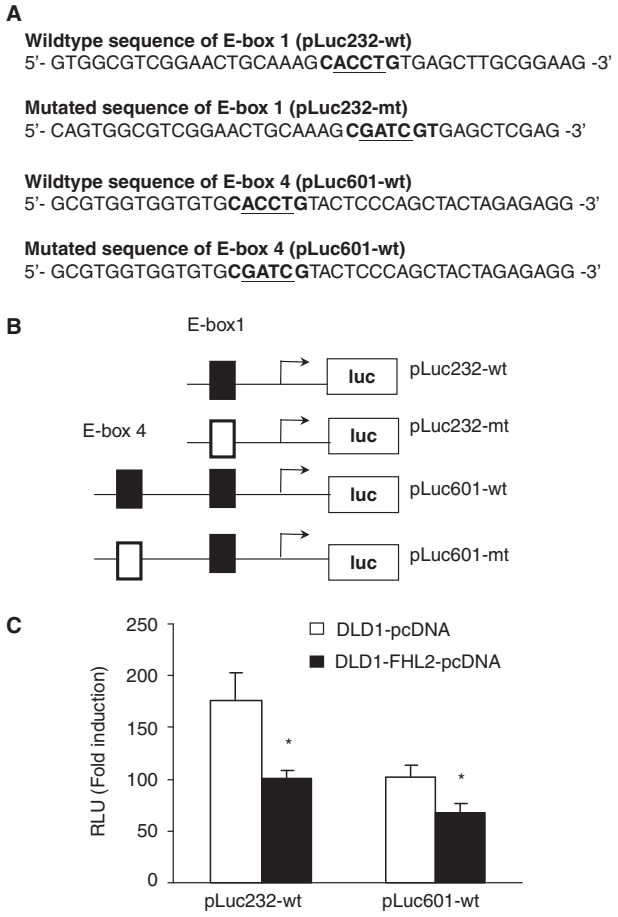

Fig. 4 – FHL2 over-expression inhibited E-cadherin transcription. (A) Primer sequences used for site-directed mutations. A 232 bp (containing E-box 1) and a 601 bp (containing both E-box 1 and 4) E-cadherin promoter fragments were cloned into pGL3 basic to generate pLuc232-wt and pLuc601-wt. Site-directed mutagenesis of E-box 1 in pLuc232 and E-box 4 in pLuc601 were performed to generate pLuc232-mt and pLuc601-mt. The locations of E-boxes were labelled in bold while the mutated nucleotides were underlined. wt: wildtype and mt: mutant. (B) Diagram showing the E-box status in the four constructs. ■, wildtype E-boxes and □, mutated E-boxes. (C) Transcriptional activities of the two wildtype reporters in stable transfectants. Dual luciferase assay were carried out with the result being expressed as fold induction of RLU. *$p < 0.05$ between the two stable transfectants.

pLuc601-wt which contained two functional Snail1-binding E-boxes was lower than that of pLuc232-wt which had only one E-box (Fig. 4C). After FHL2 was over-expressed (DLD1-FHL2-pcDNA), the RLU of pLuc232-wt and pLuc601-wt were all decreased (100.40 ± 7.47 and 68.08 ± 7.93, Fig. 4C). Significant difference was found between DLD1-pcDNA and DLD1-FHL2-pcDNA cells ($p < 0.05$).

3.5. *Effect of FHL2 on the transcription of wildtype or mutant E-cadherin promoter*

To examine the effect of FHL2 inhibition on E-cadherin expression, we suppressed FHL2 expression by siRNA transfection. As shown in Fig. 5A, we first showed that FHL2-knockdown was accompanied with the decrease of Snail1, while there is a significant increase of E-cadherin. On the molecular mechanism, FHL2 siRNA could increase the transcriptional activities of pLuc232-wt and pLuc601-wt in SW480 cells in which the RLU of both reporters were lower than that in DLD1 cells. The RLU of pLuc232-wt in cells treated with control or FHL2 siRNA were 76.36 ± 18.32 and 147.61 ± 16.63 while that of pLuc601-wt were 61.8 ± 12.18 and 84.3 ± 8.9, respectively (Fig. 5B and C).

To verify whether the effect of FHL2 on E-cadherin transcription is associated with Snail1, we mutated the E-box elements in pLuc232-wt and pLuc601-wt. As reported,[7] mutation

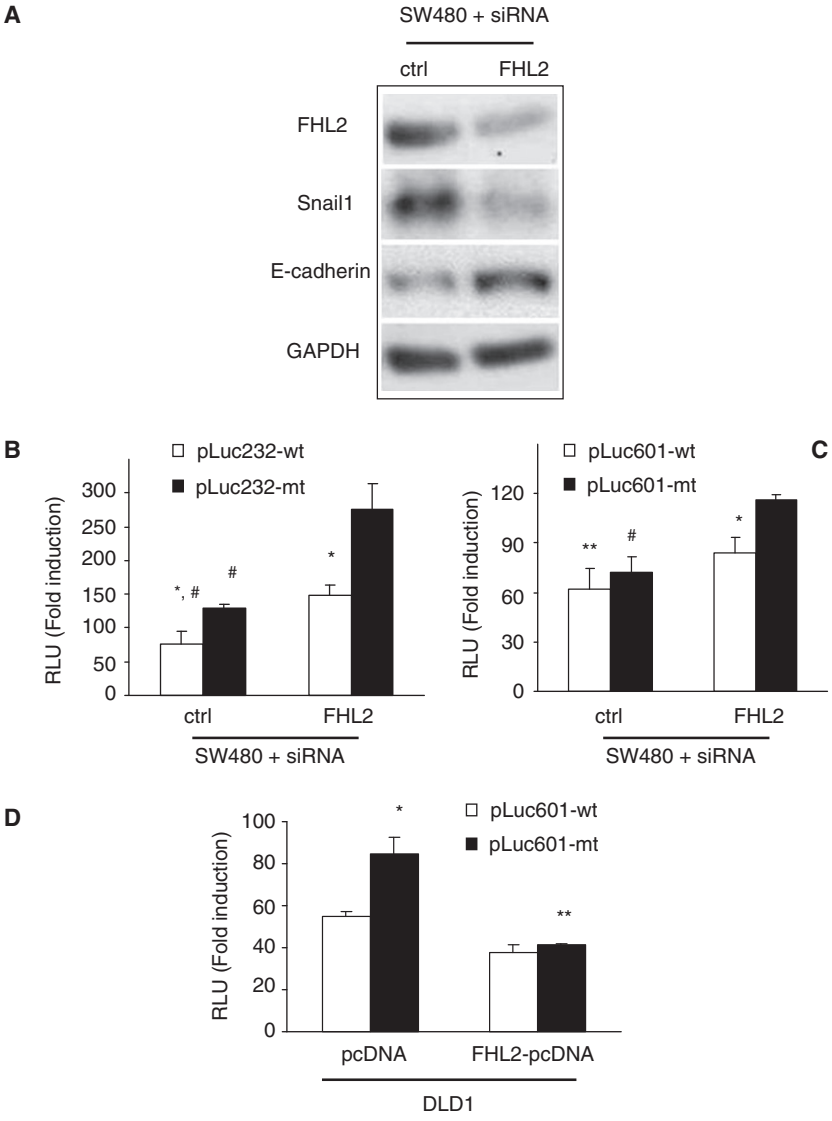

Fig. 5 – Effect of FHL2 on wildtype or mutant E-cadherin promoter activities. (A) SW480 cells were transfected with control (ctrl) or FHL2 siRNA for 48 h, FHL2, Snail1 and E-cadherin expressions were detected by Western blot with GAPDH being used as the internal control. (B) Transcription activities of pLuc232-wt and pLuc232-mt after transfecting into SW480 cells 24 h post the transfection with control (ctrl) or FHL2 siRNA. $^{*}p < 0.05$, between the wildtype and mutated constructs and $^{\#}p < 0.05$ between ctrl and FHL2 siRNA. (C) Transcription activities of pLuc601-wt and pLuc601-mt after transfecting into SW480 cells 24 h post the transfection with control (ctrl) or FHL2 siRNA. $^{*}p < 0.05$, $^{**}p > 0.05$, between the wildtype and mutated constructs and $^{\#}p < 0.05$ between ctrl and FHL2 siRNA. (D) Transcription activities of pLuc601-wt and pLuc601-mt in stable transfectants. $^{*}p < 0.05$ and $^{**}p > 0.05$ between the wildtype and mutated constructs.

of E-boxes increased the transcription activities of E-cadherin promoter. Significant difference was found between pLuc232-wt and pLuc232-mt (76.36 ± 18.32 versus 128.84 ± 6.92, $p < 0.05$) but not between pLuc601-wt and pLuc601-mt (61.8 ± 12.18 versus 72.24 ± 9.39, $p > 0.05$) in SW480 control cells (Fig. 5B and C). However, FHL2 siRNA could magnify the effect of both E-box 1 and E-box 4 mutations. In contrast, FHL2 over-expression decreased the magnitude of E-box mutation-induced E-cadherin transcription. The RLU of pLuc601-wt and pLuc601-mt were 54.92 ± 2.38 and 84.71 ± 7.82 in DLD1-pcDNA cells ($p < 0.05$) respectively and were 37.85 ± 3.49 and 41.23 ± 0.79 in DLD1-FHL2-cDNA cells ($p > 0.05$) respectively (Fig. 5D).

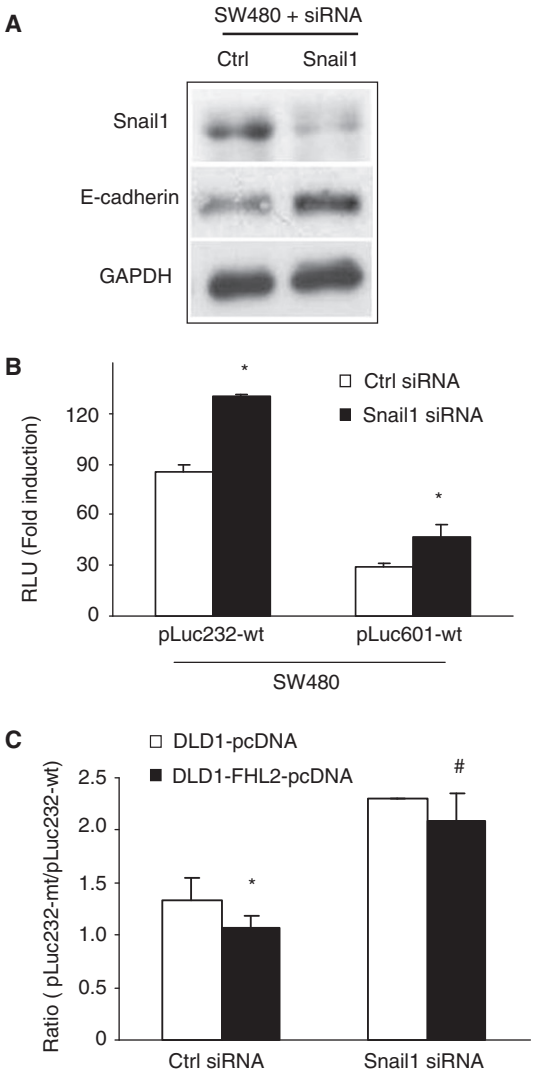

Fig. 6 – Transcriptional repression of E-cadherin by FHL2 is mediated by Snail1. (A) SW480 cells were transfected with control (ctrl) or Snail1 siRNA. The expressions of Snail1 and E-cadherin were detected by Western blot with GAPDH being used as the internal control. (B) SW480 cells were transfected with siRNAs followed by promoter-reporter constructs. Dual luciferase was performed to assess the transcription activities. $^{*}p < 0.05$ between the two siRNAs. (C) The wildtype and mutant of pLuc232 were transfected into DLD1 stable transfectants 24 h post the transfection of siRNAs. The RLU ratios of pLuc232-mt/pLuc232-wt were calculated. $^{*}p < 0.05$ and $^{\#}p > 0.05$ between the two stable transfectants.

3.6. *FHL2 modulated E-cadherin transcription is mediated by Snail1*

To further examine the effect of FHL2 on Snail1-mediated E-cadherin transcription, we first assessed the effect of Snail1 siRNA on E-cadherin. Consistent with other studies, the inhibition of Snail1 increased the expression of E-cadherin on both the protein level (Fig. 6A) and the transcription level (Fig. 6B). The RLU of pLuc232-wt and pLuc601-wt were 130.12 ± 1.19 and 47.10 ± 7.32, respectively, in cells transfected with Snail1 siRNA compared to cells treated with control siRNA, 85.38 ± 4.34 and 29.4 ± 1.42, respectively. Next, a combinational analysis with FHL2 over-expression and Snail1 knockdown was carried out. Snail1 siRNA was transfected into DLD1-FHL2-pcDNA and vector control transfectant, DLD1-pcDNA, and then dual luciferase assay was performed 24 h later. In order to express legibly, we defined the Y-axis as the ratio of pLuc232-mt/pLuc232-wt in Fig. 6C. We found that the magnifying effect of FHL2 on E-box mutation was minimised by the transfection with Snail1 siRNA.

4. Discussion

Downregulation of E-cadherin is an essential step in the progression of EMT. Here we demonstrate that FHL2 can form a complex with Snail1 which functions to repress E-cadherin. There are three members of the Snail family: Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc).[32] Although all of them are transcriptional repressors of E-cadherin through binding the E-box elements in the promoter region, Snail1 has the highest affinity.[33] Furthermore, Snail1 promotes tumour growth, invasion and metastasis, while Snail2 only has a milder effect in mouse skin carcinoma.[34] The effect of Snail1 on E-cadherin has been highlighted during EMT progression. On the other hand, the association between Snail1 and LIM protein has been verified.[30,31] FHL2 interacts with multiple transcriptional factors with different domains, for example, the association with FHL2 can be mediated by the full-length protein for the interaction with β-catenin, by LIM domains 1/2–2 for FOXO1, by LIM domains 1/2–3 for CDC47 and by the C-terminal LIM domains LIM 2–4 for BRCA1.[35] Interestingly, β-catenin can not only bind to FHL2 in its middle region (132–512 amino acid) but also mediates its binding with zinc finger protein Snail1 through its N-terminal segment (1–89 amino acid).[23] Therefore, it is possible that FHL2 directly or indirectly interacts with Snail1 and then affects the expression of E-cadherin. In this study, co-immunoprecipitation experiments showed that the four and a half LIM domains mediated the FHL2–Snail1 interaction. On the contrary, all of the FHL2 deletion mutants analysed here had lost the capacity to bind Snail1. This situation is similar to the interaction pattern between FHL2 and β-catenin, but different from the interaction between AJUBA LIM protein and Snail1 which is only mediated by LIM1 and/or LIM2. These findings indicate that the different LIM domains can have different regulatory effects

on their interacting partners and also might contribute to a cell-specific manner.

Snail1 is very unstable with a short half-life; it cycles between the nucleus and the cytosol of cells. Zhou and colleagues reported that the stability of Snail1 was dependent on its interaction with β-Trcp and its subcellular localisation was regulated by the phosphorylation, which were both regulated through interaction with GSK-3β.[16] In our study, we found FHL2 was able to interact with Snail1. Therefore, there may exist a competitive mechanism between FHL2 and GSK-3β for their respective interaction with Snail1. When FHL2 is over-expressed, the potential binding capacity between GSK-3β and Snail1 is attenuated resulting in the decreased phosphorylation of Snail1. Moreover, GSK-3β is involved in the phosphatidylinositol 3-kinase/Akt (PI3K/Akt), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), Wnt/β-catenin and Hedgehog pathways, all of which are engaged in EMT.[36] The activation of FHL2 in colon cancer cells promotes the invasive potential and EMT.[25] Interestingly, FHL2 can interact with ERK and β-catenin and then influence their activation.[37,25] So, it is possible that FHL2 directly or indirectly inhibits GSK-3β and thus in turn results in the stabilisation and nuclear localisation of Snail1 to trigger the process of EMT. However, many further investigations need to be performed to characterise the relationship between FHL2 expression and the phosphorylation and cellular localisation of Snail1.

In this study, we revealed the inverse relation between FHL2 expression and E-cadherin promoter activities, which were consistent with our previous findings of that upregulation of FHL2 inhibited E-cadherin expression at the levels of both mRNA and protein. Except for the E-box element, there also exist specificity protein 1- (Sp1-), acute myeloid leukaemia- (AML1-) and p300-binding sites in the longer plasmid, pLuc601-wt. The Sp1 transcription factor has been reported to upregulate the human E-cadherin gene.[9] Consistent with other study,[9] our mutation experiments on Snail1-binding elements indicated that the E-box 1 and E-box 4 were both functional on regulating E-cadherin expression.

In conclusion, our results uncovered a novel and unexpected regulatory function of FHL2 and identified Snail1 as a new FHL2-binding protein. It also shed light on the elucidation of the molecular mechanism of FHL2 for the regulation of EMT process in colon cancer.

Conflict of interest statement

None declared.

Acknowledgements

Supported by the Simon K.Y. Lee Gastroenterology Research Fund, the Gordon Chiu stomach cancer research fund, University of Hong Kong, the grants from the Research Grant Council of Hong Kong Special Administrative Region to Dr. J. Wang (HKU7785/09M) and the grants to C. Sardet from the French Agence National de la recherche (ANR), the Fondation pour la Recherche M'dicale (FRM) and from the National Natural Science Foundation of China (30973404).

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