Proteinase-activated Receptor 2 Promotes Cancer Cell Migration through RNA Methylation-mediated Repression of miR-125b*

Lan Yang[‡], Yiming Ma[‡], Wenxiao Han[‡], Weiwei Li[‡], Liang Cui[§], Xinhua Zhao[‡], Yantao Tian[§], Zhixiang Zhou[¶], Wengong Wang[‖], and Hongying Wang[‡1]

From the [‡]State Key Laboratory of Molecular Oncology, [§]Department of Abdominal Surgical Oncology, and [¶]Department of Gastrointestinal Surgical Oncology, Cancer Institute/Cancer Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, 100021 Beijing, PR China and [‖]Department of Biochemistry and Molecular Biology, Peking University Health Science Center, 100191 Beijing, PR China

Background: Proteinase-activated receptor 2 (PAR$_2$) correlated with cancer metastasis.
Results: Down-regulation of miR-125b, which targets Gab2, mediates PAR$_2$-induced cancer cell migration.
Conclusion: PAR$_2$ promotes cancer cell migration through RNA methylation-mediated repression of miR-125b.
Significance: This study suggests a novel epigenetic mechanism by which miRNA expression is altered to regulate cancer cell migration.

Proteinase activated-receptor 2 (PAR$_2$) participates in cancer metastasis promoted by serine proteinases. The current study aimed to test the molecular mechanism by which PAR$_2$ promotes cancer cell migration. In different cancer cells, activation of PAR$_2$ by activating peptide (PAR$_2$-AP) dramatically increased cell migration, whereas knock down of PAR$_2$ inhibited cellular motility. The PAR$_2$ activation also repressed miR-125b expression while miR-125b mimic successfully blocked PAR$_2$-induced cell migration. Moreover, Grb associated-binding protein 2 (Gab2) was identified as a novel target gene of miR-125b and it mediated PAR$_2$-induced cell migration. The correlation of PAR$_2$ with miR-125b and Gab2 was further supported by the findings obtained from human colorectal carcinoma specimens. Remarkably, knock down of NOP2/Sun domain family, member 2 (NSun2), a RNA methyltransferase, blocked the reduction in miR-125b induced by PAR$_2$. Furthermore, PAR$_2$ activation increased the level of N^6-methyladenosine (m^{6}A)-containing pre-miR-125b in NSun2-dependent manner. Taken together, our results demonstrated that miR-125b mediates PAR$_2$-induced cancer cell migration by targeting Gab2 and that NSun2-dependent RNA methylation contributes to the down-regulation of miR-125b by PAR$_2$ signaling. These findings suggest a novel epigenetic mechanism by which microenvironment regulates cancer cell migration by altering miRNA expression.

Metastasis is the main cause of mortality in cancer patients. Besides the genetic background of cancer cells, alteration in microenvironment has emerged as a vital factor to regulate the progression of cancer. Proteases are important component in microenvironment. Serine proteases, such as trypsin, mast cell-derived tryptase, matriptase, tissue factors, and KLKs, are strongly associated with cancer metastasis (1–3). Because of the complexity of microenvironment, the molecular mechanisms by which proteinases promote metastasis have not been fully understood. Notably, all these proteinases can selectively activate protease-activated receptor 2 (PAR$_2$)[2] through proteolysis of the receptor (4).

PAR$_2$ belongs to the PAR family of G-protein-coupled receptors (GPCRs). PAR$_2$ is highly expressed in various cancers (5–7), and blocking PAR$_2$ activity with monoclonal antibodies effectively suppresses tumor growth *in vivo* (8). It has been reported that PAR$_2$ activation not only promotes cell proliferation (7, 9–12) but also contributes to hypoxia-induced angiogenesis as well (13). Interestingly, PAR$_2$ and its activating proteinases are also expressed at the leading edge of cancer invasion (10, 14), and its expression levels are tightly correlated with lymphatic metastasis in breast cancer and colorectal carcinoma (15, 16). All these observations suggest that activation of PAR$_2$ promotes cell motility and facilitates tumor cell invasion. However, the mechanism by which PAR$_2$ regulates cancer metastasis has not been fully understood.

MicroRNAs (miRNAs) are small ~22 nucleotide (nt) RNA molecules that modulate gene expression by binding target mRNA usually in the 3′-untranslated region, leading to translational repression and/or degradation of mRNA (17). Increasing evidence indicate that given miRNAs including miR-125b are actively involved in the progress of tumorigenesis including metastasis and invasion (18–20). Expression of the miR-125b is suppressed in different cancers, and decreased level of miR-125b is associated with increased metastasis and the poor prog-

nosis in patients with hepatocellular, breast cancers, and small cell carcinoma of the cervix (21–23). In addition, miR-125b is shown to modulate cell migration by targeting specific genes such as MTA1 (metastasis-associated gene 1) (24), Erbb3 (erythroblastic leukemia oncogene homolog 3) (25), and Lin28B(Lin-28 homolog B) (21).

In the present study, we demonstrated that miR-125b mediates cell migration induced by PAR_2 activation and identified Gab2 (Grb2-associated-binding protein 2) as a novel target gene of miR-125b. Moreover, it was found that PAR_2 decreased the level of miR-125b through NSun2 (NOP2/Sun RNA methyltransferase family, member 2)-mediated pre-miR-125b2 methylation. Our findings not only reveal a novel epigenetic mechanism underlying promotion of cancer metastasis by PAR_2 but also provide potential target for development of new therapeutic approaches in human cancer.

Experimental Procedures

Cell Culture and Treatment—Human colonic epithelial cell lines HT-29, SW620, HCT-116, RKO, and human lung adenocarcinoma A549 cells were purchased from ATCC (Manassas, VA). These cells were grown in Dulbecco's modified Eagle's medium/F12 (Hyclone) supplemented with 10% fetal bovine serum (Gibco). Stable transfectant cell lines with PAR_2 knockdown were developed and cultured as described previously (16).

To selectively activate PAR_2, cells were treated with PAR_2 activating peptide (SLIGRL-NH$_2$, 100 μM) (Shanghai Apeptide Co. Ltd., China). Same amount of reverse peptide (LRGILS-NH$_2$) was used as control. MiR-125b mimic, miR-125b inhibitor, and negative control oligos were obtained from GeneCopoeia (GuangZhou Ribobio Co. Ltd., China). siRNA targeting Gab2(5′-GAG ACA GCG AAG AGA ACU AUU-3′); siRNA targeting BMPR1B (5′-GGG CAA ACU UCC UUG AUA AUU-3′) and NSun2 (5′-GCC UCA UCA UAA GAU CUU AUU-3′) were purchased from Genepharma (Suzhou, China). For 5′-azacytidine (5-aza-CR) treatment, A549 cells were seeded at 2×10^5 per well in 6-well plates and incubated with 5 μmol/liter 5-aza-CR (Sigma-Aldrich) for 72 h before the treatment with PAR_2-AP. The medium containing 5-aza-CR was replaced every 24 h.

RNA Isolation and Real-time PCR—Total RNA was isolated from cell lines, human, or animal samples by using Trizol reagent (Invitrogen). After treatment with DNase I, RNA was reverse transcribed into cDNA with Thermo scientific maxima first strand cDNA synthesis kit for mRNA or pri-miR-125b detection, or with Takara™ microRNA transcription kit for mature and pre- microRNA detection. Real-time quantitative PCR was carried out on Bio-Rad S1000 PCR instrument, and each sample was analyzed in triplicate. PCR data were normalized to GAPDH or U6 snRNA expression for mRNA and miRNA, respectively.

Primers for mature miR-125b and PAR_2 were obtained from GeneCopoeia. Other primers were as follows: Gab2 sense, 5′-CGC TGC TA5′-GAC AAC AGC CGA CTT CAC C-3′ and antisense, 5′-GCC CAC AAT CAT TTT CCC T-3′; BMPR1B sense, 5′-TGA TGG ACC TAT ACA CCA CAG G-3′ and antisense, 5′-ATA GTC CTT TGG ACC AGC AGA G-3′;

NSun2 sense, 5′-CGC TGC TAC CTG CTC GTC-3′ and antisense, 5′-TTT CTC ATA GTG CCG TCT CCA-3′; pri-miR-125b1 sense, 5′-CCA TAC CAC CTG TTT GTT GCA TCT-3′ and antisense, 5′-CTG AGA GGA GCG CAA CAA TGT-3′; pri-miR-125b2 sense, 5′-GAA GAA TTC TAC CGC ATC AAA CCA-3′ and antisense, 5′-CTG CAG ACA ATC AAT AAG GTC CAA-3′; pre-miR-125b1 sense, 5′-TGG GAG CTG CGA TGC T-3′; pre-miR-125b2 sense, 5′-GCT CTT GGG ACC TAG GCG GA-3′. The antisense primer for miRNAs was bought from Takara (Dalian, China).

For overexpression study, full-length of Gab2 and BMPR1B sequence were purchased from Vigene (Rockville, MD, USA) and subcloned into pENTER expression vector, respectively.

Western Blot—Western blot was conducted as described earlier (16). Briefly, proteins were extracted from cultured cells with RIPA buffer (0.01% EDTA, 0.1% Triton X-100, and 10% proteinase inhibitor mixture). Protein concentrations were quantified using a protein assay kit (Bio-Rad). Lysates were separated on 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. The membranes were probed overnight at 4 °C with primary antibody against human Gab2 (Cell Signaling Technology, 1:1000), β-catenin (Cell Signaling Technology, 1:1000), or NSun2 (Abcam, 1:2000), followed by incubation with peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h. The signal was visualized with ECL (Millipore).

Migration Assay—After starving in serum-free media for 12 h, 5×10^4 cells were seeded on the top chamber of transwell (8.0-μm pore; Corning, MA) and incubated with 100 μM PAR_2-AP, while 600 μl media containing 10% FBS was added to the bottom chamber. Ten hours later, the cells remaining in the upper chamber were removed with cotton swabs, and the cells on the undersurface of transwells were fixed and stained with crystal violet solution. The number of migratory cells was calculated by counting five different fields under a phase-contrast microscope of each transwell filter.

Gab2 3′-UTR Luciferase Reporter—3′-UTR sequence of Gab2 containing the putative miR-125b target sites was amplified from A549 genomic DNA using Gab2 sense, 5′-CGC TAG CCG TTC TTC CTC CCA TCC AC-3′ and antisense, 5′-CTC TAG ACC ACT GCT GAG CCT CCG TC-3′ and cloned immediately into the downstream of the luciferase reading frame in the plasmid PIS0-Report-vector. To generate the 3′-UTR fragments mutating the miR-125b recognition elements by KOD-plus-mutagenesis kit (TOYOBO Co., Ltd. Japan), the following primer pairs were used: 5′-TGA CAA CTG CGT CCC TGA ACC CTT CCC CTG GGA GGT G-3′ and antisense, 5′- AGT GCT TTG AAG CTT GAG AC-3′. The sequence of PCR product was confirmed by DNA sequencing.

Transient Transfection and Luciferase Assay—The cells in 24-well plates were transfected with or without miR-125b mimic (100 nM) together with wild type or mutant Gab2 3′UTR reporter (200 ng) and pRL-TK (2 ng). The transfection agent Lipofectamine 2000 (Invitrogen) was incubated with DNA or RNA in serum-free medium for 20 min before being added to cells and then incubating for an additional 2 h. After treatment with PAR_2-AP, cell lysate was collected for luciferase activity
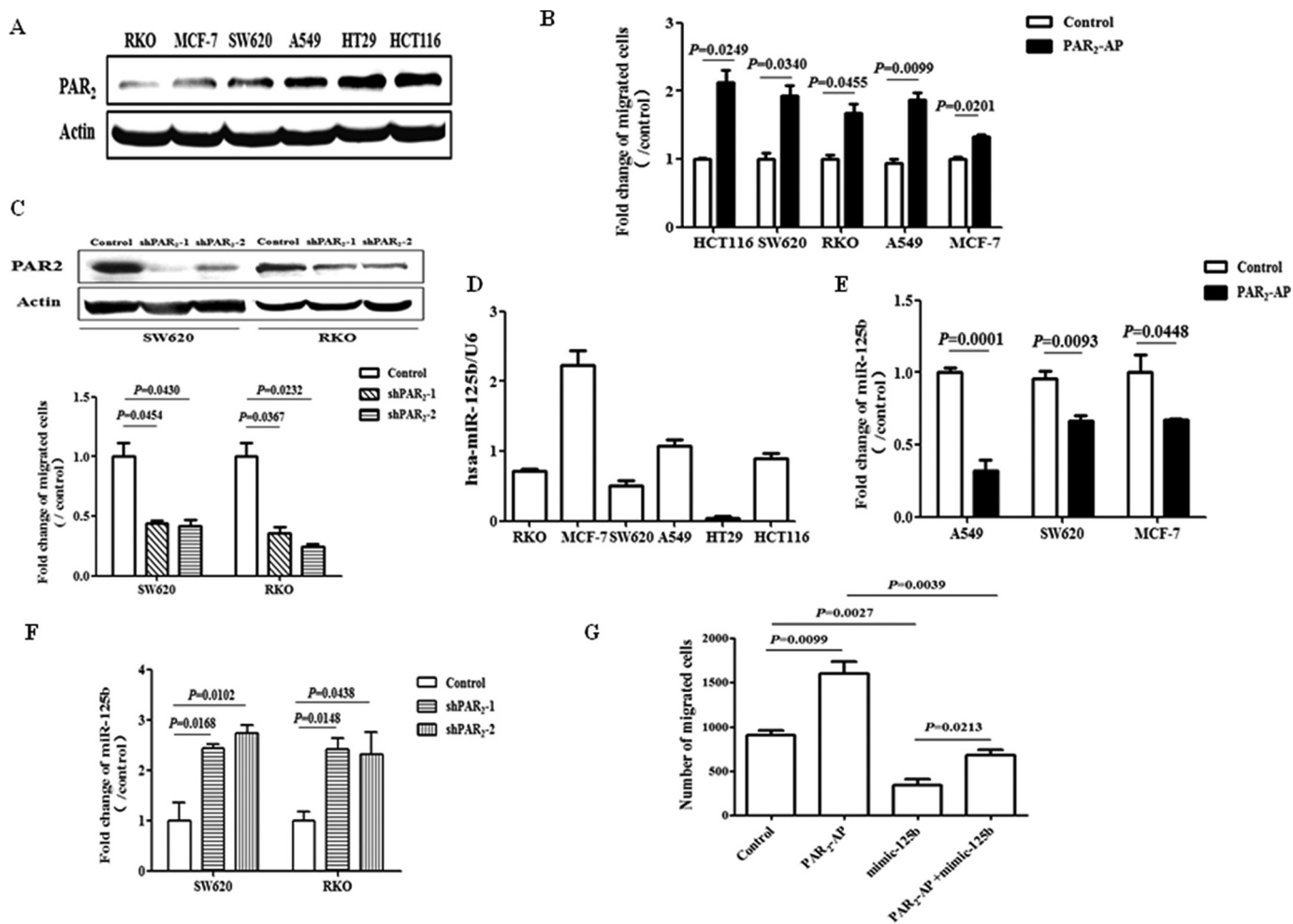
FIGURE 1. **PAR₂ activation promotes cell migration through the down-regulation of miR-125b.** *A*, expression of PAR₂ protein was measured with Western blot in different cell lines. *B*, cell migration was evaluated with transwell assay. Cells were treated with the activating peptide of PAR₂ (PAR₂-AP, 100 μM) for 10 h and then migrated cells were stained and quantified. *C, upper panel*, Western blot shows the protein level of PAR₂ in SW620 and RKO cells stably transfected with or without shPAR₂. ShPAR2–1 and shPAR2–2 represent different clones from the same parental cells. *Lower panel*, cell migration was tested with transwell assay in these cells. *D*, expression level of miR-125b was measured with real time PCR in different cell lines. *E*, after the treatment with PAR₂-AP (100 μM) for 6 h, cells were collected and tested for miR-125b expression by real-time PCR. *F*, level of miR-125b was measured in SW620 and RKO cells stably transfected with or without shRNA-PAR₂ (shPAR₂). *G*, cells were transfected with miR-125b mimic (50 μM). For the migration assay, thirty-six hours later, cells were seeded into transwell and treated with PAR₂-AP for another 10 h. The data showed the means ± S.D. from three independent experiments.

assay, which was performed with a Dual-Luciferase assay kit (Promega). All data were normalized by pRL-TK.

Measurement of m⁶A RNA—To detect M⁶A RNA, cell extract was prepared with EZ-Magna RIP™ Kit (Millipore) according to the manufacturer's instructions. Briefly, after treatment with PAR₂-AP for a half hour, A549 cells were collected and lysed in RIP lysis buffer. To pull down m⁶A RNA, whole-cell extract was immunoprecipitated with anti-m⁶A antibody (Synaptic Systems) conjugated with magnetic beads. After incubation with proteinase K, RNA was recovered followed by treatment with DNase I to avoid DNA contamination. The RNA samples were then subjected to RT and real-time quantitative PCR to detect pri-miR-125, pre-miR-125b and miR-125b. Normal mouse IgG was used as negative control. Sample without immunoprecipitation was used as input and positive control.

Human Subjects Tissue Specimen—All the colorectal cancer tissues and the paired normal tissues (≥5 cm from cancer tissue) were obtained from Cancer hospital, Chinese Academy of Medical Sciences, Beijing, China. All specimens were classified according to the tumor node metastasis (TNM) classification system. No patients received chemotherapy or radiotherapy before resection. Tissues were submerged in RNA later™ (Ambion) for 24 h and then stored at −70 °C until use. The use of human tissues was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute.

Statistical Analysis—Statistical analyses were performed on data collected from at least three independent experiments. Data were presented as means ± S.D. and analysis using GraphPad Prism 5 software. Comparison of >2 groups was made using ANVOA with Tukey test. Comparison of 2 groups was made using Student's *t* test for unpaired data. The differences were considered statistically significant when *p* value less than 0.05.

Results

MiR-125b Mediates PAR₂-induced Cell Migration—In the first set of experiments, we found that PAR₂ protein was
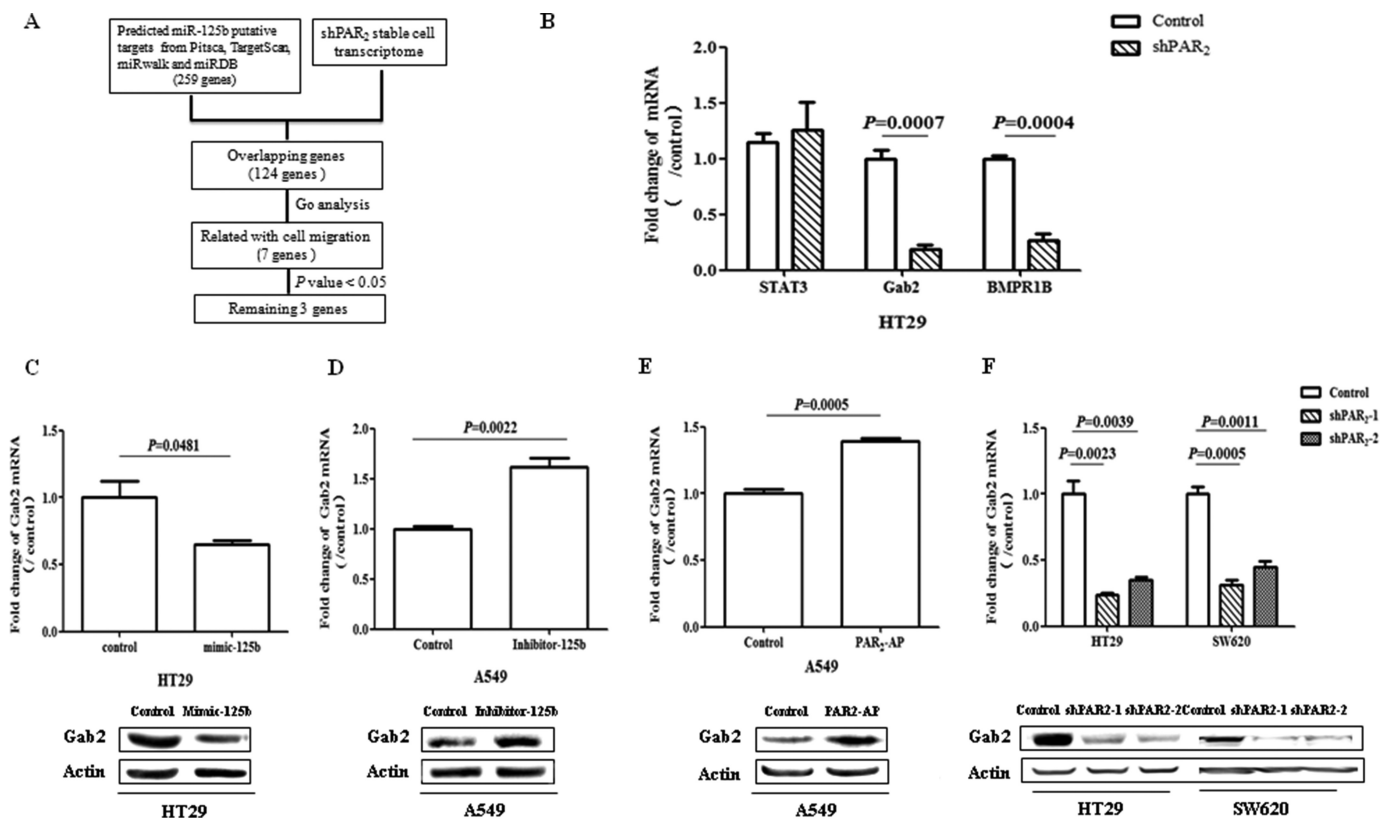
FIGURE 2. **miR-125b target genes associated with PAR₂ signaling.** *A*, schematic illustration of experimental setup. *B*, mRNA expression of potential target genes were measured with real time-PCR in HT29 cells with or without shPAR₂. *C*, after the transfection with miR-125b mimic (mimic-125b, 50 μM), the level of Gab2 was measured by real-time PCR and Western in HT29 cells. *D*, after the transfection with miR-125b inhibitor (Inhibitor-125b, 100 μM), the mRNA and protein levels of Gab2 were measured in A549 cells. *E*, Gab2 mRNA and protein expression was measured in A549 cells after the treatment with PAR₂-AP for 6 h. *F*, levels of Gab2 mRNA and protein were measured in HT29 and SW620 with or without shPAR₂. The data showed the means ± S.D. from three independent experiments.

expressed in all the human cancer cell lines we tested (Fig. 1*A*). To determine whether PAR₂ activation induce cell migration, activating peptide (PAR₂-AP) was used to selectively activate PAR₂ on cell surface. As shown, activation of PAR₂ by PAR₂-AP for 10 h significantly stimulated cell migration as measured by the transwell assay in different cancer cell lines (Fig. 1*B*). And there was no any effect on cell proliferation as examined by MTT assay (data not shown). In addition, knockdown of PAR₂ expression with shRNA remarkably attenuated cell migration compared with that observed in scramble RNA control group in different cancer cells (Fig. 1*C*). These results strongly suggested that PAR₂ activation promotes migration in different cell lines.

To test whether miR-125b involves in PAR₂-related migration, we measured the level of miR-125b and found that it negatively correlated with PAR₂ expression in all cell lines we used (Fig. 1*D*). In addition, the expression of miR-125b was significantly repressed after the stimulation with PAR₂-AP (Fig. 1*E*). On the contrary, inhibition of PAR₂ expression dramatically elevated miR-125b expression compared with scramble RNA control group (Fig. 1*F*). Importantly, over expression of miR-125b blocked cell migration induced by PAR₂ activation in A549 cells (Fig. 1*G*). Similar results were observed in SW620 cells (data not shown). Taken together, these findings indicate that down-regulation of miR-125b mediates PAR₂-induced cell migration.

Prediction of miR-125b Target Genes—To inquire into the mechanism by which miR-125b mediates PAR₂-induced cell migration, we identified the downstream targets of miR-125b. First, the target mRNAs of miR-125b were predicted with different database including Pitsca, TargetScan, miRwalk, and miRDB. There were 259 potential target transcripts by at least two databases. And 124 of them were overlapped with PAR₂-related transcriptome, which was conducted with Human Gene Expression Microarray V3.0 in HT-29 cells. Among them, 7 genes were annotated involved in cell migration according to the DAVID Annotation Bioinformatics Database. Furthermore, 3 of them showed significant difference between stably knockdown of PAR₂ group and scramble control group ($p < 0.05$) (Fig. 2*A*). At last, validating with real-time PCR, Gab2, and BMPR1B were significantly down-regulated with the knock down of PAR₂ in HT29 cells (Fig. 2*B*).

Since BMPR1B has been reported as the target gene of miR-125b (Ref. 49), we verified the regulation of Gab2 by miR-125b. It was found that forced expression of miR-125b achieved by the transfection with the mimic of miR-125b inhibited Gab2 expression at both mRNA and protein levels (Fig. 2*C*). In contrast, the inhibitor of miR-125b elevated the expression of Gab2 (Fig. 2*D*). Furthermore, activation of PAR₂ increased Gab2 expression (Fig. 2*E*) while disruption of PAR₂ signaling repressed Gab2 (Fig. 2*F*) at both mRNA and protein levels. All

A

H.Sapiens	UGACA**UCAGGGA**CCCUGAACCCUUC
P.Trogiodytes	UGACA**UCAGGGA**CCCUGAACCCUUC
B.Taurus	UGGCA**UCAGGGA**CCCUGACUCCUUC
C.Familiaris	UGAUG**UCAGGGA**CCCUGAACCCUUC
M.Musculus	CAACA**UCAGGGA**CCU-GACCCUUUC
R.Norvegicus	CAACG**UCAGGGA**CCU-GACCCUUUC

B

Lucerferase Gab2 3'UTR

Wild type 3'UTR (nt 137-143)   5'... AAAGCACUUGACAUCAGGGA

Mature-miR-125b   3'... UGUUCAAUCCCAGAGUCCCU

Mutant 3'UTR   5'... AAAGCACUUGACAACUGCGU

C

$P < 0.0001$ $P < 0.0001$

Luciferase Activity (/control)

Control mimic-125b | Control mimic-125b
Wild Type | Mutation

D

$P = 0.0017$

Luciferase Activity (/control)

Control PAR₂-AP | Control PAR₂-AP
Wild Type | Mutation

E

$P = 0.0177$ $P = 0.0086$

Fold change of Gab2 mRNA (/control)

control PAR₂-AP | control PAR₂-AP
| mimic-125b

A549

Gab2
Actin

Control PAR2-AP Control PAR2-AP
Mimic-125b

F

$P = 0.0093$ $P = 0.0031$ $P = 0.0031$

Fold change of Gab2 mRNA (/control)

control shPAR₂ | control shPAR₂
| Inhibitor-125b

SW620

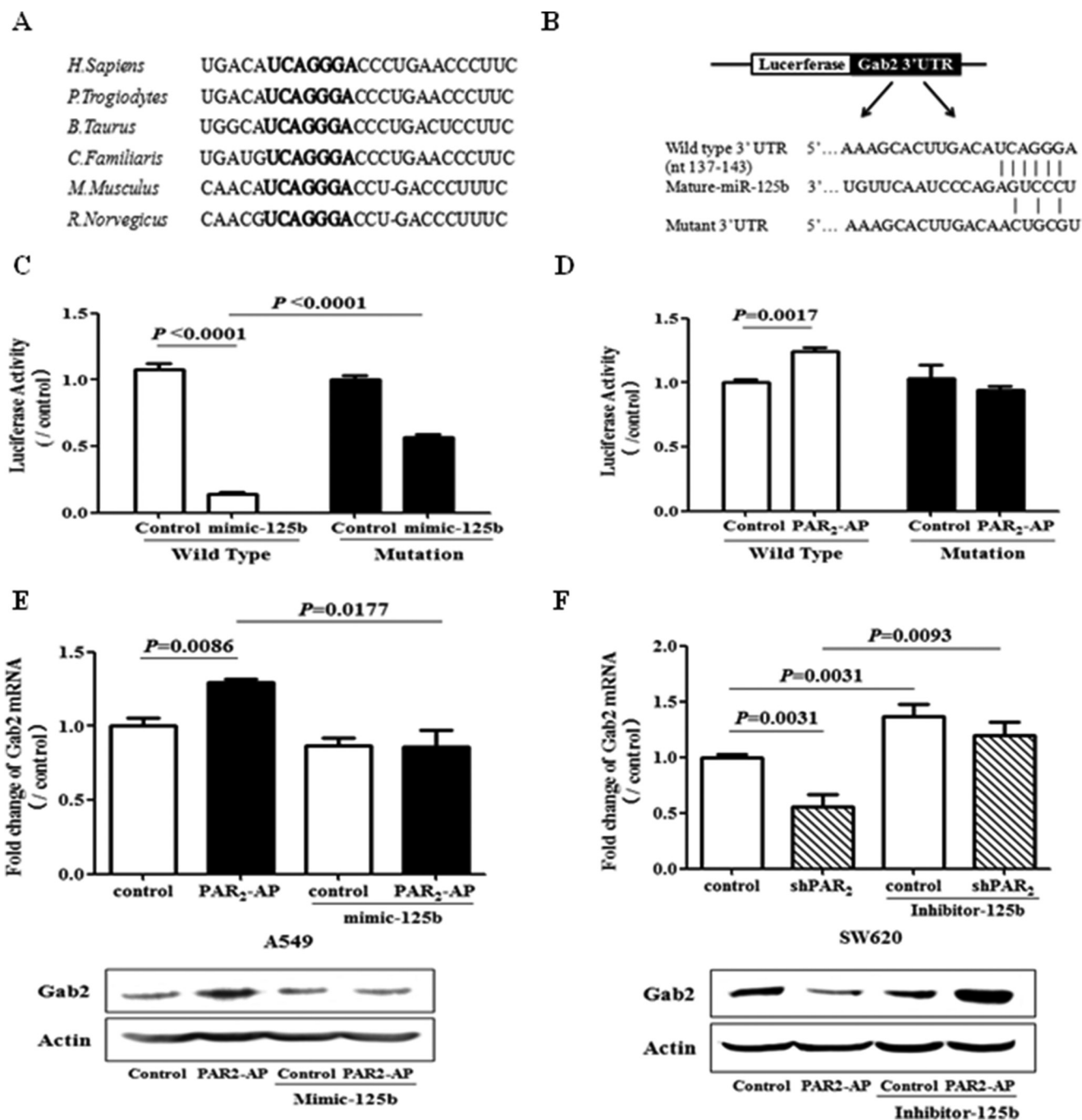Gab2
Actin

Control PAR2-AP Control PAR2-AP
Inhibitor-125b

FIGURE 3. **Gab2 is a direct downstream target of miR-125b.** *A*, potential binding site of miR-125b in Gab2 3'-UTR in different species. *B*, diagram showed the putative miR-125b-targeting seed sequence on Gab2–3'-UTR and illustrated the structure of wild type and mutant luciferase reporter of Gab2 3'-UTR. *C*, A549 cells were transfected with or without miR-125b mimic combined with wild type or mutant luciferase reporter of Gab2–3'-UTR. Twenty-four hours later, the cells were collected for luciferase assay. *D*, after the transfection with wild type or mutant luciferase reporter of Gab2–3'-UTR, A549 cells were stimulated with PAR₂-AP for 6 h and then collected for luciferase assay. *E*, A549 cells were transfected with miR-125b mimic (mimic-125b) or control RNA. One day after transfection, the cells were treated with PAR₂-AP for 6 h, and the cell lysates were collected for Gab2 mRNA and protein expression assay. *F*, SW620 cells with or without stable transfection of shPAR₂ were transfected with inhibitor of miR-125b (Inhibitor-125b) or control RNA. Twenty-four hours later, the cell lysate was collected for Gab2 mRNA and protein expression assay. The data showed the means ± S.D. from three independent experiments.

these results strongly suggest that Gab2 is a possible target gene of miR-125b.

Gab2 Was Identified as a Novel Target Gene of miR-125b— To confirm Gab2 is a direct target gene of miR125b, the possible binding site of miR-125 in the Gab2 mRNA was analyzed. TargetScan analysis indicated that Gab2 contains miR-125b binding site within its 3'-UTR, and the sequence of the binding

site is highly conserved across different species (Fig. 3*A*). To further validate that Gab2 is regulated by miR-125b through interaction of the complementary 3'-UTR region, luciferase reporters containing wild-type or mutant 3'-UTR of Gab2 were constructed (Fig. 3*B*). MiR-125b mimic significantly reduced the activity of the luciferase reporter, which was partially abolished by the mutation of miR-125b binding site in 3'-UTR of
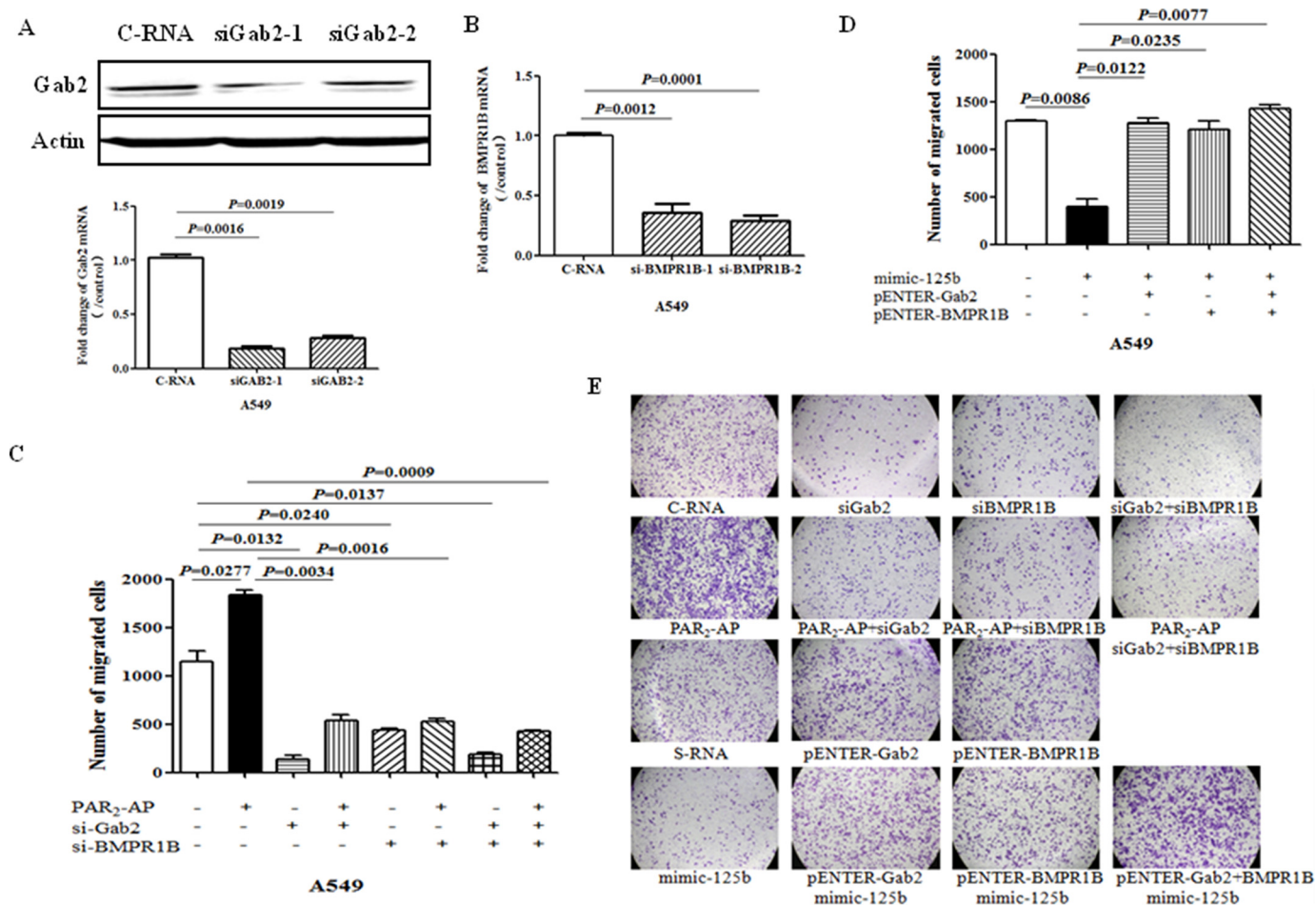
ASBMB

FIGURE 4. **Gab2 and BMPR1B mediate PAR₂-induced cell migration.** A549 cells were transfected with siRNA against Gab2 or BMPR1B. Forty hours after transfection, the cells were collected to measure the expression of (*A*) Gab2 or (*B*) BMPR1B at protein and/or mRNA levels. *C*, for cell migration assay, forty hours after transfection, the cells were seeded on transwell and treated with PAR₂-AP. After 10-h treatment, migrated cells were quantified. *D*, after transfection with mimic miR-125b with or without Gab2 or/and BMPR1B overexpression plasmid, the ability of cell migration was measured with transwell assay. *E*, representative pictures for the migration assay shown for 10 h. The data showed the means ± S.D. from three independent experiments.

Gab2 (Fig. 3*C*). Furthermore, PAR₂ activation up-regulated the activity of wild type Gab2 3′-UTR-derived luciferase reporter but not the mutated one (Fig. 3*D*). Consistent with luciferase activity, PAR₂ activation also elevated Gab2 mRNA and protein levels, which were reduced by overexpression of miR-125b (Fig. 3*E*). Contrarily, inhibition of PAR₂ repressed the levels of Gab2 mRNA and protein, which was recovered by the inhibitor of miR-125b (Fig. 3*F*). All these results suggest that miR125b partially regulates Gab2 expression through the binding site within 3′-UTR of Gab2 mRNA.

Gab2 Mediates PAR₂-induced Cell Migration—To test whether Gab2 mediates PAR₂-induced cell migration, specific siRNAs against Gab2 were applied to reduce the expression of Gab2 at both mRNA and protein levels (Fig. 4*A*). Knockdown of Gab2 significantly blocked PAR₂-induced cell migration (Fig. 4, *C* and *E*). Since BMPR1B, a known target gene of miR-125b, had been changed after PAR₂ activation, we checked the effect of BMPR1B on PAR₂-induced cell migration. Knockdown of BMPR1B with specific siRNA (Fig. 4*B*) also blocked cell migration stimulated by PAR₂ activation (Fig. 4, *C* and *E*). In addition, overexpression of Gab2 or/and BMPR1B totally reversed the repression of cell migration induced by miR-125b (Fig. 4, *D* and

E). These findings indicate that repression of miR-125b mediates cell migration induced by PAR₂ through targeting Gab2 and BMPR1B expression *in vitro*.

Correlation of PAR₂, miR-125b, and Gab2 in CRC Specimens—To confirm the findings *in vivo*, we tested the expression of PAR₂, miR-125b and Gab2 in 40 paired human CRC tissue specimens measured by real-time PCR. As shown in Fig. 5*A*, both PAR₂ and Gab2 were up-regulated while miR-125b was down-regulated in lymphatic metastasis group compared with those of non-metastasis group. Importantly, in lymphatic metastasis group, the level of PAR₂ was negatively correlated with that of miR-125b and positively correlated with that of Gab2 (Fig. 5*B*). Furthermore, miR-125b expression was negatively correlated with Gab2 levels (Fig. 5*B*). In addition, the level of PAR₂ was also positively correlated with Gab2 in gene expression profiling of lung adenocarcinoma (NCBI GEO accession number GSE31546) uploaded by Hamon Center for Therapeutic Oncology Research of UT Southwestern Medical Center, which provided 17 primary lung cancer specimens expression profiling (Fig. 5*C*). These results strongly suggest that PAR₂ mediates metastasis through miR-125b/Gab2 in human CRC.
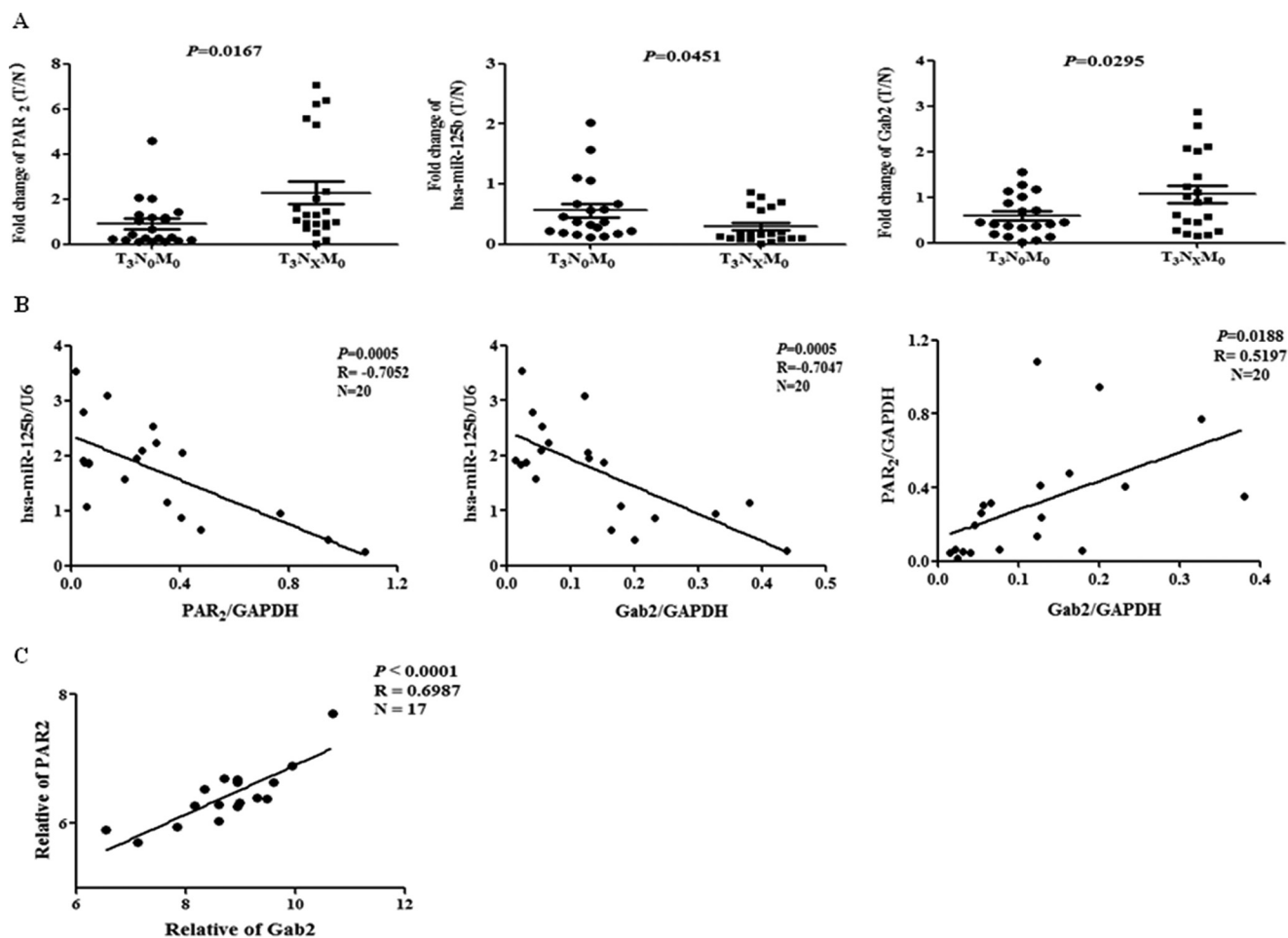
FIGURE 5. **Correlation of PAR$_2$, miR-125b and GAB2 in human specimens.** *A*, expression of PAR$_2$, Gab2 and miR-125b in human CRC samples was tested with real time PCR. The data are shown as the fold change of tumor sample (*T*) to paired normal tissue (*N*). *B*, Pearson's correlation analysis was conducted for PAR$_2$/miR-125b, Gab2/miR-125b and PAR$_2$/Gab2. *Black line* represents linear regression line. *C*, Pearson's correlation analysis was conducted for PAR$_2$/Gab2 in human lung cancer specimens microarray, which provided 17 primary lung cancer specimens gene expression profiling (NCBI GEO accession number GSE31546).

PAR$_2$ Suppresses miR-125b through NSun2-mediated Repression of miR-125b Processing—Since miR-125b mediate PAR$_2$-induced cell migration both *in vitro* and *in vivo*, it is important to understand how PAR$_2$ signaling regulates the level of miR-125b. Recent studies showed that the methylation state of CpG-rich regions is correlated with the expression pattern of miR-125b in breast cancer (22). To investigate whether hypermethylation of miR-125b promoter contribute to PAR$_2$-induced repression of miR-125b, 5-azacytidine, a demethylation agent, was used. The data showed that pretreatment with 5-azacytidine completely reverse the level of miR-125b reduced by PAR$_2$-AP (Fig. 6*A*). However, there was no obvious change in methylation status of pri-miR-125b1 promoter as measured by MSP after PAR$_2$ activation (Fig. 6*B*). Moreover, there was no significant change of primary miR-125b after PAR$_2$ activation (data not shown).

Actually, mature miR-125b comes from two primary forms, pri-miR-125b1 and pri-miR-125b2. Most recently, NSun2, a RNA methyltransferase, is shown to interfere in the mature processing of miR-125b from pri- and pre-miR-125b2. Interestingly, we found that pri- and pre-miR-125b2 was the predominant form of miR-125 precursor in A549 cells but not pri- and

pre-miR-125b1 (Fig. 6*C*). Furthermore, knockdown of NSun2 with specific siRNA (Fig. 6*D*) blocked the repression of miR-125b induced by PAR$_2$ activation (Fig. 6*E*).

To test whether PAR$_2$ activation induces methylation of miR-125b and its precursors, methylated RNA was pull down with anti-m^{6}A antibody and miRNA and its precursors were measured with qPCR. The data showed that PAR$_2$ activation induced m^{6}A methylation of pre-miR-125b2 (Fig. 6*F*) but not pri-miR-125b2 (Fig. 6*G*). Moreover, knockdown of NSun2 completely blocked both PAR$_2$-induced m^{6}A methylation of pre-miR-125b2 (Fig. 6*F*) and PAR$_2$-induced Gab2 mRNA expression (Fig. 6*H*). These findings strongly suggest that PAR$_2$ reduces miR-125b expression through an NSun2-dependent and m^{6}A methylation-involved mechanism.

Discussion

Both PAR$_2$ and its-activating proteinases, including tissue factor, trypsin, KLKs, are aberrantly overexpressed in a variety of malignancies, and shown to be involved in many aspects of cancer biology including cell proliferation, angiogenesis, and metastasis (26, 27). In addition, PAR$_2$ contributes to the tumorigenesis through the induction of Cox-2 (28), MMP 2/9 (29),
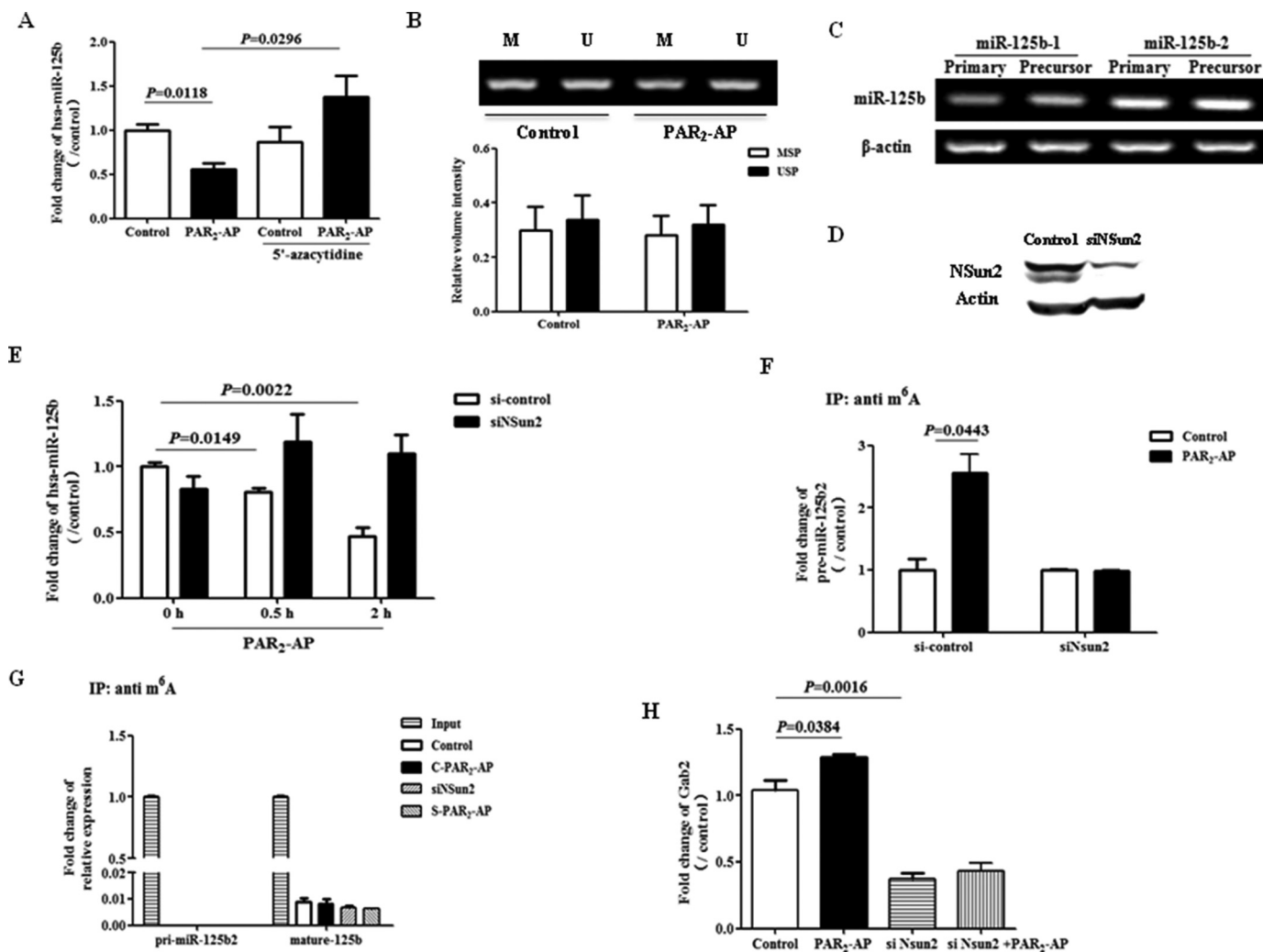
FIGURE 6. **PAR$_2$ regulates miR-125b expression through NSun2-dependent mechanism.** *A*, after the pretreatment with 5′-azacytidine for 72 h, A549 cells were stimulated with PAR$_2$-AP for 6 h. The level of miR-125b was measured by real time PCR. *B*, A549 cells were treated with PAR$_2$-AP for 6 h and then collected for MSP PCR to detect the methylation of miR-125b1 promoter. *Upper panel*, representative pictures of MSP PCR; *lower panel*, quantification of three independent MSP PCR. *M*, methylated form. *U*, unmethylated form. *C*, levels of pri- and pre-miR-125b1, pri- and pre-miR-125b2 were measured with PCR. *D*, Western blot shows the expression of Nsun2 after transfection with control RNA or siRNA against Nsun2 (siNSun2) in A549 cells. *E*, 40 h after transfection with si-Nsun2, cells were stimulated with PAR$_2$-AP for 0.5 or 2 h and then collected to measure miR-125b expression. *F* and *G*, after transfection with siNSun2, A549 cells were treated with PAR$_2$-AP for 0.5 h. RNA was isolated and pulled down with an anti-m^{6}A antibody. Pull down material was collected to measure the levels of (*F*) pre-miR-125b2 and (*G*) pri-miR-125b2 and miR-125b with real-time PCR. *H*, after transfection with siNSun2, A549 cells were treated with PAR$_2$-AP for 6 h and then collected to detect Gab2 mRNA by real time PCR. The data showed the means ± S.D. from three independent experiments.

and interleukin-8 (30). Inhibition of PAR$_2$ not only effectively suppresses tumor growth (31) but also prevents tumor formation in mouse model (8). Therefore, PAR$_2$ is an attractive and potential therapeutic target for tumor. Here, we demonstrated that miR-125b mediates PAR$_2$-promoted cell migration by targeting Gab2 in cancer cells. Moreover, PAR$_2$ suppressed the processing of miR-125b in Nsun2-dependent and miRNA methylation-mediated manner (Fig. 7). Thus, the interaction between PAR$_2$ signaling and miRNAs provides novel intrinsic molecular mechanism by which PAR$_2$ participates in tumorigenesis.

In the last decade, miRNAs have emerged as critical regulators in various cancers, including colorectal carcinoma (33, 34). The role of miR-125b in tumorigenesis is controversial since it can act as oncogene or tumor suppressor, depending on types of different tumors. Deregulations of miR-125b are observed commonly in breast (22, 32), ovarian (36), and liver cancers

(21), while its overexpression is implicated in pancreatic (37), and prostate (38) cancers. With regard to the expression of miR-125b in CRC, the evidence is conflicting. A study from Japan shows that high levels of miR-125b expression are associated with reduced survival rates in 89 colorectal tumors specimens (39). However, due to the absence of paired adjacent non-tumor tissue for each CRC sample, it is unclear whether the level of miR-125b in tumor is lower than that in non-tumor tissue. Compared with paired non-tumor tissue, our results clearly showed that miR-125b expression was down-regulated in tumor site, and it was also associated with lymphatic metastasis in CRC specimens (Fig. 5*A*).

Although deregulation of miR-125b plays a role in tumor development, the regulation of miR-125b expression is not well understood. Promoter methylation is a common mechanism for silencing miRNAs in cancer (40). Hypermethylation of miR-125b-1 promoter is involved in miR-125b down-regulation in
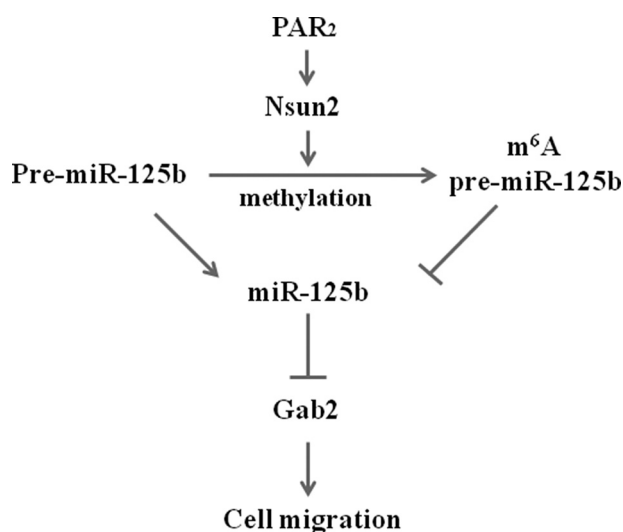
FIGURE 7. **Diagram of signaling pathways responsible for PAR₂-regulated miR-125b expression and function.** PAR₂ (Proteinase activated receptor 2) activation promotes Nsun2 (NOP2/Sun domain family, member 2)-dependent m⁶A methylation of pre-miR-125b2, which interferes with the processing of miR-125b from pre-miR-125b and results in the down-regulation of miR-125b. Gab2 is a downstream target of miR-125b and mediates the cell migration induced by PAR₂. According to the ending of lines, "⊥" represents inhibitory effect while "↓" represents stimulatory effect.

invasive breast cancer (22). Although the inhibition of methylation by 5-aza recovered the level of miR-125b (Fig. 6A), the expression of pri-miR-125b-1 and pri-miR125b-2 were not altered by PAR₂ activation (data not shown). Thus, our evidence does not support the transcriptional suppression of miR-125b by PAR₂.

Except for DNA methylation, RNA methylation is also implicated in the control of miR-125b processing (41). Methylation is a prevalent modification of almost all species of RNA. Methylation of RNA regulates the stability of RNAs and the biogenesis/processing of RNAs (42). Recently, the processing of miRNA-125b was found to be regulated by Nsun2-mediated RNA methylation in mammalian cells (41). NSun2 (NOP2/Sun domain family, member 2 is a nucleolar RNA methyltransferase implicating in cell proliferation, stem cell differentiation, and human cancers (42). NSun2 is highly expressed in cancer (43) and validated as a transcriptional target of the Lef1/β-catenin complex in hair follicles (44) and c-myc in skin (43). NSun2 methylates the 3′-untranslated region (UTR) of p16 mRNA and induces the stabilization of p16 mRNA (45). Methylation by Nsun2 decreases the levels miR-125b and disrupts its function. NSun2 is also shown to methylate precursor of miR-125b, interferes with its processing and reduces the level of mature miR-125b (41). In our study, Nsun2 silencing not only inhibited m⁶A-containing pre-miR-125b-2 (Fig. 6F) but also rescued the down-regulation of miR-125b induced by PAR₂ (Fig. 6E). These findings indicate that PAR₂ signaling interferes miR-125b processing through miRNA methylation in Nsun2-dependent manner.

Gab2 was identified as a novel target of miR-125b in the current study. Gab2, as a scaffolding protein, integrates and amplifies signals in a variety of sources including growth factors, cytokine, and antigen receptors as well as cell adhesion molecules (46). Recently, Gab2 is emerging as an oncoprotein,

and its protein is frequently overexpressed in various cancers (46). Here we showed that Gab2 expression was associated with lymph node metastasis in CRC, and the findings are consistent with other recent reports (46, 47). Besides gene amplification, E2F and estrogen signaling have also been reported to transcriptionally up-regulate Gab2 expression (35). In the present study, we provided a novel epigenetic mechanism by which Gab2 was up-regulated by PAR₂ signaling. Our previous study demonstrated that PAR₂ signaling also activates the transcriptional activity of E2F in colon cancer cells (16). Therefore, our results strongly suggest that PAR₂ up-regulates Gab2 expression at both transcriptional and post-transcriptional levels *in vivo*. Moreover, as a scaffolding protein, Gab2 mediates the crosstalk between various signaling pathways (46). This also provides the basis for the synergistic action of PAR₂ signaling. For example, Gab2 acts as a downstream of EGFR (35, 47), which is transactivated by PAR₂ (7). However, further study is needed to test whether Gab2 mediates transactivation of EGFR by PAR₂ activation. Therefore, considering the simultaneous exposure to plenty of stimuli in the real *in vivo* condition, Gab2 is considered as a key molecule for the crosstalk and integration of PAR₂ signaling and other pathways.

In conclusion, our results indicate that activation of PAR₂ promotes cell migration through the down-regulation of miR-125b via m⁶A-related mechanisms. Our study provides a novel epigenetic mechanism by which microenvironment regulates the level of miRNAs and promotes cancer cell migration.

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