Elimination of Ras Suppressor-1 from Hepatocellular Carcinoma Cells Hinders their *In Vitro* Metastatic Properties

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Abstract. *Background/Aim: Extracellular matrix (ECM) is of great significance for homeostasis in the liver. In fact, one of the stages leading to hepatocellular carcinoma (HCC) includes accumulation of excess ECM. Ras Suppressor-1 (RSU-1) is localized in the cell-ECM adhesions but its role in HCC is unexplored. Materials and Methods: We investigated the expression and role of RSU-1 in two HCC cell lines that differ in aggressiveness; non-invasive Alexander cells and highly invasive HepG2 cells. Results: Our results showed that RSU-1 expression is elevated in HepG2 cells both at the mRNA and protein level, while its silencing leads to increased cell proliferation in both cell lines. Interestingly, RSU-1 depletion from highly invasive HepG2 cells reduces cell adhesion and invasion. Conclusion: This is the first study to provide in vitro evidence for the involvement of RSU-1 in HCC cell invasive behavior.*

Extracellular matrix (ECM) is of great significance for cell survival, differentiation and tissue homeostasis. Specifically in the liver, hepatocytes are greatly dependant on communication with ECM and related proteins. This is evident by the fact that, although hepatocytes have restricted proliferative capacity in culture and tend to lose patterns of hepatocyte-specific gene expression, as well as their characteristic cellular micro-architecture, their differentiation is rapidly restored following overlay of hydrated matrix preparations (1).

Hepatocellular carcinoma (HCC) occurs as the last stage of a series of events that include replacement of the injured or impaired liver tissue by fibrotic, scar tissue and regenerative nodules (cirrhosis), generation of phenotypically altered hepatocytes and generation of dysplastic hepatocytes, which serve as precursors to HCC (2).

Ras suppressor-1 (RSU-1) (~33 KDa) was originally identified in a screening for genes that suppressed Ras-dependent oncogenic transformation (3) and it is thought to be ubiquitously expressed. Interestingly, it was recently determined that it is localized in the cell-ECM adhesion sites of the cell where it interacts with multiple cell-ECM adhesion proteins (4, 5).

However, the involvement of RSU-1 in hepatocellular carcinoma (HCC) has not been to date investigated. Thus, we hypothesized that the cell-ECM adhesion protein RSU-1 plays a critical role in HCC and we sought to investigate the expression and role of RSU-1 in two HCC cell lines that differ in aggressiveness (the non invasive Alexander cells and the highly aggressive and invasive HepG2 cells).

Materials and Methods

Cell lines. Two cell lines were used in this study; the hepatoma cell line PLC/PRF/5 (Alexander) and HCC cell line HEPG2. Both cell lines were purchased from ATCC (Manassas, VA, USA).

Antibodies and reagents. Anti-β-actin antibody (Sigma-Aldrich, St.Louis, MO, USA) was used as loading control. The Anti-RSU-1 antibody was purchased from Assay Biotechnology (Sunnyvale, CA, USA). The alamar Blue reagent was obtained from Invitrogen (Carlsbad, CA, USA).

Real time polymerase chain reaction PCR (RT-PCR). The *RSU-1* mRNA expression level was assessed by RT-PCR, as described previously (6). Briefly, total RNA was extracted using TRIzol

Abbreviations: ECM, Extracellular matrix; HCC, hepatocellular carcinoma; NSC, non-specific control; PBGD, Porphobilinogen deaminase; PFA, paraformaldehyde; RSU-1, Ras suppressor-1.

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reagent (Gibco, Waltham, MA, USA). Preservation of 28S and 18S rRNA species was used to assess RNA integrity. Only samples with prominent 28S and 18S rRNA components were included in the study. Total RNA was reversed-transcribed to cDNA using SuperScript first-strand synthesis (Invitrogen). RT-PCR was performed using SYBR Green from Invitrogen in a Mini J Opticon Real Time PCR machine (Biorad, Hercules, CA, USA). Reactions were always performed in triplicate, while human porphobilinogen deaminase (*PBGD*) was used as housekeeping gene. The sequence of the primers used for *RSU-1* was: Reverse 5' CGT GCA ATC TCA AAA GCT CA 3' and Forward: 5'-AGG CCA CAG AGC AAG GTC TA-3'.

Western blotting. Protein expression was assessed by Western blot analysis using the standard Western blotting protocol. Total protein was isolated from the two cell lines using 1% sodium dodecyl sulfate in RIPA buffer (20 mMTris/Cl pH7.5, 150 mM NaCl, 0.5% NP-40, 1% TX-100, 0.25% sodium deoxycholated, 0.6-2 μg/ml aprotinin, 10 μMleupeptin, 1 μM pepstatin). Protein concentration in the samples was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amount of protein was loaded on each lane of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and detection of the antibodies was performed using super-signal ECL (Pierce).

Transfection with siRNAs. Both Alexander and HepG2 cells were treated for 48 h with 100 nM siRNA non-specific control (NSC) siRNA or *RSU-1* siRNA using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the company's guidelines. The sequence of the NSC siRNA used was 5'AAA CUC UAU CUG CAC GCU GAC3', while the *RSU-1* siRNA sequence was 5'UCA ACG GCC UCU UUA CCU UdTdT 3'. Silencing efficiency prior to any experiment performed was tested by western blot and/or RT-PCR.

Cell adhesion assay. The cell adhesion assay was performed as described previously (7). Briefly, cells were transfected with a control NSC siRNA or *RSU-1* siRNA. Forty eight hours post-transfection, 10^4 cells/well were seeded in six (6) wells of a 96-well plate pre-coated with 0.1% gelatin. After a 60-min incubation at 37˚C, three of the wells were washed three times with phosphate-buffered saline, while the remaining three were fixed with 4% paraformaldehyde (PFA). Washed wells were also fixed with PFA and then cells in all wells were quantified using crystal violet (7). Crystal violet was washed using ddH20 and cells were solubilized using acetic acid. Absorbance was measured at 570 nm using a PerkinElmer Enspire plate reader (PerkinElmer, Waltham, MA, USA). Adhesion was presented as the ratio of the absorbance at 570 nm of adhered cells (washed) divided by the absorbance at 570 nm of the total seeded cells (not washed). The data from two (2) independent experiments were analyzed using the Student's *t*-test. *p*-values <0.05 were considered statistically significant.

Cell proliferation assay. Twenty four hours following siRNA treatment, cells were subjected to alamarBlue assay (Invitrogen) (8) according to the company's guidelines. Briefly, cells were seeded in 96-well plates at a concentration of 10^4 cells/ml and alamarBlue was added at a volume of 1/10 of the volume of medium in the well. Following incubation at 37˚C for 2, 4 and 24 h post-plating, fluorescence was measured using a Perkin Elmer Enspire plate reader at 560/590 nm. Three independent experiments were performed.

Cell invasion. Cell invasion was assessed using the QCM™ Collagen Cell Invasion Assay in 24-well invasion chambers (8 μm) (Cat.#ECM551; Merck-Millipore, Billerica, MA, USA) following the manufacturer's protocol. Briefly, cells 24 h post-trasnfection with siRNA were trypsinized and a suspension of 0.5×10^6 cells/ml was prepared in serum-free Dulbecco's modified Eagle's medium (DMEM) medium. DMEM supplemented with 10% Fetal Bovine Serum was used as chemoattractant at the bottom of the transwell. Cells were incubated at 37˚C for another 24 h. At the end of the incubation period, the transwells were placed in staining solution and incubated for 20 min. Transwells were then washed several times with distilled water. The non-invaded cells that remained on the upside of the filter were removed, while the stained insert with invaded cells was transferred to a well containing 0.2 ml of extraction buffer. After 15min incubation at room temperature, the transwell was removed and the dye mixture was transferred to three wells of a 96-well plate. Optical density (OD) was measured at 560 nm using an automated Enspire Perkin Elmer spectrophotometer. Two independent experiments were performed.

Statistical analysis. Comparison of means using the Statgraphics software was used for the statistical analysis. The *t*-test was performed and a *p*-value <0.05 was considered statistically significant.

Results

RSU-1 expression is elevated in HepG2 cells compared to Alexander cells both at the mRNA and protein level. To study the role of RSU-1 in HCC cell lines, we first examined the expression of RSU-1 mRNA and RSU-1 protein levels in the two human HCC cell lines studied. As shown in Figure 1, the expression of the RSU-1 protein (Figure 1A) and the RSU-1 mRNA level (Figure 1B) was dramatically elevated in the highly invasive HepG2 cells compared to the non-invasive Alexander cells.

RSU-1 was effectively silenced. In order to study the importance of RSU-1 in the HCC cells, we inhibited its expression by siRNA silencing. Cells transfected with a non-specific control (NSC) siRNA were used as the transfection control. Forty-eight hours post-transfection cells were harvested and RSU-1 expression was assessed by western blotting. As shown in Figure 2A, RSU-1 was effectively silenced in both cell lines (compare lane 2 with lane 1 and lane 4 with lane 3).

RSU-1 silencing leads to increased cell proliferation. Following RSU-1 silencing, we investigated its possible interference with basic cellular properties. Thus, Alexander and HepG2 cells transfected with NSC or *RSU-1* siRNA were subjected to proliferation assay using alamar Blue. This dye was added to the cells 24 h post-transfection and measurements of fluorescence were taken at 2, 4 and 24 h.

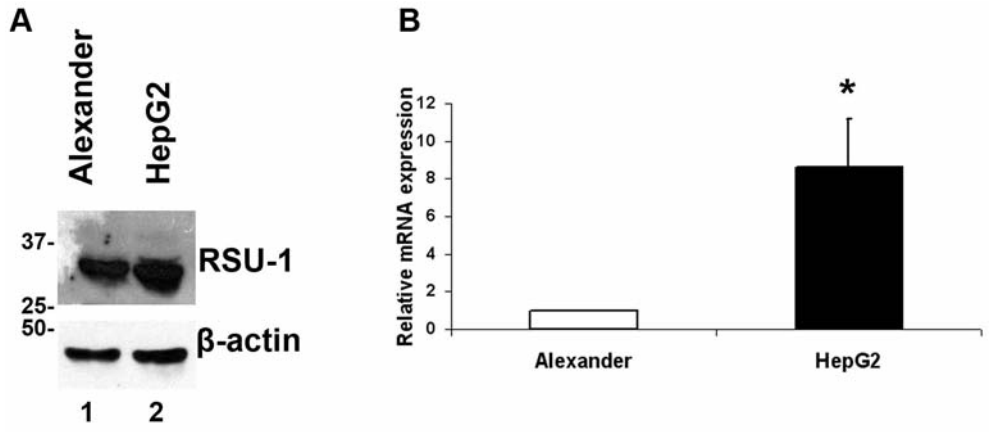

Figure 1. *RSU-1 expression at the mRNA and protein level in Alexander and HepG2 cells. A) Western blot analyses of RSU-1 in Alexander and HepG2 cells. B-actin was used as loading control. B) Real time PCR analysis of RSU-1 mRNA expression in both cell lines.*

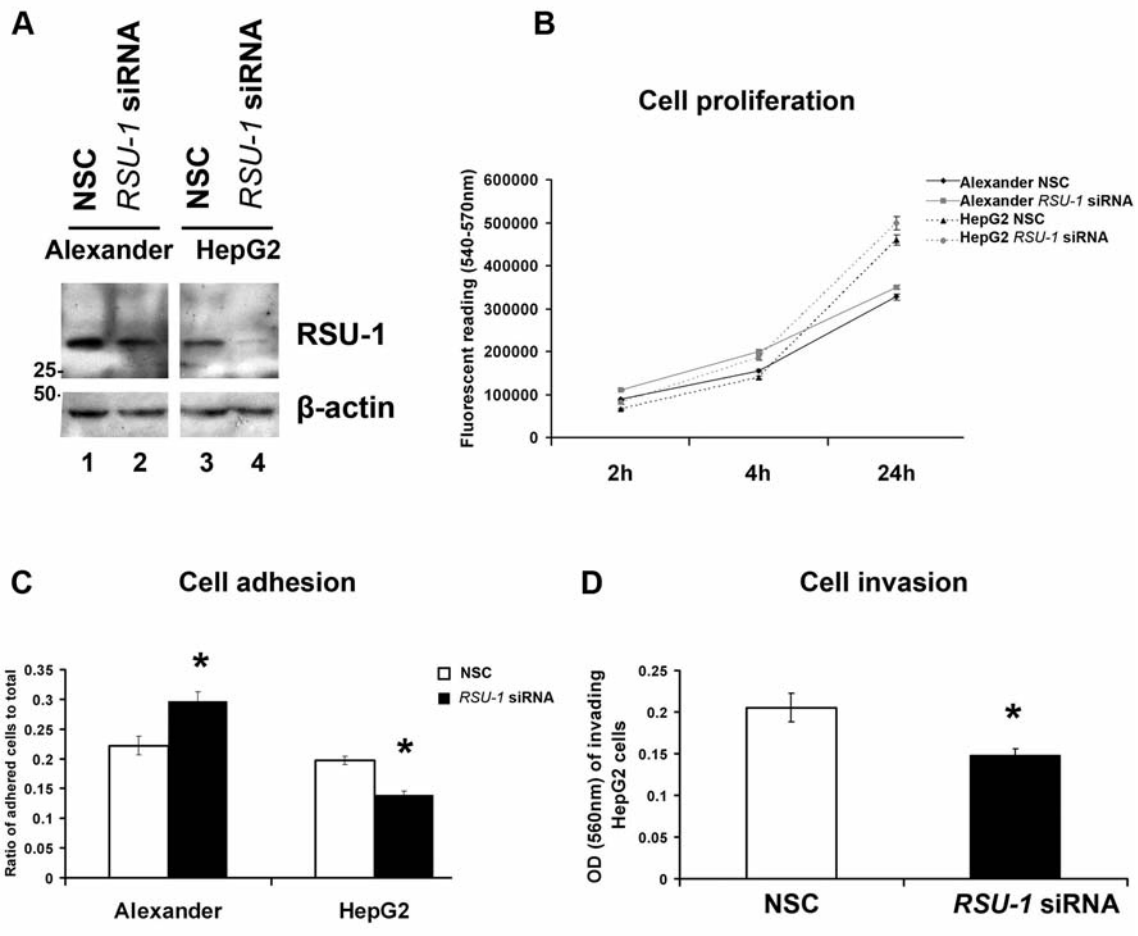

Figure 2. *Effect of RSU-1 silencing on basic metastasis-related in vitro cellular properties. A) Western blot analysis proving the efficiency of the knockdown in both cell lines. B) Cell proliferation following RSU-1 silencing in both cell lines 2, 4 and 24 h post-addition of alamarBlue (which was added 24h post-siRNA transfection). C) Cell adhesion on 0.1% gelatin following RSU-1 silencing. D) Cell invasion through collagen following RSU-1 depletion from HepG2 cells.*

As shown in Figure 2B, there was no significant difference in terms of cell proliferation in the two cell lines at 26 or 28 h post-silencing. However, at 48h post RSU-1 silencing, the proliferation of HepG2 cells was dramatically increased compared to that of Alexander cells. Moreover, in both cell lines, cells transfected with *RSU-1* siRNA proliferated at higher rates than the cells transfected with NSC indicating that RSU-1 silencing increases cell proliferation.

RSU-1 depletion has differential effect on cell adhesion properties of the two cell lines. As RSU-1 is a cell adhesion protein found at cell-ECM adhesion sites and as HCC is greatly dependent upon the connection of cells with the ECM, we evaluated the effect of its knock-down on cell adhesion properties on gelatin. As shown in Figure 2C RSU-1 silencing in Alexander cells leads to increased cell adhesion, while RSU-1 silencing in HepG2 cells leads to reduced cell adhesion.

Cell invasion is reduced following RSU-1 elimination from HepG2 cells. Since cell adhesion is closely associated with migration and invasion of cancer cells (7), we next tested the effect of RSU-1 silencing on cell invasion. As expected, Alexander cells had no invasive properties, while HepG2 invaded through collagen-coated chambers within 24 h. However, as shown in Figure 2D, RSU-1 depletion from HepG2 cells leads to significantly reduced cell invasion.

Discussion

In the present study we investigated the expression of RSU-1 in two human liver cell lines that differ in terms of their invasive capacities; the non-invasive Alexander hepatoma cell line and the highly invasive HCC cell line, HepG2. Although cancer cell lines have limitations in modeling the physiological complexity in human cancer, they are also useful as convenient tools to study the molecular mechanism involved and give a first indication of the importance of certain biological molecules in cancer therapeutics.

We found that RSU-1 is up-regulated in HepG2 cells both at the protein (Figure 1A) and the mRNA level (Figure 1B) indicating a possible involvement of RSU-1 in HCC pathogenesis.

We next utilized an siRNA-mediated approach to inhibit RSU-1 expression in both HCC cell lines and evaluate the effect of the inhibition on basic *in vitro* cellular properties related to metastasis, namely cell proliferation, cell adhesion and cell invasion. Following successful RSU-1 silencing in both cell lines (Figure 2A), proliferation was found to be increased in the RSU-1-depleted cells compared to the control in both cell lines suggesting that *RSU-1* inhibits cell proliferation (Figure 2B). Moreover, further analysis of the cellular properties of cells transfected with *RSU-1* siRNA showed that RSU-1 silencing reduces cell adhesion (Figure 2C) and cell invasion (Figure 2D) in the aggressive HepG2 cells, while it increases cell adhesion in Alexander cells and has no effect on its invasion status, as these cells are non-invasive. These results suggest that *RSU-1* enhances adhesion and invasion in the aggressive HepG2 cells and, thus, should be further evaluated as a potential therapeutic target in HCC. Thus, based on our findings, further analysis is required to elucidate the implicated molecular mechanism, while a study of RSU-1 expression in human HCC samples is deemed imperative.

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