Overexpression of Hyaluronan Binding Protein 1 (HABP1/p32/gC1qR) in HepG2 Cell Leads to Increased Hyaluronan Synthesis and Cell Proliferation by Upregulation of Cyclin D1 in AKT-Dependent Pathway.

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Capsule

Background: Hyaluronan (HA) levels regulate cell behavior, tumor invasion and migration through interactions with hyaladherins.

Result: Elevated expression of Hyaluronan Binding Protein 1 (HABP1) leads to enhanced HA synthesis, HA cable formation and activation of cell survival pathways in HepG2 cells.

Conclusion: Constitutively elevated expression of HABP1 leads to enhanced tumorigenic potential by HA-mediated pathways.

Significance: HABP1 modulates cell survival through enhanced HA synthesis.

Overexpression of mature form of Hyaluronan Binding Protein 1 (HABP1/gC1qR/p32), a ubiquitous multifunctional protein involved in cellular signaling, in normal murine fibroblast cells leads to enhanced generation of reactive oxygen species (ROS), mitochondrial dysfunction and ultimately apoptosis with the release of cytochrome C. In the present study, human liver cancer cell line HepG2, having high intra-cellular anti-oxidant levels was chosen for stable overexpression of HABP1. The stable transformant of HepG2, overexpressing HABP1 does not lead to ROS generation, cellular stress and apoptosis, rather it induced enhanced cell growth and proliferation over longer periods. Phenotypic changes in the stable transformant were associated with increased ‘HA pool’, formation of ‘HA cable’ structure, upregulation of HA synthase-2 and CD44, a receptor for HA. Enhanced cell survival was further supported by activation of MAP kinase and AKT-mediated cell survival pathways which leads to increase in cyclin D1 promoter activity.

Compared to its parent counterpart HepG2, the stable transformant showed enhanced tumorigenicity as evident by its sustained growth in low serum conditions, formation of ‘HA cable’ structure, increased anchorage-independent growth and cell-cell adhesion. The study suggests that overexpression of HABP1 in HepG2 cells leads to enhanced cell survival and tumorigenicity by activating HA-mediated cell survival pathways.

Hyaluronan (HA), an important mucopolysaccharide of extracellular matrix (ECM) in vertebrates, is now known to play a critical role in tumorigenesis and malignant transformation. HA forms part of the ECM by linking HA binding proteins, ‘hyaladherins’ into cell matrix network, thereby regulating their behavior like cell adhesion, motility, proliferation and differentiation (1).

Our laboratory has identified one such hyaladherin, Hyaluronan Binding Protein 1 (HABP1) from rat liver using HA-affinity column chromatography (2). HABP1 is a conserved eukaryotic protein ubiquitously present from yeast to mammals. HABP1, a highly acidic protein with pI 4.5, has a native molecular weight of 68 kDa, generating sub-units of 34 kDa on SDS-PAGE (3). The cDNA sequence of HABP1 shows complete identity with p32, a protein co-purified with the human pre-mRNA splicing factor SF2 and gC1qR, receptor for the globular head of complement sub-component 1q (4-6). The gene encoding HABP1 (accession no. AF275902) was mapped at chromosome 17p12-p13 by fluorescence in situ hybridization (FISH) analysis (7). The open reading frame of HABP1 encodes a pro-protein of 282 amino acid residues which after post-translational cleavage of first 73 amino acids...
generates the mature protein of 209 amino acid residues (8). The mature protein has a predicted molecular weight of 23.7 kDa from its amino acid sequence but migrates ambiguously at 34 kDa on denaturing gels due to high ratio of polar to hydrophobic amino acid residues. The crystal structure of HABP1 shows it to be a trimer having a doughnut shaped quaternary structure with an asymmetric charge distribution along its surface which attributes to its functional diversity (9). HABP1 also exhibits structural flexibility influenced by the ionic environment which plays an important role in its binding towards different ligands (10). HABP1 has been detected in a number of cellular compartments including the mitochondria, nucleus and cytoplasm and cell surface where it is shown to interact with many different cellular proteins (11). The diverse subcellular localization of HABP1, coupled to its various interacting proteins suggest that it could be a component of the trafficking pathway connecting the nucleus, mitochondria and cytoplasm and the export pathway to the cell surface (11).

HABP1 is highly phosphorylated in transformed fibroblasts and is shown as an endogenous substrate for MAP kinase which translocates to the nucleus upon mitogenic stimulation (12). Constitutive expression of HABP1 in parent fibroblast cell line has shown to inhibit cell growth, formation of vacuoles and induction of apoptosis at 60 hours in absence of media replacement (13). Transient expression of HABP1 and its N- and C-terminus truncated variants in COS-1 cells were found to induce autophagic vacuoles and disruption of F-actin network indicating a stress condition (14). Upon constitutive overexpression of HABP1 in fibroblast cell line F111, HABP1 gets accumulated in the mitochondria which lead to the generation of reactive oxygen species (ROS), mitochondrial dysfunction and apoptosis (15). These observations indicate an important role of HABP1 in cell growth, proliferation and apoptosis induction mediated by excess ROS generation.

In view of the existing literature, in the present study, we have chosen an alternative human liver carcinoma cell line (HepG2), which displays high levels of important protective enzymes such as Mn-superoxide-dismutase and Cu/Zn-superoxide-dismutase, as well as catalase, glutathione peroxidase, glutathione reductase and thioredoxin reductase (16), as a model system to examine the function of HABP1. Furthermore to substantiate our data, we have analysed HA level, cell survival pathways and tumor inducing potency of HABP1 in this distinct cellular model system that is constitutively overexpressing HABP1.

Experimental procedures

Reagents- Dulbecco’s modified Eagle’s medium (DMEM), Fetal bovine serum and all antibiotics were from Invitrogen Co. (USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (USA), Sigma Aldrich Chemicals Pvt. Ltd. (USA) and Cell Signaling Technologies (USA). All chemicals were procured from Sigma Aldrich Chemicals Pvt. Ltd. (USA) unless otherwise specified. Antibody to HABP1/p32/gC1qR was generated in our laboratory as previously described (5).

Plasmids- Plasmid having myc-tagged full-length HABP1, pHVL 22 was a gift from Dr. Peter O’hare (Marie Curie Research Institute Surrey, UK) and has been previously described (12). CyclinD1–Luciferase reporter construct was provided by Dr Edith Wang (University of Washington, Seattle, USA). CyclinB1–Luciferase reporter construct (hB1-Luci) was gifted by Dr Kurt Engeland (University of Leipzig, Germany). Dominant negative AKT construct was a kind gift from Prof. S. Dimmeler (University of Frankfurt, Germany), cdc-25–Luciferase construct was a kind gift from Dr. Giulia Piaggio (Istitute Regina Elena, Italy).

Cell Culture, Transfection and Growth Kinetics- All cell lines were cultured and maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, streptomycin and gentamycin and 50 µg/ml fungizone in tissue culture flask or dishes. The culture was grown in humidified CO₂ incubator at 37°C with 5% CO₂. Transient and stable transfections in cell lines were done using Lipofectamine™ 2000 (Invitrogen Inc.) following manufacturer’s instructions. The samples were collected 40 hours post-transfection and processed for lysate preparation for immunoblotting or for immunofluorescence. Cell proliferation assay was done using CellTiter™ 96 Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA) following manufacturer’s protocol.

Immunoblot analysis- The protein samples electrophoresed by SDS-PAGE were electro-
blotted on either nitrocellulose membrane or PVDF by applying 0.8mA/hour current in a semi-dry transfer unit or wet-transfer unit. Following transfer, the membrane was blocked with 5% non-fat dry milk in TBS for 2 hours at room temperature and incubated with desired primary antibody for overnight at 4°C. The PVDF membrane was then washed three times with TBST (TBS with 0.1% Tween-20) and incubated for 1 h with 1:2000 dilution of horseradish peroxidase or alkaline phosphatase conjugated secondary antibody. The bound antibody complexes were detected using the NBT/BCIP system or enhanced chemiluminescence (ECL) system.

**Immunofluorescence microscopy**- Cells were grown on coverslips for 24 hours in CO₂ incubator and were then fixed with 4% paraformaldehyde in PBS for 15 minutes followed by permeabilization with 0.1% Triton-X100 for 1 min. The cells were washed thoroughly with PBS and blocked with 3% BSA-PBS for 1 h at room temperature followed by incubation with primary antibody in 1% BSA-PBS for 1 h at RT. Cells were washed thrice with PBS and then incubated with secondary antibody. The secondary antibodies used were tagged either with cy3 (Sigma, U.S.A.) or AlexaFluor 488 and 546 (Molecular Probes). Hoechst 33342 was co-incubated with the secondary antibody to facilitate the visualization of the nucleus. Cells were again washed with PBS thrice, 5 minutes each and mounted in 20% glycerol in PBS. The imaging was done under Zeiss fluorescence microscope equipped with epifluorescence and axiocam camera system coupled with axiovision software (Carl Zeiss, Germany) or by Nikon 90i microscope (Nikon Instech Co. Ltd. Parale Mitsui Bldg. Japan) using Evolution QEi Digital Camera (Media Cybermatric, U.S.A.).

**Cell Surface Biotinylation**- Cell surface biotinylation was done with Sulfo-NHS-LC-biotin (Pierce) as per manufacturer’s protocol.

**Generation of HepG2-HABP1 cell line stably expressing full-length myc-tagged HABP1**- Plasmid pHVL22 was linearized by overnight digestion with Bgl II restriction enzyme. The linearized plasmid was gel eluted and used for transfection of HepG2 cells. HepG2 cells were transfected with HABP1 expression plasmid pHVL22 and pCDNA3.1myc/His (as vector control) and allowed to double once under non-selective conditions. Both the plasmids carry neomycin resistance gene. Later, the cells were supplemented with the complete medium containing 400 µg/ml of Geneticin and medium was replaced every third day. After two weeks of selection period individual colonies were isolated and further propagated under selective conditions. Individual clones were screened for the stable expression of HABP1 by indirect immunodetection and Western blotting analysis using anti-HABP1 and anti-myc antibodies.

**Haematoxylin-Eosin staining of cells**- The cells were grown overnight on coverslips and were then washed twice with PBS and fixed with chilled methanol for 15 minutes followed by staining with haematoxylin-eosin as described earlier (14). For viewing the cells, coverslips were mounted in glycerol, sealed with nail enamel and observed under phase contrast microscope (Nikon) fitted with Nikon FX-35W camera.

**Electron Microscopy**- Cells growing on culture dishes were washed thrice with PBS and fixed in 3% glutaraldehyde at 4°C for minimum of 4 hours to overnight. After washing, cells were again fixed for 2 hours in 1% osmium tetraoxide in phosphate buffer at 4°C. After several washes with PBS, the cells were dehydrated in graded acetone solutions and embedded in CY212 araldite resin. Ultra-thin sections of 60-80nm thickness were generated using Ultracut E Ultramicrotome and the sections were stained with alcoholic uranyl acetate and lead citrate for appropriate time intervals. The grids were then examined with Transmission Electron Microscope (Morgagni 268 Model, Philips) operated at 80kV.

**Soft agar colony assay**- 1% agarose was melted and mixed with an equal volume of 2X DMEM containing 20% Fetal Bovine Serum to get 0.5% agarose in 10% FBS-DMEM. 1.5 ml of this 0.5% agarose in FBS-DMEM was poured in each 35 mm dish as base agar. The dishes were left at room temperature to allow the base agar to solidify. For top agar, 0.7% agarose was melted and cooled to 40°C before mixing with 2X DMEM as higher temperatures could lead to cell death. In between, the cells were trypsinised and counted. The cell count was adjusted to 2×10⁵ cells/ml. For plating, 0.1 ml of cell suspension (2×10⁵ cells/ml) was added to 6 ml DMEM-agarose mix at 40°C. 1.5 ml of this was added to each quadruplet plate. The assay plates were incubated at 37°C in a humidified incubator for 10-14 days. The plates were then stained with 0.5 ml of 0.005% crystal
violet for about 3-4 hours and the colonies were counted using a dissecting microscope.

**Assay of intracellular ROS in cells**- Intracellular H_2O_2 production was detected by fluorescence of 2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester, H_2DCFDA (10 μM) incubated under various conditions for 10 minutes in dark as previously reported (15).

**Glutathione Assay**- Cells were counted and 1×10^6 cells were suspended in 0.5 ml of cold sulphosalicylic acid, prepared in MQ water containing 0.5 mM EDTA. Cells were kept on ice for an hour and then centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected and used for the assay. 50 μl of the sample was mixed with 1ml PBS containing 20 μg NADPH, 60 μg DTNB and one unit of glutathione reductase. The reaction rate was monitored by measuring absorbance at 412 nm over a period of time in spectrophotometer. The concentration of glutathione in the samples was calculated from the standard curve made out of different dilutions of glutathione.

**Cellular adhesion to immobilized HA**- Flat bottom 96 well tissue culture plates (Corning) were coated overnight with 5 mg/ml HA. 40 μg/ml BSA was used as a negative control. Cells were counted and 2×10^5 cells in serum free medium (SFM) were added to each well in triplicates. The plates were incubated at 37°C in CO_2 incubator for 60 minutes.

**Detection of F-actin**- The cells cultured on sterile cover-slips were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and washed with 0.1 M glycine for 5 min to quench excess aldehyde. Cells were permeabilized using 0.1% Triton X-100 (v/v) for 1 min and the excess detergent was washed off with PBS. Following permeabilisation, the cells were washed once with PBS and incubated with rhodamine conjugated phalloidin along with Hoechst for 30 min. After incubation the cells were rinsed with PBS several times and mounted in 15% glycerol in PBS.

**Competitive ELISA for HA quantification**- A competitive enzyme-linked immunosorbent assay (ELISA)-like method was adapted as reported earlier (17) to measure HA concentrations in the medium of the cultured cells and the cell lysates. Briefly, flat bottom 96 well tissue culture plates were pre-coated with 0.1 mg/ml HA (in 0.1M NaHCO_3, pH 9.6) by overnight incubation. Next day, excess coating solution was removed and the plate was dried with warm air to ensure the adhesion of HA to the wells. Blocking was done for 1hr at RT by using 100 μl of 1% BSA in PBS. The plates were then washed with PBS-T and dried with warm air. The samples and HA standards were diluted with 6% BSA in PBS and pre-incubated at RT for 1hr with an equal volume of 0.05 μg/ml of biotinylated HA-binding protein (Seikagaku) in 50 mM Tris, pH 8.6. 100 μl of samples and standards at multiple dilutions and blanks were added in triplicates to the wells and incubated at RT for 1hr. The wells were then washed 3 times with PBS-T and dried. 100 μl of streptavidin AP antibody (diluted 1:3,000 in PBS) was added to each well followed by 1hr incubation at RT. The wells were washed 3 times with PBS-T and dried. For colour development, 200 μl of 1mg/ml of PNPP solution (in 100 mM Tris-Cl pH 9.3, 100 mM NaCl, 5mM MgCl_2) was added to the wells. The plates were incubated in dark for 30 min or till yellow colour appears. The absorbance was taken in ELISA Reader (BD) at 405 nm. The concentration of HA in the samples was calculated against a standard curve of HA.

**Purification of HA and separation on a gradient polyacrylamide gel**- HA purification was carried out using a procedure described earlier (18). Equal number of HepG2 and HepR21 cells were seeded in culture dishes and allowed to grow for 48 hrs after which the media was removed and the monolayer washed gently with PBS. Subsequently, the cells were treated with 200 μl of 50 mM sodium acetate (pH 6.0) containing 250 μg/ml of proteinase K, 5 mM EDTA and 5 mM cysteine. After 10 minutes of incubation, the cells were scraped and collected into microcentrifuge tubes followed by incubation for 5 hrs at 60°C. Proteinase K was inactivated by incubation in a boiling water bath for 10 minutes followed by centrifugation at 13,000 rpm. Supernatant was collected and treated with 4 volumes of 1% cetylpyridinium chloride in 20 mM NaCl, for 1hr at room temperature and centrifuged at 13,000 rpm for 15 minutes. After discarding supernatant,
precipitate was washed with 1 ml water, centrifuged again and dissolved in 50 µl of 4M guanidine-HCl. Further, 900 µl of ethanol was added and the tube was kept at -20°C for an hour after which each sample was centrifuged and precipitate retained and dissolved in 50 µl of 50 mM sodium acetate (pH 6.7). Hyaluronan digestion was carried out using 50 µg/ml bovine testicular hyaluronidase (BTH) for 3 hrs at 37°C. Equal volumes of both undigested and BTH-digested products derived from two cell lines were loaded onto a 5-20% gradient gel (19), along with BTH-digested and undigested pure polymeric HA from human umbilical cord acting as positive controls. The gel was then stained with 1% Alcian blue in 3% acetic acid, destained and subsequently stained with silver nitrate (19).

**Reporter gene assays**- Cells were transfected with different promoter-reporter constructs and harvested 24 hours post-transfection. Luciferase and β-galactosidase assays were performed in the lysates according to the kit protocol (Promega, Madison, WI, USA).

**Semi-quantitative Reverse Transcriptase-PCR and Real-time PCR**-Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) as per manufacturer’s guidelines and was subjected to reverse transcription reaction for the first strand synthesis using MuMLV reverse transcriptase (Invitrogen Life Technologies) with oligo dT as the primer. The cDNA concentrations were normalized by amplification of a 323 bp GAPDH fragment (used as internal control) using gene specific GAPDH primer. The amplification conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification. Real-time PCR using gene-specific primers was used to quantitate the relative mRNA levels in parental HepG2 and HepR21 cells. The PCR reaction was done in a final volume of 20 µL and consisted of SYBR Green mix, 2 µl of cDNA, 100 nM of each forward and reverse primer, PCR amplification was done by denaturation for 10 minutes at 95°C followed by 40 cycles of 15s at 95°C, 30s at 60°C and 30s at 72°C. Thermocycling and fluorescence measurements were done in Mastercycler, Realplex (Eppendorf). Relative quantitation was done by parentizing threshold cycle (Ct) values of each sample gene with Ct values of GAPDH. \( \Delta C_t \) corresponds to the difference between the Ct of the genes of interest and the Ct of GAPDH. Data are presented as fold-change difference (derived by 2\(^{-\Delta\Delta C_t} \) method) relative to HepG2. The sequence of primers used in the study are given below:

| Primer       | Sequence                  |
|--------------|---------------------------|
| HABP1 Forward| 5’ ATCAACTCCCAATTTCGTGGTT 3’ |
| HABP1 Reverse| 5’ TCCTCTGGATAATGACAGTCCAA 3’ |
| Hyl2 Forward | 5’ GGCAGAGCTGGTGTCATC 3’ |
| Hyl2 Reverse | 5’ CCGTGTCAGGTAATCTTTGAGGACT 3’ |
| Hyl3 Forward | 5’ GATCTGGAGGTTCTCTGTC 3’ |
| Hyl3 Reverse | 5’ AGATGCCAGACTCCTCCT 3’ |
| HAS1 Forward | 5’ TCTGTAATCGCAACAAAGGT 3’ |
| HAS1 Reverse | 5’ CTACCCAGATCGACGACT 3’ |
| HAS2 Forward | 5’ TTTCTATTGTGACTCATCTGTC 3’ |
| HAS2 Reverse | 5’ ATGGTTGGCTACCAGTTATCCAAACGG 3’ |
| HAS3 Forward | 5’ CGCGCAACTTCCATGAGG3’ |
| HAS3 Reverse | 5’ AGTCGACACACCTGGATGTAG 3’ |
| GAPDH Forward| 5’ CGAGATCCCTCCAAATCAAG 3’ |
| GAPDH Reverse| 5’ GTCTTTGAGGTGGCAGTGAT 3’ |

**RESULTS**

Overexpression of HABP1 does not lead to growth inhibition. Stable transfectants expressing HABP1, tagged at C-terminus with myc were derived by G418 selection over a period of 3-4 weeks and confirmed by immunofluorescence and Western blotting using anti-myc and anti-HABP1 antibodies. While propagating the clones, frequent media change was not required as was seen in case of F111 cells (13). Two of the stable transfectants, named clone 6 and clone 21 showed most appropriate expression of myc-tagged HABP1. As the two clones were identical in morphology and growth kinetics, we chose clone 21 for further studies and referred it as HepR21 hereafter. The clone expressing vector alone was named Hep.
To confirm the expression of myc-tagged HABP1 in HepR21, the lysates of HepG2 and HepR21 cells were prepared, equal amounts of protein were loaded on to 12.5% SDS-PAGE followed by Western blotting. Increased expression of HABP1 (2.6 folds) is evident in HepR21 cells as compared to the HepG2 cells when probed with anti-HABP1 antibody. As the full-length transfected HABP1 is myc tagged, on probing with anti-myc antibody, a band on the position of HABP1 can be seen in HepR21 but not in HepG2 cells (Fig. 1A). The clone was confirmed by transfecting the HepR21 cells with psil 570, siRNA against HABP1 using Lipofectamine reagent (which is reported to be a highly efficient transfection reagent for HepG2 cells). A decrease in the expression level of myc-tagged HABP1 was seen in HepR21 cells as compared to mock transfected cells (Fig. 1B) confirming the enhanced expression of HABP1 in the clone due to genomic integration of full-length HABP1. mRNA quantification by real-time PCR too showed ~ 1200 fold increase in HABP1 mRNA levels in HepR21 cells as compared to parent HepG2 (Fig. 1C) To explore the effect of overexpression of HABP1 on growth kinetics, HepG2, Hep-Vec, HepR21 and HepR6 cells were grown in complete medium and cell growth was monitored by performing MTT assay for cell proliferation at different time points. HepG2 and Hep-Vec cells had similar growth characteristics in complete media; while HepR21 and HepR6 cells had better survival rates (Fig. 1D) over long periods of growth without the change of media. In HepG2 cells, it was observed that the cell growth reaches a plateau after 48 hours and about 75% of cells die after 144 hours of growth. Whereas, in HepR21 and HepR6 cells, the cell growth gets saturated after 120 hours and only about 20% of cells die beyond this time period.

**Morphological changes in the HepR21 stable clone expressing myc-tagged HABP1.** After confirming the expression of full-length myc-tagged HABP1 in HepR21, we investigated whether overexpression of HABP1 in HepR21 and HepR6 cells has some effect on the cellular morphology. Interestingly, we observed a change in morphology in HepR21 cells under phase contrast microscope, while they were being routinely cultured. Another observation which we made while culturing the cells was that HepR21 cells took longer time to get trypsinized as compared to HepG2 cells. Thus, haematoxylin and eosin staining was done to observe cellular morphology of HepG2 and HepR21 cells. Differential Interface Contrast microscopy (DIC) images were also recorded for both the clones. Interestingly, we observed that in HepR21 and HepR6 cells, the nucleus size and cytoplasmic area was larger and the cells gave a bulky/bloated appearance as compared to HepG2 and HepG2 vector-control cells (Fig. 2A and 2B). HepR21 and HepR6 cells proliferated in close proximity to each other as if the cell-cell adhesion was preferred by cells for their propagation. Previous studies from our laboratory have shown ultrastructural changes like autophagic vacuoles, abnormal mitochondria with ruptured membranes upon HABP1 overexpression in fibroblast cell line F111 (15). To study if any such changes occur in the stable clone HepR21, we performed transmission electron microscope (TEM) studies and scanning electron microscope (SEM) studies on HepG2 and HepR21 cells. Transmission electron micrographs retrieved from the two cell lines showed similar morphology with a distinct nucleus, intact mitochondria and abundant ER (Fig. 2C, Panel a and b). No autophagic vacuoles were observed in HepR21 cells. SEM studies too supported the difference in size between the two cell types. In addition, HepR21 cells had a more spread out appearance than HepG2 cells suggesting that HepR21 cells may be more adherent to each other (Fig. 2C, Panel c). The only morphological difference seen was that HepR21 cells were bigger in size as compared to HepG2 cells. No significant change in nuclear to cytoplasmic ratio was observed in the two cell lines.

**Overexpression of HABP1 in HepG2 cells does not induce cellular stress.** To determine whether overexpression of HABP1 induces cellular stress in HepG2 cells as it does in F111 and HeLa cells, we checked the F-actin localization in HepG2 and HepR21 cells by rhodamine-phalloidin staining as actin depolymerization is indicative of generation of oxidative stress. When compared with HepG2 cells, no change in actin polymerization was observed in HepR21 cells indicating that HABP1 overexpression does not induce actin depolymerization (Fig. 3A). To investigate if the overexpression of HABP1 leads to the generation of ROS, we assayed the intracellular H₂O₂ generated in HepG2 and HepR21 cells. The cells
were assayed for ROS formation at different time points from 24 to 72 hours. No significant increase in ROS formation in HepR21 was seen at all the tested time points (Fig. 3B). The cells were treated with different concentrations of H$_2$O$_2$ (0, 10, 20 and 50 µM) and then the ROS formation was assayed. There was no significant increase in ROS formation even after treatment with 50 µM of H$_2$O$_2$ (Fig. 3C) suggesting that this cell line is resistant to oxidative stress. Increase in ROS formation leads to induction of heat shock proteins like Hsp70 and ER stress marker GRP78. So next, we examined the levels of Hsp70 and GRP78 in HepG2 and HepR21 cells by Western blotting. No change in the expression levels of Hsp70 and GRP78 was observed in HepR21 cells, confirming that overexpression of HABP1 does not lead to induction of cellular stress (Fig. 3D). The proper redox state of the cell is maintained by intracellular thiols such as glutathione, which are capable of scavenging ROS. An increase in ROS generation, a decrease in antioxidant capacity, or both will lead to oxidative stress. Thus, we tested the levels of glutathione and oxidant guards of intracellular systems like superoxide dismutase (SOD). In HepR21 cells, more than two fold increase in glutathione levels were seen (Fig. 3E) whereas the activity levels of superoxide dismutase (Fig. 3F) and catalase (results not shown) were almost similar in all the three cell lines tested.

Enhanced levels of HABP1 are expressed on cell surface of HepR21 cells. To determine whether HABP1 is present on the surface of HepG2 and HepR21 cells, we performed permeabilized immunofluorescence studies on both the cell types. Our results showed that HABP1 was localized on the surface of both HepG2 and HepR21 cells. In HepG2 cells, HABP1 was found to be distributed evenly on the surface of the cell while in HepR21 cells, an interesting profile of HABP1 was seen on the cell surface. We observed that HABP1 localized more on the specific areas on the cell rather than being present uniformly on the entire cell surface. This observation became more clear when HepR21 cells were probed with anti-myc antibody, which can detect only the myc-tagged HABP1. HABP1 was seen to be concentrated on the cell periphery suggesting that it may play an important role there in cell-cell adhesion (Fig. 4A). The augmented cell surface localization of HABP1 in HepR21 cells was confirmed by cell surface biotinylation followed by western blotting with anti-HABP1 and anti-myc antibodies (Fig. 4B).

Stable overexpression of HABP1 in HepG2 cells leads to an increase in tumorigenic potential. Tumor cells adapt themselves to survive in low nutrient conditions. So, growth of cells in low serum conditions can be indicative of the tumorigenic potential of the cells. HepG2 and HepR21 cells were grown in DMEM supplemented with 2% FBS and the cell growth was monitored from 0 to 196 hours by MTT assay. Interestingly, HepG2 cells showed growth saturation between 72 and 96 hours and after 196 hours most of the cells die, whereas in HepR21 cells, the growth gets saturated after 120 hours but even after 196 hours, more than 75% of cells were surviving (Fig. 5A). To examine tumorigenic index of the stable clone HepR21, we did soft agar colony assay and found a marked increase in the colony count in HepR21 cells as compared to HepG2 cells (Fig. 5B).

While culturing HepG2 and HepR21 cells, we observed enhanced cell-cell adhesion in HepR21 cells, resulting in more proximal cell cultures. Also HepR21 cells appeared to adhere better onto the surface of the culture plates and took longer time to get trypsinized as compared to HepG2 cells. To examine this, we performed a two hour assay to see the cell adhesion of HepR21 and HepG2 cells, in complete media and serum-free media, on to the surface of 96 well plates. We observed that in complete media, the number of cells of HepR21, sticking to the plate in two hours was about four times more as compared to HepG2 cells (Fig. 5C) confirming that HepR21 cells are more adherent. In subsequent experiments we tested the adherence of both the cell lines on to the HA coated wells. As expected, we found that HepR21 cells are about twice more adhesive to plates coated with HA as compared to the HepG2 cells (Fig. 5D), which indicate activation or upregulation of cell adhesion molecules that play a pivotal role in development of invasive and metastatic cancers.

These results suggest that upon overexpression of HABP1, the cells become more tumorigenic as indicated by better survival rates in low serum conditions with concomitant increase in the anchorage independent growth and enhanced cell-cell adhesion.
Upregulation of HA synthesis and formation of ‘HA cables’ in HepR21 cells. HA levels are linked to cell proliferation and tumorigenic potential of cells with high HA levels present in tumor cells. High stromal HA is associated with poorly differentiated tumors and aggressive clinical behavior in human adenocarcinomas (20). HA is reported to accumulate into the stroma of various human tumors and modulates intracellular signaling pathways, cell proliferation, motility and invasive properties of malignant cells. The dynamic turnover of HA is tightly regulated by altering the expression profiles of HAS isoenzymes. Increased expression of HAS2 and HAS3 has been shown to result in increased HA production leading to malignant tumor progression (21). Therefore, to investigate whether increase in cellular proliferation and tumorigenic potential in HepR21 cells correlates with increased HA levels, we quantitated in both the cell lines, the levels of HA in the cell lysate and the HA secreted in to the culture supernatant. Higher levels of HA (~2 folds) were found to be secreted in the media by HepR21 cells as compared to HepG2 cells and the levels of HA in the cell lysate were about 20 folds higher in HepR21 cells as compared to HepG2 cells (Fig. 6A). When using purified HA preparations, an increase in HA levels in HepR21 cells was clearly observed with 5-20% gradient polyacrylamide gel electrophoresis. It was evident that subsequent to BTH treatment, high levels of polymeric HA in HepR21 disappears with appearance of increased levels of oligomeric HA (Fig. 6B). As increase in HA levels are linked to cell survival and increased tumorigenicity of the cells and our observation that HepR21 cells were more ‘adhesive’ than HepG2 cells, we subsequently attempted to examine whether some of these phenomena could be attributed to alteration in HA distribution between the two cell types. HA localization, as seen by immunofluorescence by probing with b-HABP followed by streptavidin-cy3, gave us a very interesting observation. In HepR21 cells, ‘HA cable’ like structures were seen connecting the cells unlike HepG2 where very little or no ‘cable’ like structure was evident (Fig. 6C). To confirm whether the ‘cable’ like structure as seen in HepR21 cells was indeed HA or HA-enriched, we treated both the cell types with different concentrations of HA degrading enzyme, *Streptococcal pneumoniae* hyaluronate lyase (SpnHL) for 30 minutes and then observed the localization of HA by immunofluorescence. In HepG2 cells, it was observed that upon treatment with SpnHL, the cell-cell adhesion reduced to the extent that the cells were detached from each other (results not shown). As anticipated, upon treatment with SpnHL, all the ‘cable’ like structures were disrupted in HepR21 cells confirming that they were indeed ‘HA cables’ (Fig. 6D). To determine whether increase in HA levels in HepR21 is associated with increased levels/activity of HAS isoenzymes, we performed RT-PCR using HAS1, HAS2 and HAS3 specific primers and observed a 2.5 fold increase in the HAS2 RNA transcripts in HepR21 cells as compared to HepG2 cells whereas HAS1 and HAS3 levels remained unchanged thus, confirming transcriptional upregulation of HAS2 in HepR21 (Fig. 6E). Western blot using anti-HAS2 antibody too shows 1.5 fold increase in HAS2 expression in HepR21 cells (Fig. 6F). To observe if some of the other genes involved in HA metabolism are also affected by HABP1 overexpression, real-time PCR was performed using gene specific primers for HAS1, HYAL2 and HYAL3, with GAPDH as control. The mRNA expression levels of all the three genes were found to decrease in HepR21 cells (Fig. 6G).

Cell survival pathways are activated upon stable HABP1 overexpression in HepG2 cells. Increased HA synthesis as observed in HepR21 cells made us look further into HA-mediated survival pathways. CD44, a cell surface HA receptor, is implicated in a variety of physiological and pathological processes, including lymphocyte activation, cell-matrix interactions, and regulation of tumor growth and metastasis (22). Various studies have established its role in cell survival signaling as well as regulation of cellular invasion and metastasis. HA-CD44 interactions are reported to activate ERK and ERK-dependent cyclin D1 gene expression leading to increased cell proliferation (23). So we wished to study the effect of HABP1 overexpression on CD44 and its downstream effectors. Interestingly upon probing the lysates of the two cell lines with anti-CD44 antibody, we observed two bands, an upper band at ~85kDa and a lower band at ~40 kDa. The intensity of bands in HepR21 was about 2.5 folds more as compared to the HepG2 (Fig. 7A and 7G) indicating that overexpression of HABP1 leads to increase in HA levels and activation of HA.
mediated signaling pathways. It is known that HA, via activation of Ras activates MAP kinase signaling pathway which is responsible for HA-mediated cell survival (24). To further investigate whether MAP kinase pathway is activated in HepR21 cells, ERK and activated ERK (p-ERK) levels were examined in both the cell lines. Immunoblot analysis of equal amounts of lysates from HepG2 and HepR21 showed increase in the levels of p-ERK in HepR21 cells as compared to HepG2 cells, but the levels of ERK remain same in both the cell types (Fig. 7B and 7G) indicating an enhancement in activation of MAP kinase signaling pathway in HepR21 cells. To ascertain whether HA mediates ERK signaling through Ras activation, we subsequently checked Ras levels in HepG2 and HepR21 cells as activation of Ras signaling is believed to augment cell growth, differentiation and survival. Western blot analysis using anti-Ras antibody shows an increased expression of Ras in HepR21 cells when compared to HepG2 cells indicating that HA mediates its downstream signaling events through Ras activation (Fig. 7C and 7G). Subsequently, we attempted to examine the levels of AKT, p-AKT and β-catenin in the stable clone as HA is reported to induce cell survival pathway via the activation of AKT and β-catenin. Immunoblot analysis of equal amounts of lysates of HepG2 and HepR21 showed increase in the levels of both AKT (~4 folds), p-AKT (2.4 folds) and β-catenin in HepR21 cells as compared to HepG2 cells (Fig. 7D, E and G) indicating the activation of AKT-mediated cell survival pathways in HepR21 cells. To further explore the signaling events involved in cell survival in HepR21 cells, expression level of cyclin D1, which is a downstream effector in MAP kinase and AKT pathway, was examined in both the cell lines. Cyclin D1 levels were found to be upregulated (1.5 folds) in HepR21 cells (Fig. 7F). Cyclin–CDK complexes are precisely regulated by cell cycle inhibitors that block their catalytic activity. One of such inhibitors is p21 which following anti-mitogenic signals or DNA damage, binds to Cyclin–CDK complexes to inhibit their catalytic activity and induce cell-cycle arrest. p21 thus functions as a regulator of cell cycle progression at G1 phase. Western blot analysis by using p21 and cyclin D1 antibody shows a six fold decrease in the levels of p21 in HepR21 cells as compared to HepG2 cells (Fig. 7F and 7G) indicating the activation of cyclin D1 mediated survival pathways in HepR21 cells.

Overexpression of HABP1, specifically enhances the promoter activity of cell cycle regulatory gene Cyclin D1 in AKT dependent manner. Since we observed increased cellular proliferation of HepR21 cells, we sought to determine the molecular and mechanistic details underlying this phenomenon. We have already shown an activation of both ERK and AKT in HepR21 cells. It has been recently reported ERK activation and AKT dependent cyclin D1 gene expression is stimulated by HA binding to CD44 selectively; thereby enhancing cell cycle progression and mitogenesis (24). As cellular proliferation is a direct consequence of enhanced functional activity of cell cycle regulatory genes, hence, we determined promoter activity of several cell cycle regulatory genes and compared their activities in HepR21 cells as well as in control HepG2 cells using promoter-reporter gene constructs for Cyclin D1 (CD1), elongation factor 2F (E2F), cell division cycle 25 (Cdc25) and Cyclin B1 (CB1).

For this purpose, control HepG2 cells and HepR21 cells were transiently transfected with CD1-Luc, Cdc25-Luc and CB1-Luc promoter-reporter constructs. Reporter gene activities were determined 24 hours post-transfection. In our results, approximately 3 to 4 fold enhancement in the activity of reporter genes from the promoter of CD1 was observed, where as no change was evident for Cdc25 and CB1 promoter-reporter constructs (Fig. 8 A and B). On the basis of these findings, we can speculate that overexpression of HABP1 attributes to enhanced activity of cell cycle regulatory genes CD1 and as a consequence increased cellular proliferation is observed in the stable clone HepR21.

As we have demonstrated elevated levels of AKT and phosphorylated AKT forms in HepR21 cells, subsequently we wished to determine the role of AKT in modulating the promoter activity of cell cycle regulatory genes. We therefore co-transfected the vectors encoding dominant negative AKT (DN-AKT), which interferes with the function of endogenous AKT and CD1-Luc promoter-reporter genes into HepG2 cells and in HepR21 cells. Reporter gene activities were determined after 24 h of expression period. In our results, a significant 50% reduction in CD1 promoter activity was observed when dominant
negative AKT form was co-transfected into cells stably expressing HABP1, where as in control HepG2 cells only a marginal reduction was evident (Fig. 8C).

Taken together, our results highlight the role of elevated levels of AKT in maintaining the higher promoter activity of cell cycle regulatory genes like cyclin D1 in HepR21 cells.

DISCUSSION

The human hepatoma cell line, HepG2 is known to possess elevated levels of anti-oxidant enzymes which shield these epithelial cells from oxidative assault (16). Also, HA and other glycosaminoglycans (like chondroitin sulfate) are reported to be elevated in hepatic carcinomas (25). Additionally, HA is reported to have anti-oxidant properties and it is known to act as a scavenger of chemical reactive oxygen intermediates (ROI) in the extracellular space (26). In view of these facts, a liver cell line HepG2 appeared to be most suitable model to study the effects of HABP1 under the conditions where endogenous anti-oxidant levels are maintained at relatively higher levels.

The present study has shown that overexpression of HABP1 in human liver cancer cell line HepG2 (HepR21), induces high endogenous glutathione levels and enhanced cellular proliferation over longer periods of cell growth, contrary to F111 cells, where HABP1 overexpression leads to ROS mediated apoptosis (13,15). A marked increase in the total ‘HA pool’ and ‘HA cable’ formation was seen in HepR21 cells, as reflected by HAS2 upregulation and downregulation of HYAL2 and HYAL3. Upregulation of CD44, the most important cell surface receptor for HA was also seen in HepR21 cells suggesting that its interaction with HA might be a possible mechanism for activation of HA-mediated signaling pathways in the stable clone. Activation of MAP kinase and AKT-mediated cell survival pathways with upregulation of their downstream effectors like β-catenin, Ras and cyclin D1 was shown in HepR21 cells. Interestingly, HepR21 cells had a higher tumorigenic potential as compared to HepG2 cells as indicated by their elevated cell surface HABP1 expression, increased survival rates in low serum conditions, and increase in the anchorage-independent growth and enhanced cell adhesion.

Present study indicates that increased levels of GSH in HepR21 cells may lead to cellular proliferation. In this context it has already been reported that high intracellular GSH levels lead to proliferation of lymphocytes and fibroblast (27,28). Enhanced levels of HA and GSH in HepR21 cells, could be a mechanism by which this cell line becomes resistant to oxidative stress and undergoes cell proliferation.

It is now well known that HA levels are elevated in most malignancies and are related to tumor invasion and migration. Stromal microenvironment having elevated HA levels in colon epithelia is conducive for development and progress of cancer (29). HA production is increased at tumor-stroma interface in invasive and metastatic human breast cancers when compared with benign or premalignant lesions (21). HA-induced signaling pathways reportedly direct the migratory phenotypes of stromal cells via interaction with HA receptors like CD44 and RHAMM which are responsible for HA-dependent cell migration and invasion of stromal fibroblasts (30). These reports support our observation that elevated HA levels are associated with enhanced tumorigenicity by activation of HA-mediated signaling.

In mammals, HA is synthesized by membrane-bound synthases on the inner surface of the plasma membrane by three hyaluronan synthases (HAS1–3) (31). Several growth factors like EGF, KGF, PDGF, and cytokines regulate the expression of the HAS isoenzymes (32). HA synthesis can also be regulated by post-transcriptional events, such as phosphorylation or ubiquitination of HAS, and/or dimerization of the enzyme, as well as by availability of the cytosolic UDP-sugar substrates. Different HAS isoenzymes are reported to generate HA of different molecular weights. HAS3 is known to synthesize HA with a smaller molecular mass than HA synthesized by HAS1 and HAS2 (33). By evaluating the HA secreted into the culture media by stable HAS transfectants, it has been demonstrated that HAS1 and HAS3 generate HA with broad size distributions (2×10⁵ to ~2×10⁶Da), whereas HAS2 generates HA with a broad but extremely large size (~2×10⁶ Da) (33). But another study has reported that all three HAS enzymes can drive the biosynthesis and release of high-molecular-mass HA (1×10⁶Da) (34).
Several studies have shown the association of HAS2 levels in the cells with enhanced tumorigenicity. Ectopic expression of HAS2 in mammary tumor cells has been reported to form HA-rich ECM which recruits stromal cells inside the tumors leading to the formation of intratumoral stroma (35). Overexpression of HAS2 in human fibrosarcoma cell line has been reported to increase HA synthesis, and to promote anchorage-independent growth and tumor formation (36). In a different study, it has been shown that antisense suppression of the HAS2 expression significantly decreases HA production in the cells transformed by the oncogenic v-Ha-ras which eventually leads to a reduction in tumorigenicity in the rat peritoneum (37). Overexpression of HAS2 alone in prostate cancer cell lines was shown to be sufficient to enhance the in vivo tumorigenic potential of prostate tumor cells (38). These studies are in line with our observation that increased HA synthesis mediated by upregulation of HAS2 enhances the tumorigenic potential in HepR21 cells.

CD44 is most widely expressed and extensively studied hyaluronan receptor and HA binding to CD44 has been reported to selectively activate ERK and ERK-dependent cyclin D1 gene expression thereby stimulating cell cycle progression and mitogenesis (29). The Ras/ERK signaling cascade (Raf, MEK and ERK) has been implicated to play an important role in the transcriptional activation of cyclin D1 gene in response to a variety of mitogenic stimuli. Cyclin D gene is amplified in a subset of hepatocellular carcinomas and is a downstream target of β-catenin. HA-CD44 interaction can also activate Rac1 signals, which regulate actin assembly thereby promoting cell survival and motility (39). Activation of AKT, a downstream effector in HA signaling, in HepR21 cells can be correlated with an earlier report which indicates that interactions between elevated HA and CD44 receptors on epithelial tumor cells activate HA-receptor tyrosine kinase mediated cell survival pathway. HA-ErbB2-PI3-kinase/AKT-β-catenin-COX-2 signaling axis has been reported to lead to intestinal epithelial and colon tumor cell division and proliferation (29). Furthermore, AKT-mediated cyclin D1 promoter activity enhancement, as demonstrated in HepR21 cells may promote cell proliferation and tumorigenesis. Consistent with its critical role in cell cycle progression, increased expression of cyclin D1 has been observed in several tumors (40). Ectopic overexpression of cyclin D1 in transgenic mice has been shown to induce formation of tumors (41) and cyclin D1 null mice show remarkably decreased development of tumors (42). Based on these reports, it is evident that activation of HA-CD44 interaction leads to activation of MAP kinase and AKT, and their downstream signaling targets, which induces cell survival pathways in HepR21 cells.

We have observed a higher expression of HABP1 on the cell surface in HepR21 cells, along with its increase in tumor potentiality. HABP1 could not be detected on the cell surface when it was overexpressed in F111 (mouse fibroblast) and HeLa (human cervical carcinoma) cells earlier in our laboratory. Significantly higher HABP1 expression is common in human cancers and the HABP1 levels are often greatly elevated compared with the corresponding normal tissue (43). In an extensive study using combinatorial immunoglobulin libraries and phage display, it was further shown that HABP1 is preferentially overexpressed in adenocarcinoma cells and it contributes to the malignant phenotype (44). It has been recently reported that HABP1, particularly its cell-surface-expressed form, is a new marker of tumor cells and tumor-associated macrophages/myeloid cells in hypoxic/metabolically deprived areas of tumors (45). HABP1 was identified as the receptor for a tumor-homing peptide, LyP-1, which specifically recognizes an epitope in tumor lymphatics and tumor cells in certain cancers. The tumor specificity of the peptide and anti-HABP1 antibodies was shown to be a result of higher expression of total HABP1 and the propensity of malignant and tumor-associated cells to express HABP1 at the cell surface. Hence, it is reasonable to postulate that increased cell surface expression of HABP1 as seen in HepR21 cells may be responsible for its increased tumorigenicity (45).

HA in the pericellular matrix can have both adhesive and anti-adhesive properties, which are regulated at several levels. HA that is present in the form of cable structures is pro-adhesive. HA cables promote the adhesion of monocytes in inflammatory environments in cell culture and within tissues such as inflamed intestinal mucosa and kidney (46). The high degree of cross-linking of HA within the cable structures create stable and ordered presentation of HA and/or its associated...
proteins, which can influence receptor clustering and intracellular signaling. This indicates an important role for pericellular and/or intracellular HA in the maintenance of the proper milieu for normal cell shape changes, and as part of the cellular architectural framework that regulates gene expression (47). It has been recently reported that cyclin D3 mediates synthesis of a HA matrix with adhesive properties for monocytes in mesangial cells that were stimulated to divide in hyperglycemic medium (48). In a recent study (49), it has been shown that no cable-like ECM structures of HA could be detected in Has2-deficient (Has2Δ/Δ) fibroblasts, but such structures could be seen in wild-type and Has2floxfloxfibroblasts indicating that HAS2 expression too can be correlated to formation of HA-cables as observed in our study. Microarray analysis on both the cell types has shown upregulation of epithelial marker E-cadherin and also other key cell surface adhesion proteins (P-cadherin, fibronectin, laminin etc.) in HepR21 cells which can influence cell morphology as seen in HepR21 cells and cell adhesion (data not shown). Therefore, enhanced cell adhesion as seen in HepR21 cells may be attributed to i) alterations in HA levels, ii) upregulation of CD44, iii) formation of ‘HA cable’ structure and, iv) distribution and cross-linking of HA cable with its cell surface binding proteins. Based on our consolidated observations, we conclude that stable overexpression of HABP1 in hepatic HepG2 cells leads to an increase in HAS2 levels that results in synthesis and maintenance of high levels of HA. Consequently, interaction of HA with CD44 triggers activation of downstream signaling events which leads to activation of various cell survival pathways resulting in cell cycle progression, mitogenesis and enhanced cellular adhesion and finally the regulation of tumor potential.

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FOOTNOTES

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The abbreviations used are: HA, Hyaluronan; HABP1, Hyaluronan Binding Protein1; ROS, Reactive Oxygen Species; HAS, Hyaluronan Synthase.

FIGURE LEGENDS

**Fig. 1.** Immunodetection with anti-HABP1 and anti-myc antibody to confirm the expression of full-length myc-tagged HABP1 in HepR21. (A) Lysates of HepG2 and HepR21 were made in RIPA buffer as described in ‘Materials and Methods’. Equal amounts of protein were subjected to 10% SDS-PAGE followed by Western Blotting. Proteins were immunodetected using anti-HABP1 and anti-myc antibodies. Equal amount of protein in lysates was confirmed by probing the blot with anti-actin antibody. Densitometric analysis of the bands using ImageJ software showed 2.6 fold increase in HABP1 levels in HepR21 cells as compared to HepG2 cells. (B) HepR21 cells were mock transfected and transfected with siRNA against HABP1 pSil570. The lysates were prepared 48 hours post-transfection in RIPA buffer and electrophoresed through 10% SDS-PAGE, blotted on to PVDF membrane and probed with anti-myc antibody. A decrease in expression of myc-tagged HABP1 was seen in HepR21 after transfection with siRNA thus confirming the authenticity of the clone expressing the integrated full-length HABP1. (C) Quantitation of mRNA expression of HABP1 in parental HepG2 and stable transfectant, HepR21. Total RNA was extracted from exponentially dividing cultures of HepG2 and HepR21 cells. The level of mRNA for HABP1 was quantitated by real-time RT-PCR. Enhanced HABP1 mRNA expression (1207 folds) could be observed in HepR21 cells stably expressing myc-tagged HABP1. (D) HepG2, HepR21, HepR6 and HepG2 vector integrated (Hep-Vec) cells were grown in complete media and MTT assay for cell proliferation was done at different time points from 0 to 144 hours. The media was not changed at any point of time. HepR21 and HepR6 cells were shown to have better survival rates over a period of time as compared to HepG2 cells and HepG2 vector control.

**Fig. 2.** Morphological changes in the stable clones upon HABP1 overexpression. (A) Hematoxylin- eosin staining of HepG2 cells and the stable clones show a considerable difference in their
morphology. HepR21 and HepR6 cells have a bigger cytoplasm and nucleus as compared to HepG2 cells and Hep-Vec cells thus giving a swollen appearance. (B) Differential Interface Contrast microscopy images of HepG2, Hep-Vec, HepR6 and HepR21 cells confirm the above observation. (C) Transmission and scanning electron microscopy images of HepG2 and HepR21 cells show no change at the ultrastructural levels between the two cell types. Both HepG2 and HepR21 cells have a distinct nucleus, abundant ER and intact mitochondria (‘a’ at 2200X) and (‘b’ at 7100X). Scanning electron micrograph images show that HepR21 cells give a more flattened and spread out appearance (‘c’ at 1200 X).

Fig. 3. Overexpression of HABP1 does not induce cellular stress and ROS formation. (A) Stable transfectant HepR21, overexpressing full-length HABP1 does not show F-actin polymerization defects (as seen by rhodamine-phalloidin staining) and has actin profile similar to the parent HepG2 cells indicating that overexpression of HABP1 does not induce cellular stress in HepR21 cells. The intensity of cy3 fluorescence has been decreased in the whole image to get a better contrast. (B) HepG2 and HepR21 cells were incubated with 10µM of H2DCFDA for 10 min at different time points. Cells were lysed in RIPA buffer and the fluorescence was measured in the supernatant by fluorimeter at an excitation wavelength of 488nm and emission at 530nm. The total protein in the samples was estimated by BCA method and the results were expressed as fluorescence/mg of total protein. (C) HepR21 and HepG2 cells were treated with different concentrations of H2O2 and ROS formation was assayed as described above. (D) Lysates of HepG2 and HepR21 were made in RIPA buffer. Equal amounts of protein were subjected to Western Blotting and immunodetection was done using anti-Hsp70 and anti-GRP78 antibodies. Equal amounts of protein were confirmed by probing the blot with anti-tubulin antibody. (E) and (F) Glutathione and SOD levels were quantitated in the lysates of HepG2, HepR21 and Hep-Vec according to manufacturer’s guidelines. Increased levels of glutathione were seen in HepR21 cells while SOD levels remained same in all the cell lines.

Fig. 4. Enhanced cell surface localization of HABP1 in HepR21 cells. (A) For impermeable immunofluorescence to see cell surface localization of HABP1, cells were fixed in 2% PFA for 10 min and probed with anti-HABP1 and anti-myc antibody as primary antibody and cy-3 conjugated secondary antibody. (B) The cell surface was biotinylated using EZ-NHS-LC-biotin. The cells were lysed in RIPA buffer and the biotinylated proteins were pooled down with streptavidin AP beads. The beads were washed and then boiled in SDS-PAGE sample buffer. The samples were analysed by Western blotting by probing with anti-HABP1 and anti-myc antibody. Non-biotinylated HepR21 cells were taken as negative control.

Fig. 5. HepR21 cells have enhanced tumorigenic potential. (A) Increased survival rate of HepR21 cells in low serum conditions. HepR21 and HepG2 cells were grown in DMEM supplemented with 2% FBS and MTT assay was done at different time intervals from 0 to 192 hrs. HepR21 cells as shown in the curve have better survival rates in low serum conditions as compared to HepG2 cells. (B) Soft agar colony assay to examine anchorage independent growth of stable clone HepR21. Histogram shows 1.36 fold increase in number of colonies in HepR21 cells as compared to HepG2 cells, grown on 35mm dishes on soft agar for a period of two weeks. (C) Adhesion of the cells to the surface of tissue culture plates. Adhesion of the cells to the 96 well culture plates was assayed by seeding 2x10⁵ cells in each well in complete medium (CM) and serum free medium (SFM) in triplicates for two hours. The non-adherent cells were removed by washing with PBS. The adherent cells were fixed by treating with 1% glutaraldehyde for 10 minutes and stained with 0.1% (w/v) crystal violet for 25 minutes. The cells were washed and solubilized in 1% Triton×100 for overnight and the absorbance was taken at 570 nm. HepR21 cells were shown to be more adherent to the culture plates in both CM and SFM. (D) Adhesion of the cells to immobilized HA. Flat bottom 96 well cell culture plates were coated with 5 mg/ml HA overnight. Cells were trypsinized and 2x10⁵ cells in SFM were added to each well in triplicates. The plates were incubated at 37°C in CO₂ incubator for 60 minutes. The non-adherent cells were removed by washing with PBS. The adherent cells were fixed by treating with 1% glutaraldehyde for 10 minutes and stained
with 0.1% (w/v) crystal violet in DDW for 25 minutes. The cells were washed and solubilized in 1% Triton×100 for overnight and the absorbance was taken at 570 nm. The histogram shows that HepR21 cells are twice more adherent to HA as compared to the parent HepG2 cells. The data represents the mean ±SD of at least three separate experiments. In fig C and D, asterisk (*) signifies HepR21 values that differed significantly from HepG2 values.

**Fig. 6.** Hyaluronan synthesis is augmented upon stable overexpression of HABP1 leading to formation of ‘HA cable’ structure. (A) HA was quantified in the cell lysate and culture supernatant of HepG2 and HepR21 cells by competitive ELISA as described in ‘Materials and Methods’. Histogram showing relative increase in levels of HA in HepR21 cells in culture supernatant and cell lysate as compared to HepG2 cells. (B) Hyaluronan was purified from both HepG2 and HepR21 cells as per the protocol mentioned in ‘Materials and Methods’ and then subjected to digestion with 50 µg/ml of bovine testicular hyaluronidase (BTH). Equal volumes of both the undigested and BTH digested products from the two cell lines were electrophoresed onto a 5-20% gradient polyacrylamide gel. Pure polymeric HA and pure polymeric HA treated with BTH were taken as controls. The gel was stained with 1% Alcian Blue in 3% acetic acid and then subsequently with silver nitrate. A higher level of polymeric HA in the HepR21 cells is clearly evident as compared to the HepG2 cells and upon BTH digestion the high molecular weight HA in HepR21 cells, disappear significantly giving rise to oligomeric fragments. (C) HepG2 and HepR21 cells were cultured on coverslips, fixed and probed with biotinylated HA binding protein (b-HABP) followed by detection with streptavidin-cy3. Hoechst was used to stain the nucleus. HA (red) can be seen forming cable like structure in HepR21 cells (indicated by arrows) while in HepG2 cells, no HA cables were seen. (D) HepR21 cells were treated with different concentrations of SpnHL for 30 min in SFM. The cells were then fixed and probed with biotinylated HA-binding protein followed by streptavidin-cy3. ‘HA cable’ structure was disrupted in HepR21 cells on treatment with SpnHL. (E) Steady state levels of HAS1, HAS2 and HAS3 mRNA in HepG2 and HepR21 cells was determined by RT-PCR. Elevated levels of HAS2 mRNA (2.5 folds) were seen in HepR21 as compared to HepG2 whereas no significant change was observed in the levels of HAS1 and 3 mRNA between both cell types. (F) Immunodetection using HAS2 antibodies shows a 1.5 fold increase in HAS2 level in HepR21 cells. (G) Histogram showing fold change in mRNA expression levels of Has1, Hya2 and Hya3 by real-time PCR relative to HepG2. The level of each mRNA quantitated was normalized with respect to their internal GAPDH controls.

**Fig. 7.** Activation of HA mediated cell survival pathways in HepR21 cell line. (A) By Western blot using anti-CD44 antibody, a 2.5 fold increase in CD44 levels are observed in HepR21 cells as compared to parent HepG2 cells. Tubulin level in the lysates is used as a loading control. (B) Immunodetection with anti-AKT and anti-p-AKT antibody shows an increase in AKT and p-AKT levels in HepR21 cells. Equal protein loading is confirmed by probing the blot with anti-tubulin antibody. (C) Western blot analysis with anti-β-catenin shows an increase in the expression of β-catenin. (D) Western blot with anti-pERK antibody shows an increase in activated ERK levels in HepR21 cells. However ERK levels are same in both the cell lines as is evident by immunodetection with anti-ERK antibody. (E) Western blot analysis with Cyclin D1 and anti-p21 antibody shows 1.5 folds increase in Cyclin D1 expression and a decrease in the levels of p21 (~ 6 folds) in HepR21 cells as compared to HepG2 cells. Equal amount of proteins in the samples is confirmed by immunodetection with anti-tubulin antibody. (F) Western blot analysis with anti-Ras antibody shows an increase in Ras levels in HepR21 cells. Equal amount of protein in the samples is confirmed by immunodetection with anti-GAPDH antibody. (G) The intensity of the bands in the above figures is measured by ImageJ software and plotted as histogram indicating the fold change in the protein expression levels.

**Fig. 8.** Enhanced cell survival in HepR21 cells is mediated by AKT dependent activation of cyclinD1 promoter activity. (A) Promoter activity of cell cycle regulatory protein Cyclin D1 (CD1) is increased in HepR21 cells. HepG2 and HepR21 cells were transfected with promoter-reporter plasmids for cyclin B1-Luc (CB1-Luc), cdc25-Luc and cyclin D1-Luc (CD1-Luc). β-gal plasmid was used in transfection as an internal control. 24 hrs post-transfection, the cells were used for assaying transcriptional
response as described under ‘Materials and Methods’. (B) No significant change in promoter activity of cdc25 and cyclin B1 was observed between HepG2 and HepR21 cells. (C) Dominant negative-AKT (DN-AKT) decreases Cyclin D1 promoter activity in HepR21 cells. HepG2 and HepR21 cells were transfected with CD1-Luc reporter plasmid in the absence and presence of DN-AKT plasmid. β-gal was used as an internal control. Empty pcDNA3.1 plasmid was used as a control vector to equalize the total DNA amount. 24 hrs post-transfection, cells were assayed for transcriptional response. A 50% reduction in CD1 promoter activity was observed when DN-AKT form was co-transfected into HepR21 cells whereas in HepG2 cells only marginal decrease in CD1 activity could be observed.
Figure 1

A

|          | HepG2 | HepR 21 |
|----------|-------|---------|
| 34 kDa   |       |         |
| 34 kDa   |       |         |
| 45 kDa   |       |         |

anti-HABP1

anti-myc

anti-Actin

B

|          | HepR21 mock transfected | HepR21 transfected with psil570 |
|----------|-------------------------|-------------------------------|
| 34kDa    |                         | Anti-myc                      |

C

Relative fold change in HABP1 mRNA

HepG2 | HepR21

D

O.D. units at 570 nm

Time (in hrs)
Figure 2

A  

HepG2  Hep-Vec

HepR21  HepR6

B  

HepG2  Hep-Vec

HepR21  HepR6

C  

HepG2  HepR21

a  

b  

c  

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Figure 3

A) Actin

B) Fluorescence/µg Protein

C) Fluorescence/µg of protein

D) HepG2 HepR21

E) % SOD activity

HepG2 Hep-Vec HepR21

GSH (µg)/mg of total protein

Hsp70

Tubulin

GRP78

70kDa

72kDa

50kDa

Concentration of H2O2

0µM 10µM 20µM 50µM

Time (in hours)

0 24 48 72

Fluorescence/µg Protein

0 0.01 0.02 0.03 0.04 0.05

Fluorescence/µg of protein

0 0.01 0.02 0.03 0.04 0.05

GSH (µg)/mg of total protein

0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45

% SOD activity

0 20 40 60 80 100

HepG2 Hep-Vec HepR21
Figure 4

A

Cy3  Hoechst  Merged  DIC

HepG2  Anti-HABP1

HepR21  Anti-HABP1

HepR21  Anti-myc

B

|                | HepG2 | HepR21 | HepR21 | HepG2 | HepR21 |
|----------------|-------|--------|--------|-------|--------|
| NHS-LC-biotin  | +     | +      | -      | +     | +      |
| Streptavidin-agarose | +     | +      | +      | +     | +      |

34kDa

Anti-myc  Anti-HABP1
Figure 5

A

B

C

D
Figure 6

A

B

C

D

E

F

G
Figure 7

A

HepG2  HepR21

CD44

Tubulin

82kDa

40kDa

50kDa

B

HepG2  HepR21

ERK

pERK

Tubulin

44kDa  42kDa

44kDa  42kDa

50kDa

C

HepG2  HepR21

Ras

GAPDH

20kDa

37kDa

D

HepG2  HepR21

p-AKT

AKT

Tubulin

55kDa

50kDa

E

HepG2  HepR21

β-catenin

Tubulin

92kDa

50kDa

F

HepG2  HepR21

cyclinD1

p21

Tubulin

38kDa

21kDa

50kDa

G

Relative normalized band intensity

HepG2  HepR21

CD44 (82kDa)  CD44 (60kDa)  ERK  p-ERK  Ras  p-AKT  AKT  β-catenin  CyclinD1  p21
Figure 8

A

Luciferase Activity (CD1)

HepG2  HepR21

B

Luciferase activity

cdc25  Cyclin B1

HepG2  HepR21

C

Luciferase Activity

HepG2  HepR21

CD1-Luc  +  +  +  +
Control vector  +  -  +  -
DN-AKT  -  +  -  +

Minus DN-AKT  Plus DN-AKT
Overexpression of hyaluronan binding protein 1 (HABP1/p32/gC1qR) in HepG2 cell leads to increased hyaluronan synthesis and cell proliferation by upregulation of cyclin D1 in AKT-dependent pathway
Rachna Kaul, Paramita Saha, Mallampati Saradhi, L. A. Rama Chandra Prasad, Soumya Chatterjee, Ilora Ghosh, Rakesh K. Tyagi and Kasturi Datta

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