# Down expression of *LRP1B* promotes cell migration via RhoA∕Cdc42 pathway and actin cytoskeleton remodeling in renal cell cancer

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The low-density lipoprotein receptor-related protein 1B (LRP1B) is known as a putative tumor suppressor. The decreased expression of *LRP1B* has been involved in multiple primary cancers in several studies. However, its expression and function in the carcinogenesis of renal cell cancer (RCC) remain unclear. In this study, we investigated the expression of *LRP1B* in RCC by *in situ* hybridization (ISH) and real-time polymerase chain reaction (qRT-PCR). Our results indicated that *LRP1B* was frequently downexpressed in human RCC tissue and cell lines, which involved both epigenetic events (DNA methylation and histone deacetylation) and N-terminal deletion of *LRP1B*. Moreover, we testified that knockdown of LRP1B by shRNA significantly promoted anchorage-independent growth, cell migration and invasion in HEK293 cells and renal cancer cells 127 *in vitro*. We further found that silencing of *LRP1B* altered the expression of focal adhesion complex-associated proteins, and Cdc42∕RhoA activities, which regulate the cytoskeleton dynamics. Taken together, these results strongly support that LRP1B may function as a tumor suppressor against renal cell cancer, and may regulate cell motility via RhoA∕Cdc42 pathway and actin cytoskeleton reorganization in RCC. (*Cancer Sci* 2013; 104: 817–825)

R enal cell carcinoma (RCC) is the most common kidney malignancy, and its incidence is increasing worldwide.[1] The early diagnosis and treatment of renal tumors have not reduced the mortality rate significantly, and in about 25–30% of cases, the localized tumors became spread around ultimately after surgical extirpation.[2–4] In addition, RCC is resistant to conventional therapies. Therefore, further elucidation of molecular mechanisms of RCC will be necessary for improving clinical diagnosis and effective therapeutic approaches.

*LRP1B*, a member of the low-density lipoprotein (LDL) receptor family, was identified as a putative tumor suppressor. The down-expression of *LRP1B* was observed in multiple primary cancers. Liu *et al.*[5] first reported the homozygous deletions of the N terminal part and abnormal transcripts of LRP1B in non-small cell lung cancer. Subsequently, it was found that the homozygous loss and aberrant DNA methylation contributed to *LRP1B* silencing in esophageal squamous cell carcinoma, oral squamous cell carcinoma and gastric Cancer.[6–9] Yet, there is a lack of research on the expression and function of *LRP1B* in RCC.

The LDL receptor family is a group of cell-surface transmembrane proteins.[10–12] LRP1B, along with LRP1 (LDL receptor-related protein 1) and LRP2 (megalin) are the largest members of LDL receptor family with multiple ligand-binding sites.[11] LRP1B might participate in extracellular signal transduction via the different phosphorylation status of the cytoplasmic tail.[13] LRP1B shows 59% amino acid sequence identity with LRP1, and shares a nearly identical overall structure with LRP1, except for additional exon 68 and 90. Functionally, *LRP1B* was different with LRP1, as LRP1 showed increased expression in cancer cells. It is still unclear whether LRP1B is associated with cell migration and invasion, even though Song *et al.*[14] reported that LRP1 promoted cancer cell migration and invasion.

In this study, we investigated the expression of *LRP1B* in RCC and its function on cell migration. We found that *LRP1B* was frequently down-expressed in RCC tissues and cell lines. The depletion of *LRP1B* increased the anchorage-independent growth, cell migration and invasion *in vitro*. Moreover, the expression and activation of Rho family members, actin cytoskeletons and focal adhesions complex (FAC) were also affected, indicating that down-expression of *LRP1B* led to the increase of cell migration and invasion, which is possibly mediated by actin cytoskeleton remodeling regulated through Cdc42∕RhoA pathway, and expressional alteration of FAC components.

## Materials and Methods

**Clinical specimens and *in situ* hybridization.** Renal cell carcinoma tissue samples, including 64 formalin-fixed and paraffin-embedded samples, and 38 liquid nitrogen-frozen samples with the paired normal adjacent tissues (NATs), were obtained from the First Affiliated Hospital, Harbin Medical University from 2005 to 2009. The using of samples was approved by the Medical Ethics and Human Clinical Trial Committee at the First Affiliated Hospital, Harbin Medical University.

*In situ* hybridization (ISH) was performed as described.[15] The target sites of horseradish peroxidase (HRP) labeled RNA probes were designed in exon 4 (1327–1361 bp), exon 6 (1678–1702 bp) and exon 8 (2028–2053 bp) of *LRP1B* mRNA. LRP1B expression was evaluated by the intensity of staining and the percentage of positive cells. "++++/+++": >50% cells stained and brown in tissue; "++": 20–50% of cells stained and snuff color in tissue; "+": mild to moderate staining of 5%–20% cells in tissue; "+/−": <5% of cells stained and faint in tissue; "−": negative, non-stained cells in tissue; "×": means lacking the data. Asterisks indicate obvious difference with $P < 0.05$.

**RNA extraction and qRT-PCR.** Total RNA was isolated from culturing cells or clinical samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. cDNA was synthesized using the Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qRT-PCR) was carried out using the SYBR@ Premix Ex Taq (Takara, Dalian, China). Primers are listed in Table S1.

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E-mail: liyugene@hit.edu.cn
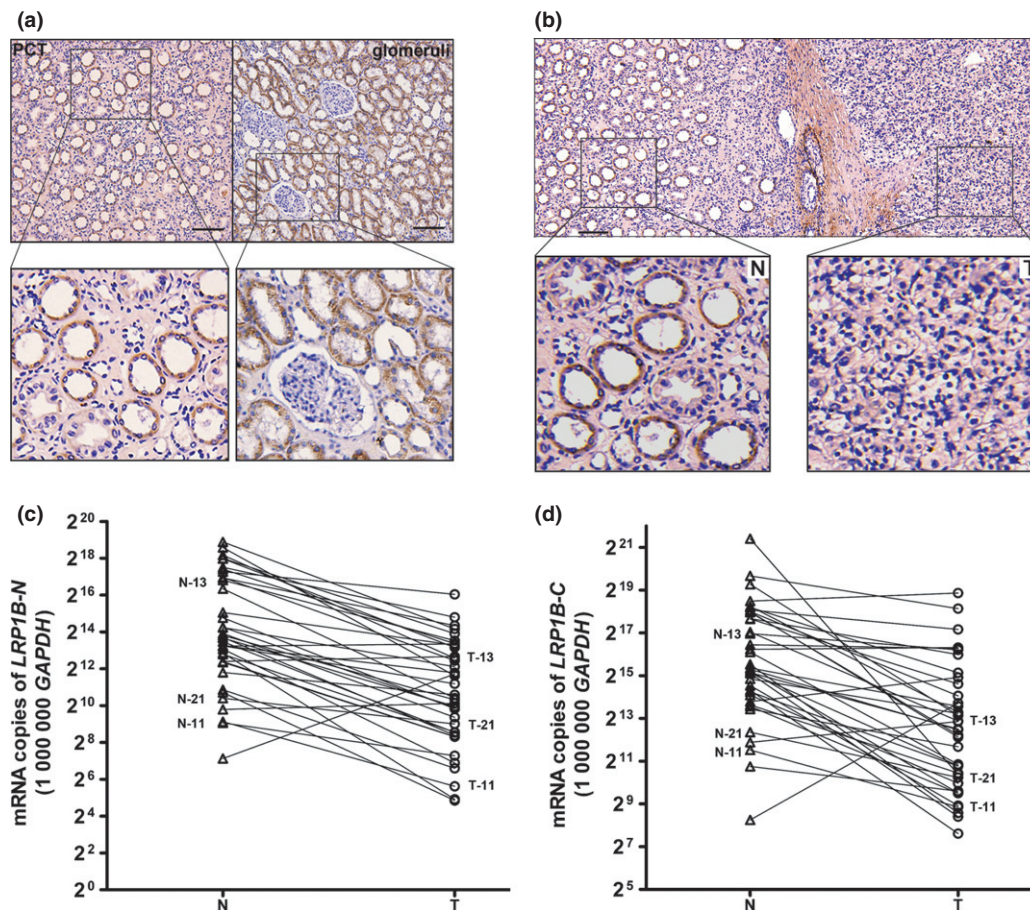[4]These authors contributed equally to this work.

**Fig. 1.** The down-expression of low-density lipoprotein receptor-related protein 1B (*LRP1B*) in renal cell cancer (RCC) tissues. (a, b) The expression level of *LRP1B* in paired normal renal tissues (a) and cancer tissue (b) was detected by *in situ* hybridization (ISH). The left and right panels in A show the expression of LRP1B in regions near the proximal convoluted tubule and the glomeruli respectively. Bar: 100 μm. (c, d) The expression level of N- and C-terminal region of *LRP1B* mRNA was detected by quantitative real-time polymerase chain reaction (qRT-PCR) in paired RCC samples (*n* = 38). The relative copy numbers of N- and C-terminal LRP1B in normal and cancer tissues were shown in left and right respectively.

**DNA bisulfite sequencing.** Three pairs of genomic DNAs from clinical samples were treated with methylSEQrTM Bisufite Conversion Kit (Applied Biosystems), amplified using nest PCR with gene specific primers (Table S1). The amplified sites were located at CpG region of *LRP1B* representing the mRNA site of 815–1573 bp. The PCR products were cloned into pMD18-T Vector (Takara), and the DNAs from randomly chosen colonies were sequenced.

**Cell culture, RNA Interference.** HEK293 cells were cultured in DMEM medium. A-704, Caki-1 and 127 cells were cultured in RPM1640 medium. Three siRNAs (Table S1) targeting *LRP1B* were designed by siRNA Target Finder systerm on the Applied Biosystems Website. The synthetic double-stranded oligonucleotides were cloned into a pSilencer 4.1-CMV vector (Ambion, Austin, TX, USA). HEK293 cells were stably transfected with the shRNA constructs or negative control using Lipofectamine 2000 (Invitrogen), and were selected with 700 μg/mL G418 subsequently. A total of 127 cells were transfected with the same strategy as with HEK293, except for that the expression of shRNA in 127 was transient.

**DNA demethylation and histone acetylation of cells.** A-704 and Caki-1 cells were treated with 5 μM 5-aza-2′-deoxycytidine (5-aza-CdR, Sigma, St. Louis, MO, USA) for 24, 48 and 72 h with or without 100 ng/mL Trichostatin A (TSA, Sigma) for 12 h. The control dishes were maintained in RPM1640 complete medium with DMSO or equal TSA. The cells were harvested for RNA analysis.

**Cell proliferation assay.** For anchorage-dependent cell growth assay, $1 \times 10^4$ cells were seeded in 24-well plate with three replicates, and cultured in 37°C incubator with 5% $CO_2$. Cell numbers were counted with an interval of 2 days, 3 days after incubation. For anchorage-independent cell growth assay, HEK293 cells expressing control vector or LRP1B shRNA were detached with trypsin. $5 \times 10^3$ cells/well with three replicates in 0.4% top agar were seeded into 6-well plates containing 0.5% of base agar (Amreso, Solon, OH, USA), and then cultured in complete medium for 2 weeks. The colony numbers were counted respectively, according to the diameter grades from 100 to 500 μm. The soft agar assay of 127 cells expressing control vector or LRP1B shRNA was performed as the same as that in HEK293 cells except for that $2 \times 10^3$ cells were seeded in each well. Colonies that were greater than 100 μm formed by 127 cells transfectants were counted as the numbers per well.

**Cell spreading, invasion and migration.** For cell spreading assay, cells were seeded into culture dishes without coating or coated with collagen I, and monitored at indicated time points. Invasion assay was performed using Transwells (Millipore, Boston, MA, USA; Corning, Corning, NY, USA) coated with 20 μg/μL of matrigel on the upper surface and 10 μg/μL of fibronectin on the lower surface of filters (8 μm pores).

$2 \times 10^5$ cells were seeded and incubated for 48 h. Then the chambers were fixed with methanol and stained by crystal violet. The invaded cells on the lower surface of the filters were captured in 10 random fields under light microscopy. Meanwhile, the chemotactic migration assay was carried out with the same procedure as shown in invasion assay except that only fibronectin was coated on the lower side of filters, and that the incubation time in migration assay is 24 h (8 μm pores).

For wound healing assay, cells were seeded in a 12-well culture plate and grown to 80% of confluence. Then the plate was scratched across the surface of the cell monolayer with a sterile pipette tip. Five random fields of wound were captured immediately 24 h after scratching.

**Immunofluorescence.** Cells were cultured on coverslips for 24 h, and then fixed with 4% paraformaldehyde, permeabilized in 0.2% TritonX-100. Then the cells were blocked in 3% BSA, and incubated with fluorescently labeled phalloidin (Sigma). The coverslips were mounted and observed using a Zeiss LSM 510 META confocal microscope.

**Western blot analysis.** Cells were directly lysed in radio-immunoprecipitation assay buffer. The equal amount of proteins was subjected to western blot with antibodies against integrin-$\beta_1$, focal adhesion kinase (FAK), α-actinin, paxillin, talin, vinvulin, GAPDH, RhoA, Cdc42 purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), or with antibodies against Tyr397-phosphorylated FAK (Invitrogen) and Rac1 purchased from Abcam (Cambridge, MA, USA). All the western blot experiments were performed three times, with the representative results shown in figures.

**RhoA/Cdc42/Rac1 activity assay.** Guanosine 5'-triphosphate-bound Cdc42 and Rac1 were determined by GST-PAK-RBD pull-down assays as described.[16] Briefly, cells were lysed in Ral buffer and incubated with GST-PAK-RBD coupled to sepharose 4B beads for 1 h. The proteins bound to beads were subjected to western blot with antibodies against Rac1 or Cdc42. To detect RhoA acivity, HEK 293 cells expressing shRNA or control vector were lysed with Ral buffer, and cell lysate was incubated with GST-Rhotekin-RBD beads for 1 h. The amount of GTP-bound, RhoA was determined by western blot.

**Statistical analysis.** All values were presented as means ± SD obtained from three independent experiments. Statistical analyses were carried out using Student's t-test. Differences were considered to be statistically significant at $P < 0.05$.

## Results

**Expression of LRP1B is frequently silenced in primary RCCs.** To investigate the expression of *LRP1B* in RCCs, *LRP1B* mRNA levels in 64 paraffin-embedded tissue samples of RCC and their NATs were detected by ISH using three RNA probes targeted to *LRP1B* N-terminal region. Clinicopathological parameters including patients' sex, age at surgery, tumor size, nuclear grade, lymph node status, local invasion, and TNM staging are

summarized in Table S2. The results of ISH showed that *LRP1B* was significantly expressed in epithelial cells of normal nephric tubule (Fig. 1a) and NATs, but expressed at very low levels in tumor tissues (Fig. 1b). The negative control was shown in Fig. S1. Forty-eight of 64 (75.5%) samples manifested low expression of *LRP1B* in cancer tissues compared with NATs (Table 1). Meanwhile, LRP1B down-expression was associated with cancer staging and local invasion (Table 2). The down-expression of *LRP1B* was observed in early stage, and most histopathologic subtypes of RCCs, such as clear-cell and papillary carcinoma, implying that *LRP1B* down-expression was a common event in RCC.

It was known that down-expression of *LRP1B* could result from homozygous deletions of N-terminal (exon 2 to 9) or epigenetic events in multiple primary cancers. Thus, to confirm whether deletions are the causative factors for the down-expression of *LRP1B* in RCC, qRT-PCR was performed with liquid nitrogen-frozen samples. Among 38 paired samples, down-expression of N-terminal LRP1B (89.5%) was found to be slightly more increased than the C-terminal LRP1B (81.6%) in tumor tissues (Fig. 1c,d). This result was consistent with the previous finding that N-terminal deletion of LRP1B could be one of the mechanisms accounting for its down-expression, besides the epigenetic mechanism.

**Table 1. Expression of low-density lipoprotein receptor-related protein 1B (LRP1B) in renal cell cancer (RCC) samples**

| Feature | n | Cases with expression | | Cases without expression | | P |
|---|---|---|---|---|---|---|
| | | (n) | (%) | (n) | (%) | |
| NATs | 44 | 39 (++++/+++) | 88.6 | 0 | 0 | <0.001 |
| Tumors | 64 | 16 (+/−) | 25.0 | 48 (−) | 75.5 | |

Forty-four pairs of 64 cases with *LRP1B* expression in normal adjacent tissues (NATs) higher than expression in tumors.

**Table 2. Summary of *in situ* hybridization (ISH) and clinical pathologic features in 64 cases of renal cell cancer (RCC)**

| Feature | n (%) | Cases with *LRP1B* expression (n) | | | | | P |
|---|---|---|---|---|---|---|---|
| | | ++++/+++ | ++ | + | +/− | − | |
| **Sex** | | | | | | | |
| Male | 39 (60.9) | 0 | 0 | 0 | 8 | 31 | 0.489 |
| Female | 25 (39.1) | 0 | 0 | 1 | 8 | 16 | |
| **Age at surgery** | | | | | | | |
| ≤65 | 52 (81.3) | 0 | 0 | 1 | 12 | 39 | 0.700 |
| >65 | 12 (18.7) | 0 | 0 | 0 | 4 | 7 | |
| × | 1 (1.6) | 0 | 0 | 0 | 0 | 1 | |
| **Tumor extent (TNM 2004)** | | | | | | | |
| T1 | 41 (64.1) | 0 | 0 | 0 | 7 | 34 | 0.033* |
| T2 | 2 (3.1) | 0 | 0 | 0 | 0 | 2 | |
| T3 | 9 (14.1) | 0 | 0 | 0 | 5 | 4 | |
| T4 | 2 (3.1) | 0 | 0 | 1 | 0 | 1 | |
| T× | 10 (15.6) | 0 | 0 | 0 | 4 | 6 | |
| **Regional lymph node metastasis (TNM 2004)** | | | | | | | |
| N0/pN0 | 52 (81.3) | 0 | 0 | 1 | 11 | 40 | 0.326 |
| pN1, pN2 | 2 (3.1) | 0 | 0 | 0 | 1 | 1 | |
| N× | 10 (15.6) | 0 | 0 | 0 | 4 | 6 | |
| **Regional invasion (TNM 2004)** | | | | | | | |
| Yes | 12 (18.8) | 0 | 0 | 1 | 5 | 6 | 0.034* |
| No | 49 (76.6) | 0 | 0 | 0 | 10 | 39 | |
| × | 3 (4.7) | 0 | 0 | 0 | 1 | 2 | |
| **Histopathologic subtype** | | | | | | | |
| Clear-cell RCC | 59 (92.2) | 0 | 0 | 1 | 15 | 43 | 0.134 |
| Papillary RCC | 5 (7.8) | 0 | 0 | 0 | 1 | 4 | |
| **Nuclear grade** | | | | | | | |
| I | 21 (32.8) | 0 | 0 | 0 | 5 | 16 | 0.596 |
| II | 34 (53.1) | 0 | 0 | 1 | 8 | 25 | |
| III–IV | 9 (14.1) | 0 | 0 | 0 | 3 | 6 | |
| **Sarcomatoid differentiation** | | | | | | | |
| Yes (extent >50%) | 1 (1.6) | 0 | 0 | 0 | 0 | 1 | 0.565 |
| No | 63 (98.4) | 0 | 0 | 1 | 16 | 46 | |

++++/+++: >50% cells stained and brown in tissue; ++: 20–50% cells stained and snuff color in tissue; +: mild to moderate staining of 5–20% cells in tissue; +/−: <5% of cells stained and faint in tissue; −: negative, non-stained cells in tissue; ×: means lacking the data.
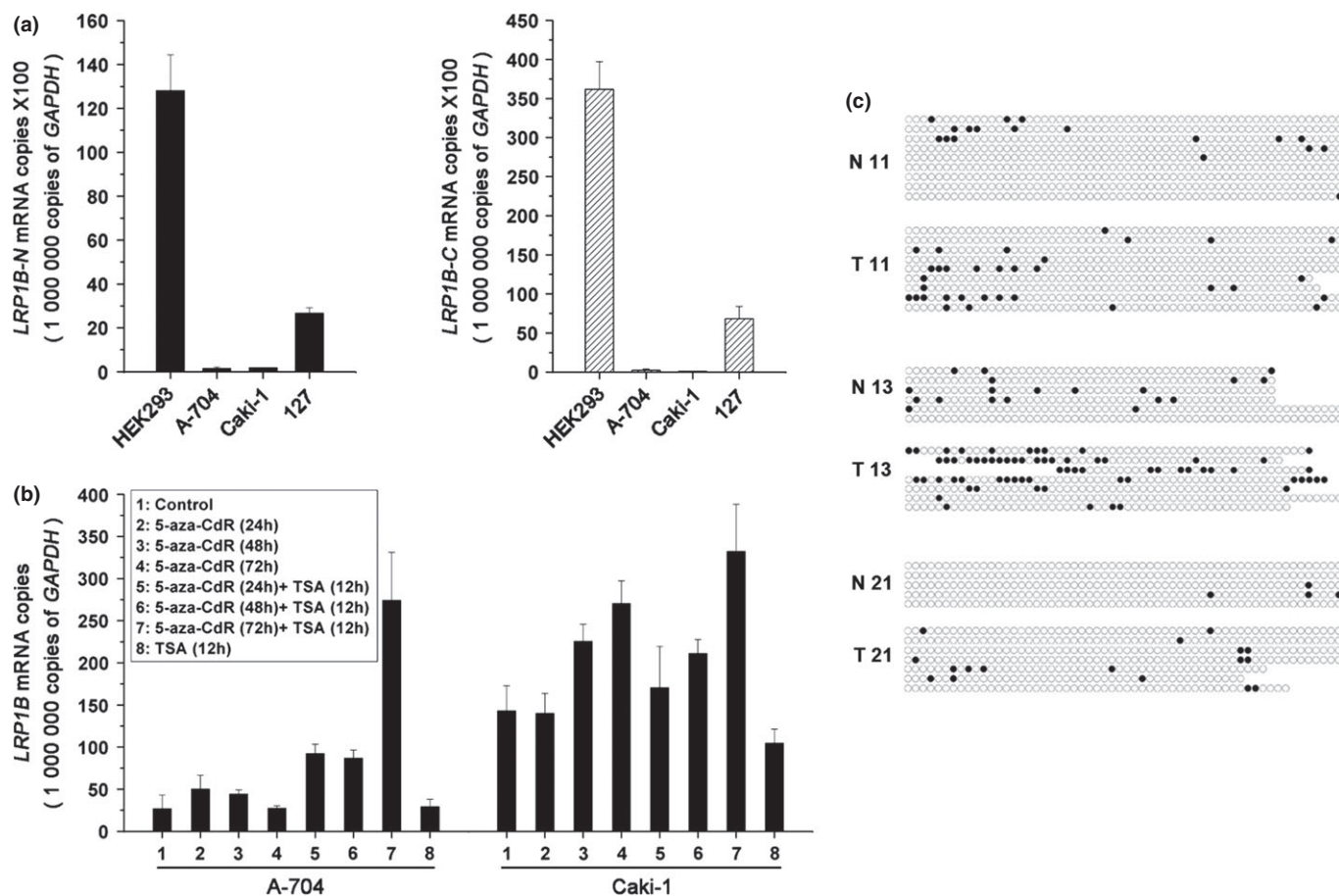*Obvious difference with P < 0.05.

**Fig. 2.** Low-density lipoprotein receptor-related protein 1B (*LRP1B*) is epigenetically silenced in renal cell cancer (RCC) cell lines. (a) The expression of N- and C-terminal region of *LRP1B* mRNA in RCC cell lines was detected by quantitative real-time polymerase chain reaction (qRT-PCR). (b) A-704 and Caki-1 cells were treated by 5-aza-CdR or Trichostatin A (TSA) for the time indicated, or treated by 5-aza-CdR and TSA. Then, *LRP1B* mRNA level was analyzed by qRT-PCR and described as means ± SD. (c) Bisulfite sequencing was performed at CpG region of *LRP1B* gene in three RCC patients. Left panel, normal tissues; Right panel, cancer tissue. Filled circles, methylated sites; Circles, unmethylated sites.

**LRP1B expression is affected by DNA methylation and histone acetylation in RCC.** To explore whether epigenetic events are involved in *LRP1B* silencing in RCC, A-704 and Caki-1 cells were treated by 5-aza-CdR, with or without TSA. Then, *LRP1B* was detected by qRT-PCR (Fig. 2a). The results showed that 5-Aza-CdR could promote the expression of *LRP1B* in A-704 and Caki-1 cells alone, and the expression of *LRP1B* was remarkably enhanced when TSA was added further (Fig. 2b). The observation suggested that histone deacetylation and DNA methylation might synergistically contribute to *LRP1B*-silencing in RCC cells. Furthermore, the methylation status of *LRP1B* CpG region in three paired RCC samples with down-expression of *LRP1B* in tumor tissues, were analyzed by bisulfite-sequencing. The results indicated that RCC tissues showed higher methylation in *LRP1B* CpG region as compared to their NATs (Fig. 2c).

**LRP1B silencing promotes anchorage-independent growth of HEK293 and RCC cells.** To understand clearly the effect of *LRP1B* silencing in RCC, HEK293 cells were first transfected by shRNA-constructs (shRNA-1, shRNA-2 and shRNA-3), and the stable transfectants were obtained with different depletion efficiency (100%, 95%, and 80%) (Fig. 3a). Then, the growth of *LRP1B*-silencing cells was examined. Unexpectedly, neither the depletion of *LRP1B* showed any effects on anchorage-dependent growth (Fig. S2), nor the colony formation on soft agar was apparently affected. However, the cell proliferation on soft agar was enhanced when *LRP1B* was depleted by

shRNA, as the mean colony diameter was increased by the expression of *LRP1B* shRNA (Fig. S3), and the colonies with diameters larger than 400 or 500 μm were dramatically increased (Figs 3b and S4). These data suggested that knockdown of *LRP1B* might enhance the anchorage-independent cell proliferation of cells, and thus promote cell transformation. To confirm the role of *LRP1B* in the renal cell transformation, the RCC cell 127 was transfected with LRP1B shRNA-constructs. Again knockdown of *LRP1B* significantly promoted anchorage-independent growth of 127 cells, indicating that *LRP1B* could regulate renal cell transformation (Fig. 3c,d).

**LRP1B silencing promotes cell migration and invasion.** The acquisition of invasive ability is a crucial step for transformation and malignant progression of cells. Therefore, cell chemotactic migration assay was carried out with HEK293/shRNAs cells to determine whether LRP1B could have the ability to regulate cell migration. In accordance with the results of soft agar assay, the number of *LRP1B*-silencing HEK293 cells migrated through the polycarbonate membrane was 3–5-fold higher than the control cells (Fig. 4a). Furthermore, the effect of LRP1B silencing on 2D migration of HEK293 cells and 127 cells was also analyzed using a wound-healing assay (Fig. 4b,c). The migration of HEK293/shRNA-1 cells and 127 /shRNA-1 cells was enhanced significantly as compared with control cells respectively. These results indicated that LRP1B had the ability to repress 2D and 3D migration in both HEK293 cells and RCC 127 cells. At the same time, cell inva-
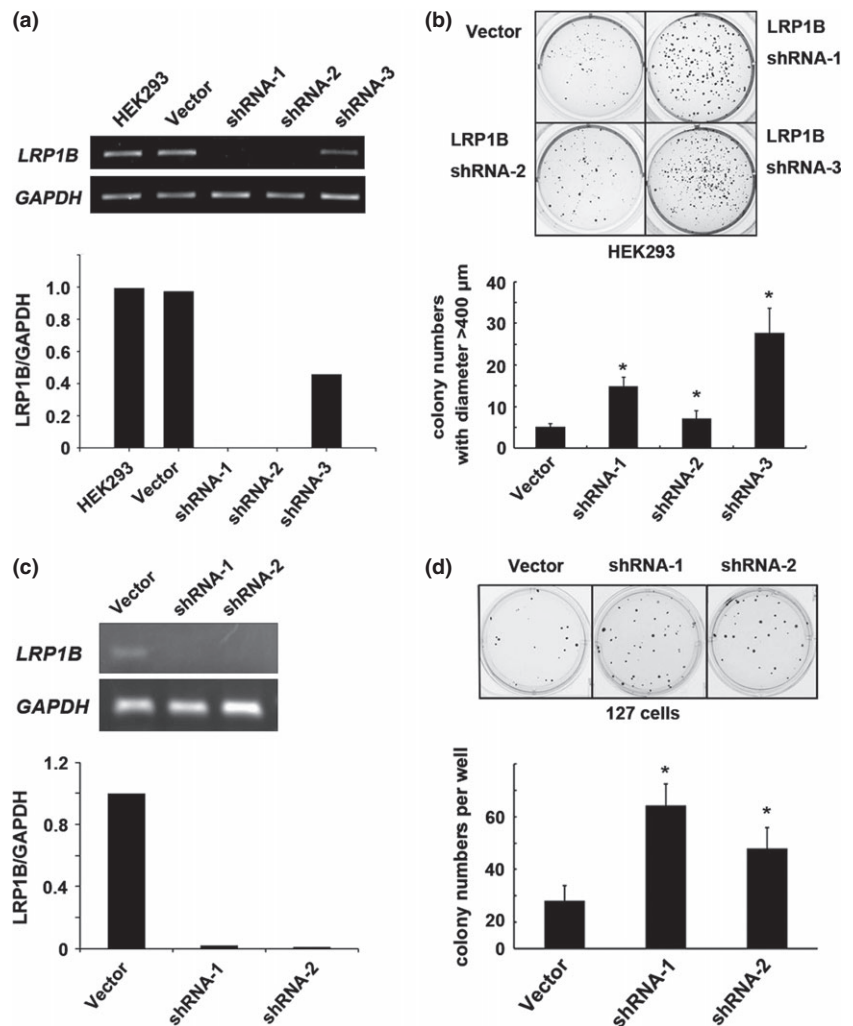
**Fig. 3.** Effects of low-density lipoprotein receptor-related protein 1B (LRP1B) on cell growth *in vitro*. (a) The mRNA levels of *LRP1B* in HEK293 cells expressing shRNA or vector were assessed by reverse transcription-polymerase chain reaction (RT-PCR) (upper panel), and plotted as a column graph (lower panel). (b) Colonies formed within 2 weeks that exceeded 100 μm in diameter were counted. Results were shown as means ± SD. *P < 0.01. (c) The silencing efficiency of *LRP1B* in 127 cells was detected by RT-PCR. (d) The knocking-down of *LRP1B* significantly increased the growth of 127 cells in soft agar. Colonies formed after 2-week growth that exceeded 100 μm in diameter were counted.

sion was determined by Boyden chamber assay. The number of *LRP1B*-silencing HEK293 cells that invaded the lower surface of the filter was 2–4-fold higher than control cells (Fig. 4d). However, MMPs-dependent ECM degradation may not contribute to this enhanced cell invasion, as the activity of MMP-2 was not changed and MMP-9 could not be detected in the HEK293 cells (data not shown).

**LRP1B regulates cell spreading and actin cytoskeleton organization via Cdc42/RhoA pathway.** To confirm that LRP1B could regulate the actin-based morphology, the cell spreading assay was carried out. Although many pseudopod had already extended in control cells 4 h after plating, its formation was delayed in HEK293/shRNA-1 cells that became spreading on matrix until 12 h after plating (Fig. 5). When plating the cells on collagen I-coated dishes, the control cells began to spread at a very early time (30 min), while the spreading of LRP1B shRNA expressing cells was delayed to 4 h after plating. The spreading area of HEK293/shRNA-1 cells was significantly smaller than that of control cells. Moreover, *LRP1B*-silencing cells showed a polarized morphology and a significant increase in the number and size of filopodia formation (Figs 6a and S5). These findings indicated that actin reorganization, and protrusion formation could be regulated with *LRP1B*-silencing.

Based on these results, we presume that LRP1B may repress cell migration and spreading through regulating Rho family expression or activity. Therefore, GST-pull down assay was performed for GTP-bound Cdc42, RhoA and Rac1. The expression levels for Cdc42, RhoA and Rac1 were not changed significantly when LRP1B expression was down. However, the Cdc42 activity was increased over 1.5-fold and RhoA activity was reduced to 0.6-fold after LRP1B was silenced (Fig. 6b), but Rac1 activity was not changed significantly. These results were consistent with the increased philopodia formation in LRP1B-silencing cells.

**LRP1B regulates the level of focal adhesion molecules.** Focal adhesions play essential roles in cell motility and invasion, and some components participate in the structural and/or functional links between transmembrane receptors and the actin cytoskeleton. In this respect, the expressions of several focal adhesion molecules were analyzed (Fig. 6c,d). The proteins level of paxillin, vinculin, integrin-β and talin were remarkably reduced, while α-actinin and paxillin levels were not changed clearly. In contrast, the expression of FAK was increased clearly in *LRP1B*-silencing cells, while the phosphorylation state of Y397 only changed slightly. The results indicated that LRP1B could regulate the protein level of focal adhesion complex; loss of function of LRP1B may serve as a mechanism to enhance cell transformation through regulating focal adhesion formation.

## Discussion

Low-density lipoprotein receptor-related protein 1B is a member of the LDLR family protein and is involved in the endocytic function and signal transductions.[10,11,13] Although the func-
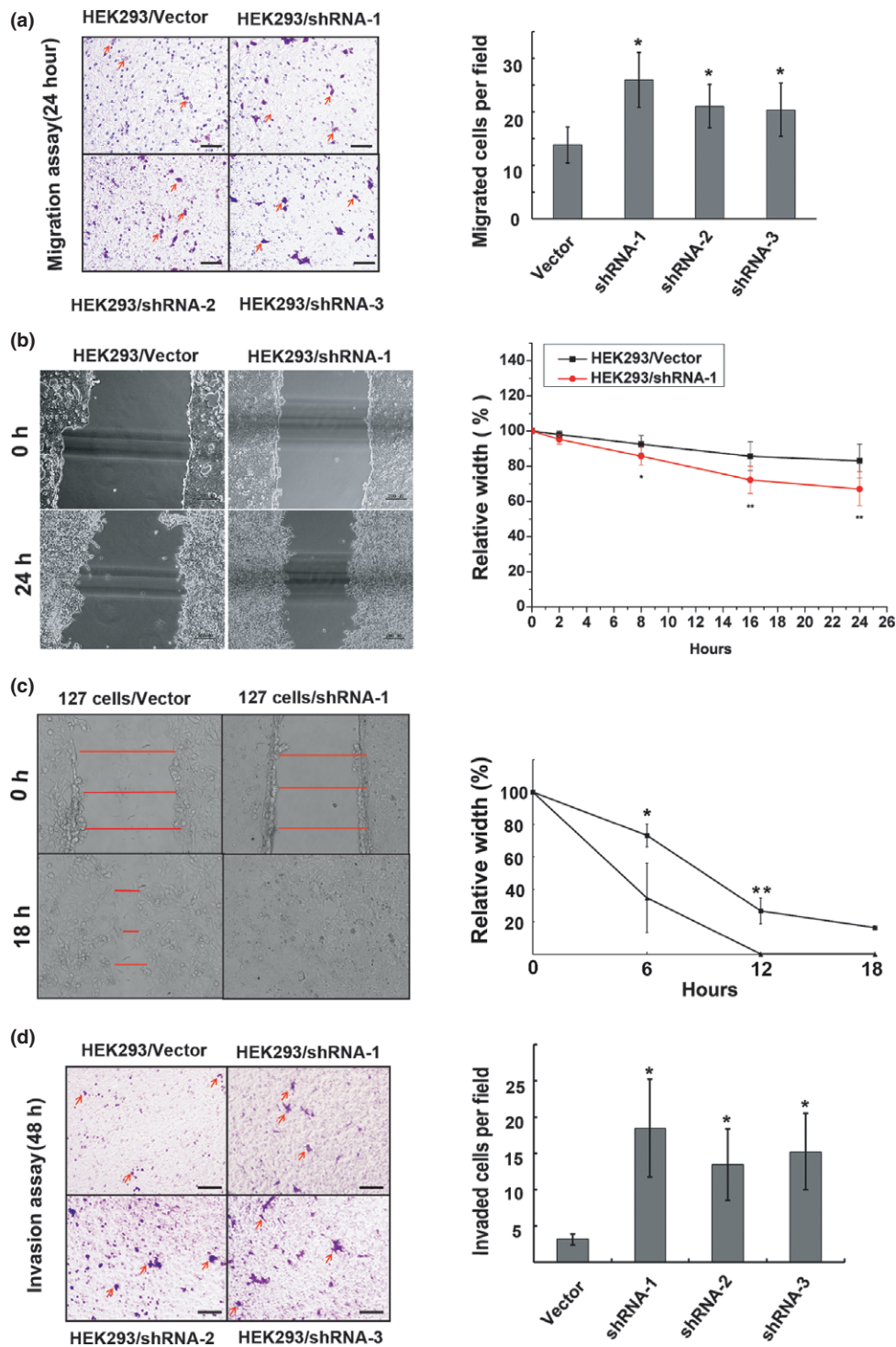
**Fig. 4.** Low-density lipoprotein receptor-related protein 1B (*LRP1B*) silencing increased HEK293 cell migration and invasion. (a) HEK293 cells stably expressing shRNAs or control vector were subjected to migration assay using Boyden chamber. Twenty four hours after incubation, cells migrated to the lower surface of the chambers were counted in 10 random fields. (b) Cell migration was determined by wound healing assay. The relative migration was measured as a rate of width of wound remained 2, 8, 16 or 24 h after scratching. (c) 127 cells expressing *LRP1B* shRNA or control vector were subjected to wound healing assay. The relative migration was measured as a rate of width of wound remained 6, 12 or 18 h after scratching. (d) HEK293 cells expressing *LRP1B* shRNA or control vector was seeded on matrigel-coated Boyden chamber. 48 h after seeding, cells invaded across the matrigel were counted, and results were described as means ± SD. *P < 0.05; **P < 0.01. 400 × . Bar: 100 μm.

tions of most LDLR family members have been described, the characteristics of several molecules in this family remain unclear.[10–12,14,17,18] It has been shown that increased expression of LRP1 promoted cancer cell migration and invasion by inducing the expression and activation of MMP2 and MMP9.[14] However, it was also reported that LRP1B may function as a putative tumor suppressor, although the mechanism for LRP1B to suppress cancer is unknown.[5]
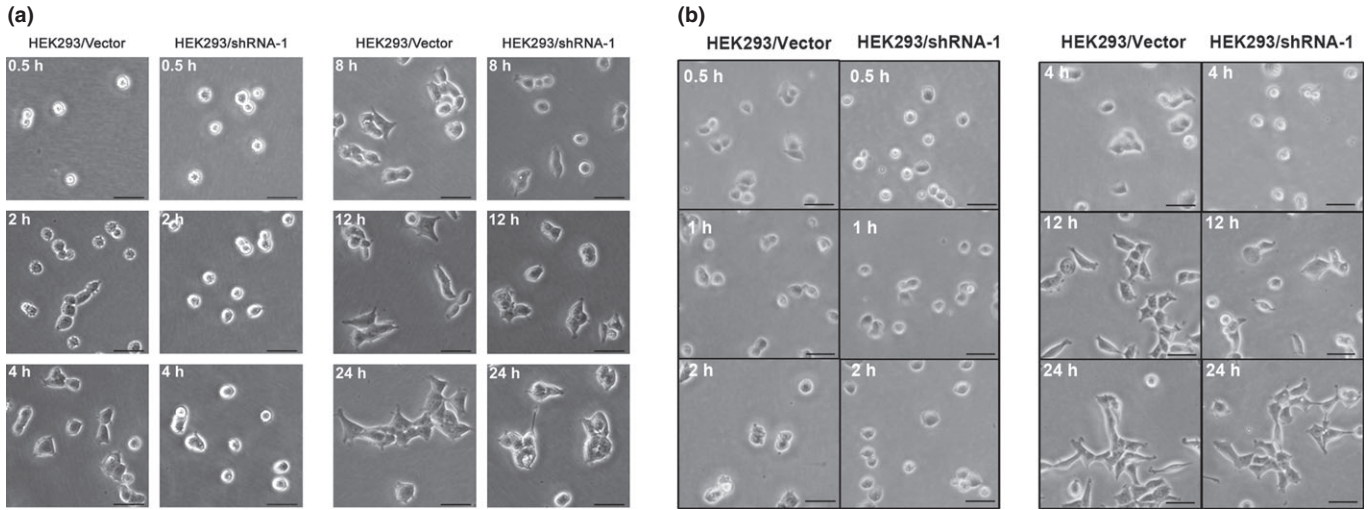
**Fig. 5.** Low-density lipoprotein receptor-related protein 1B (*LRP1B*)-silencing attenuated HEK293 cell spreading. Cells were seeded onto uncoated culture dishes (a) or dishes coated with collagen I (b) and visualized by microscopy at indicated points. Images are representative of three separate sets of cultures. Bars: 100 μm.
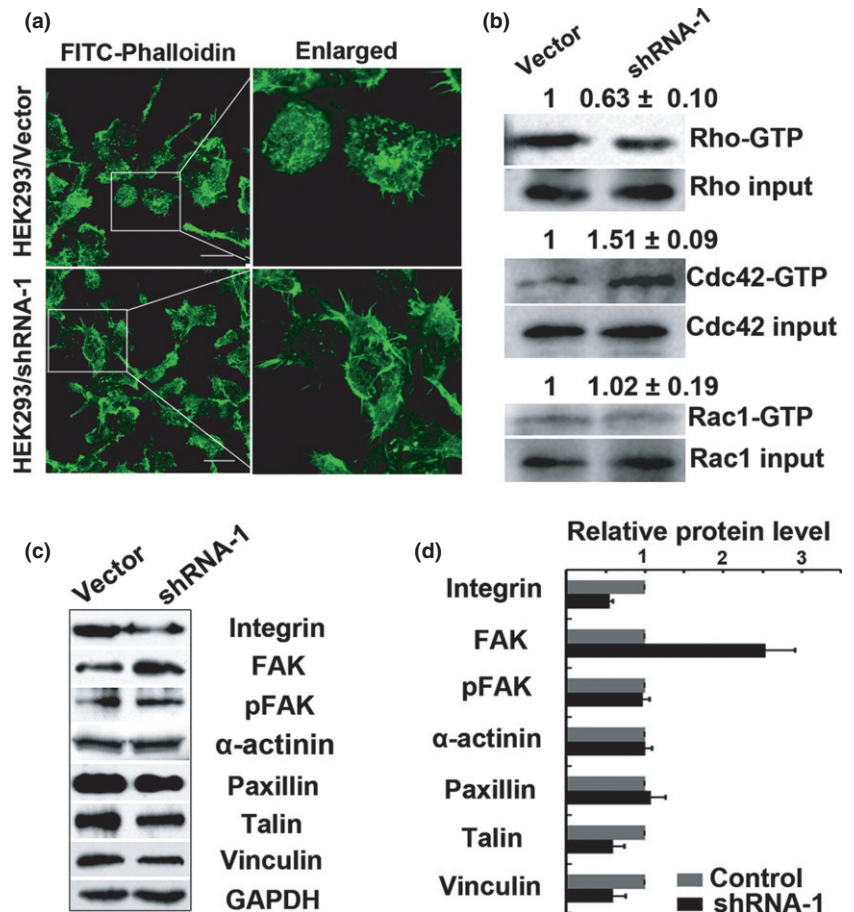


**Fig. 6.** Low-density lipoprotein receptor-related protein 1B (*LRP1B*) regulates actin cytoskeleton reorganization and focal complex expression. (a) HEK293 cells indicated were stained with fluorescein isothiocyanate (FITC)-conjugated phalloidine. (b) The activation of Cdc42, Rac1 and RhoA in HEK293 cells-expressing LRP1B shRNA or control vector was detected by GST-pull down with GST-PAK-RBD, or GST-Rhotekin-RBD. The activation extents of Rho, Cdc42 or Rac1 in LRP1B shRNA cells were indicated as the means ± SD, compared to control cells. (c, d) The levels of focal adhesion proteins or phosphorylated FAK in the cells indicated were detected by western blot (left panel). The proteins levels or phosphorylation level of FAK was measured by densitometer (right panel). Bar: 100 μm.

Here, we found that *LRP1B* mRNA was widely expressed in the normal renal tubular epithelial cells, but frequently down-regulated in RCCs, which could result from both genetic and epigenetic mechanisms. The similar mechanism has also been reported in other human cancers.[5–9] At the same time, we also found that silencing of *LRP1B* obviously occurred in T1 of TNM. The result suggests that silencing of *LRP1B* is an early event in RCC. Our observation provided an insight into the potential contribution of LRP1B to tumorigenesis, and that LRP1B may be explored as a molecular target in RCC therapy by regulated epigenetic activation means.

Rho family proteins Cdc42, RhoA and Rac1 are closely concerned with regulating cell migration, invasion and cytoskeleton assembling.[19–22] Particularly, Cdc42 functions in the formation

of philopodia through WASP-ARP2/3 pathway, and FAK.[22,23] The expression of a dominant-negative Cdc42 could impair cell spreading and inhibit the philopodia formation.[24] In our results, Cdc42 activity was increased in HEK293 cells with silencing of *LRP1B*, accompanying enhanced philopodia formation, indicating that the change of coincidence constitutes a foundation of RCC cell migration and invasion. In addition, *LRP1B*- silencing in RCC impaired RhoA activity and the FAC components expression, but increased FAK expression. It is known that FAK played a central role in cell migration.[24–26] FAK could bind to the GTPase-activating protein (GAPs) directly[26–28] and affect GAPs activity, and the latter (GAPs) could promote hydrolysis of GTP-bound Rho, Rac or Cdc42.[29,30] Therefore, our results suggested that the *LRP1B*-silencing might mediate cell spreading, cell migration and invasion through regulating Rho family proteins.

Despite high similarities between LRP1 and LRP1B, their function is not consistent. It has been reported that LRP1 had a positive effect on cancer cell migration and invasion via MMPs-independent pathway.[31] Silencing of LRP1 could prevent cell invasion.[14,32] However, LRP1B could attenuate the migration of smooth muscle cell by reducing membrane localization of urokinase and platelet-derived growth factor (PDGF) receptors,[33] and have a negative effect on migration of intimal smooth muscle cells from rabbit aortic plaques.[34] By this token, their function should be contrary on controlling cell migration and invasion. But the role of LRP1B in cancer is poorly understood. In this study, we found that silencing of *LRP1B* caused a significant increasing of cell migration and invasive capacity. We proved that the enhanced migration and invasion of cells were not due to the endocytic uptake of MMPs in RCC, but Cdc42 and RhoA activity of Rho family, actin cytoskeletal reorganization and FAC composition alteration may be involved. These functional specificities in cell spreading, migration and invasion strongly validated that LRP1B may function as a tumor suppressor, and exert opposite effects to LRP1 on cell transformation and malignant progression.

## Disclosure Statement

The authors have no conflict of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Paraffin embedded normal kidney tissue was subjected to *in situ* hybridization using LRP1B probe (A). Sense probe was used as control (B).

**Fig. S2.** Depletion of LRP1B showed no effects on anchorage-dependent growth.

**Fig. S3.** LRP1B silencing enhanced the cell proliferation on soft agar.

**Fig. S4.** LRP1B silencing enhanced the anchorage-independent proliferation of HEK293 cells.

**Fig. S5.** HEK293 cells expressing LRP1B shRNA or control vector were subjected to immunostaining with FITC-conjugated phalloidine.

**Table S1.** Sequences of oligonucleotides usded in real-time PCR, DNA bisulfite sequencing and RNAi.

**Table S2.** Summary of ISH and clinical pathologic features in 64 cases of RCC.