Chapter-I

Generation and characterization of liver cancer cell Line overexpressing full-length HABP1
INTRODUCTION

The ubiquitous glycosaminoglycan hyaluronan (HA), which is a major constituent of extracellular matrix, has diverse biological role in vertebrates. These include acting as vital structural component of connective tissues, formation of loosely hydrated matrices that allow cells to divide and migrate (e.g. during development and metastasis), immune cell adhesion and activation and a role in intracellular signalling (Tammi et al, 2002). The diverse range of activities of HA results from the large number of hyaluronan binding proteins (hyaladherins) that exhibit significant differences in their tissue expression, cellular localization, specificity, affinity and regulation.

The 34 kDa Hyaluronan Binding Protein (HABP1) is a member of hyaladherin family that binds with a high specificity to HA. It was originally isolated from rat kidney using HA-Sepharose affinity chromatography and was identified as a glycoprotein containing sialic acid (D'Souza and Datta 1985; Gupta et al, 1991). HABP1 has been shown to be identical to p32, a protein co-purified with human pre-mRNA splicing factor SF2 (Krainer et al, 1991) and the receptor of globular head of complement protein C1q (gC1qR) (Ghebrehiwet et al, 1994). Other homologues of HABP1 are Mam33p (Seytter et al, 1998), a mitochondrial matrix protein associated with oxidative phosphorylation in S. cerevisiae and YL2, an HIV-Rev binding murine homologue (Tange et al, 1996). Although the exact physiological function of HABP1 remains to be defined, in vitro and in vivo experiments have proposed it to have a role in macrophage cell adhesion, signal transduction, mammalian reproduction and pathological infection.

HABP1 appears to be a multifunctional protein that interacts with a wide range of biomolecules residing at different subcellular locations. Considerable controversy has surrounded the localization of
HABP1 since its cDNA sequence does not predict a traditional membrane-anchoring domain, and has a probable mitochondrial targeting sequence. The open reading frame of HABP1 encodes a proprotein of 282 amino acids, which is post-translationally processed by the removal of the first 73 amino acids through cleavage between serine\(^{73}\) and leucine\(^{74}\) resulting in generation of the mature HABP1 of 209 amino acid residues (Honore et al, 1993). The initial 73 amino acids are presumed to contain the mitochondrial targeting sequence. HABP1 has been found to be present in a number of cellular compartments including the mitochondria, nucleus and cytoplasm where it interacts with diverse range of other proteins. HABP1 is reported to interact with nuclear proteins including pre-mRNA splicing factor (SF2/ASF), TFIIB, lamin B receptor, CCAAT-binding factor (CBF) and cytoplasmic proteins like ERK of MAP kinase pathway, protein kinase C, cytoplasmic domain of α\(_{1B}\)-adrenergic receptor. HABP1 has been identified as a putative receptor protein for extracellular plasma proteins like high molecular weight kininogen, Factor XII, and C1 component protein C1q. HABP1 also interacts with the components of extracellular matrix like vitronectin and hyaluronan indicating its possible role in cell adhesion and motility. Several studies have shown HABP1 to be exclusively mitochondrial. Yeast homologue of HABP1, p30 is reported to play an important role in mitochondrial oxidative phosphorylation (Muta et al, 1997). A recent report has shown HABP1 to interact with BH3-only protein Hrk in mitochondria suggesting its possible role in apoptosis (Sunayama et al, 2004). In addition to cellular proteins, HABP1 also interacts with numerous pathogenic proteins like adenovirus core protein V, Epstein Barr virus nuclear antigen-1, Hepatitis C Virus core protein, HIV-1 tat, HIV-1 rev, Rubella virus capsid protein, *Listeria monocytogenes* surface protein In1B etc., indicative of its possible role under conditions of pathogenic stress (van Leeuwen et al, 2001). 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 (van Leeuwen et al., 2001). Moreover, HABP1 exhibits structural flexibility which is influenced by the ionic environment under in vitro conditions near physiological pH which may play an important role in its binding towards different ligands (Jha et al., 2003). The crystal structure of HABP1 shows it to be a trimer with a doughnut shaped quaternary structure and a non-crystallographic 3-fold symmetry, without any distinct domains. The trimer has an asymmetric charge distribution along its surface; one side has highly negatively charged residues, while the other side possesses positive polarity. This polarity in charge distribution may have functional implications for the protein (Jiang et al., 1999).

It has already been reported that HABP1 is highly phosphorylated in transformed fibroblasts and phosphorylation of HABP1 may be involved in cellular transformation and cellular signaling (Majumdar et al., 2002). 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 (Sengupta et al., 2004). Ectopic expression of HABP1 in simple eukaryote Schizosaccharomyces pombe leads to morphological aberrations and inhibition in cell growth with the interaction of cell cycle regulatory protein cdc25 (Mallick and Datta, 2005). Constitutive expression of mature form of HABP1 in normal fibroblast cell line has shown to inhibit its growth, formation of autophagic vacuoles, and induction of apoptosis at 60 hours without media change (Meenakshi et al., 2003). It has been reported from our lab that upon constitutive overexpression of HABP1 in fibroblast cell line F111, it accumulates in the mitochondria which leads to generation of reactive oxygen species (ROS), mitochondrial dysfunction and ultimately apoptosis (Roy
Chowdhury et al, 2008). Stable transfectants in HeLa cells overexpressing mature and proprotein forms of HABP1 show growth inhibition, morphological changes, and apoptosis induction as shown by increase in percentage of subdiploid population. The number of apoptotic cells was shown to reduce significantly if the expression of HABP1 was knocked down using short interfering RNA specific to the HABP1 transcript (Kamal and Datta, 2005). The above observations indicate an important role of HABP1 in cell growth, cell proliferation and apoptosis induction.

Glutathione (GSH) is the main non-protein thiol in the mammalian cells which participates in many critical cellular functions, including antioxidant defense storage of cysteine, maintenance of intracellular redox state, and modulation of cell growth. Many studies involving lymphocytes and fibroblasts have established that increased GSH level was associated with an early proliferative response and was essential for cell to enter S phase (Shaw and Chou, 1986; Hamilos et al, 1989). Increased glutathione levels have been reported during proliferation of rat hepatocytes, which stimulates hepatocytes to shift from G₀ to G₁ phase of the cell cycle (Lu and Ge, 1992). The increased GSH levels have been attributed to increased availability of cysteine and induction of γ-glutamylcysteine synthetase heavy subunit (GCS-HS). It is known that glutathione (GSH) levels increase in hepatocytes during active proliferation (Huang et al, 2000). It has been shown that GSH levels is increased in human liver cancers as a result of increased expression of GCS-HS and GSH synthetase at the transcriptional level (Huang et al, 2000). Moreover, in liver, HA is primarily metabolized by sinusoidal endothelial cells but the role of liver epithelial cells in this process is elusive.

In the present study, we have chosen an alternative human liver cancer 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 (Murakami et al., 2002), as a model system to examine the function of HABP1 under conditions of high anti-oxidant levels and to study whether the phenotypical cellular effects (like apoptosis, vacuolation) are specific to cell types of the same organ.

Results

Construction and subcellular localization of tagged mature and full-length HABP1

To see the expression of tagged HABP1 in cells, different tagged constructs of full-length and mature HABP1 were used to transfect COS1 or 293T cells. The tagged construct was used to discriminate between the endogenous and the transfected protein. The different constructs used were RFP-full-length HABP1, RFP-mature HABP1 (plasmids with DsRed tag at N-terminus), GFP-full-length HABP1 (full-length construct of HABP1 tagged with GFP at C-terminus), full-length HABP1-myc (full length HABP1 myc-tagged at C-terminus), Flag-full-length HABP1 (N-terminus FLAG tagged full length HABP1 construct) and Flag-mature HABP1 (N-terminus flag tagged mature HABP1). All these constructs were transfected into COS1 cells or 293T cells and the localization of HABP1 was examined by immunofluorescence. Punctuated mitochondrial kind of localization was seen on transfecting the cells with, myc-tagged full-length HABP1, flag tagged mature HABP1, RFP full-length HABP1 and GFP-full-length HABP1, whereas on transfection with full-length flag-tagged HABP1 and RFP-Mature HABP1 constructs, HABP1 was shown to form aggregates around the periphery of the nucleus (Fig. 6). For further studies, we took up the C-terminal myc-tagged full-length
Fig. 6: Localization of HABP1 after transfection with different tagged constructs. Cos-1 cells (A to D) and 293T cells (E and F) were transfected with different tagged constructs of HABP1 and the localization of HABP1 was observed 36 hours post-transfection. HABP1 showed punctuated distribution in the cytoplasm when transfected with full-length myc-tagged HABP1 and probed with anti-myc antibody (A), flag-mature HABP1 when detected with anti-Flag antibody (B), GFP-Full-length HABP1 (D) and, RFP-full-length HABP1. HABP1 was observed to form aggregates around nucleus when transfected with flag-full-length HABP1 (C) and RFP-mature HABP1 (F).
HABP1 plasmid (pHVL22: full length HABP1 cloned into KpnI and XbaI site of pCDNA3.1myc/his) as myc is a small tag of 10 amino acids which should not interfere with the conformation and functions of the protein as compared to RFP and GFP tags which are bigger proteins which may change the conformation of the protein. Moreover, it has been reported that HABP1 tagged at N-terminus by Flag, changes the conformation and localization of the protein whereas tag at C-terminus by myc did not change the conformation and localization of HABP1 (van Leeuwen and O hare, 2001). We selected full length construct for further studies since it was apparently similar to the endogenous full-length HABP1 and was expected to undergo processing and post-translational modifications in the ways similar to the endogenous protein.

**Generation of stable transfectants in HepG2 cell line overexpressing full-length myc tagged HABP1**

Plasmid pHVL22 (full-length myc tagged HABP1 in pcDNA3.1 vector) 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 pHVL 22 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 G418 (selective medium) 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. While propagating the clones, frequent media change was not required as was seen in case of F111 cells.
Fig. 7: 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 amounts of protein was confirmed by probing the blot with anti-actin antibody. (B) The histogram shows the relative band intensity of HABP1 (normalized with the intensity of bands of actin) in HepR21 as compared to HepG2 cells. About three fold increase in HABP1 levels can be seen in HepR21. (C) HepR21 cells were mock transfected and transfected with SiRNA against HABP1 pSil570. The lysates were prepared 48 hrs 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.
Two stable transfectants, named clone 6 and clone 21 showed most appropriate expression of myc-tagged HABP1. The level of expression of tagged HABP1 was higher and uniform in clone 21 as compared to clone 6. So clone 21 was used for further studies and it was thus named HepR21. The clone expressing vector alone was named Hep-Vec. To confirm the expression of myc-tagged HABP1 in HepR21, the lysates of HepG2 and HepR21 cells were prepared, equal amounts of the protein were loaded on to the gel followed by Western blotting. The blots were probed with anti-HABP1 and anti-myc antibody. The equal amount of protein in the lysates was confirmed by probing the blot with anti-actin antibody. A clear increased expression of HABP1 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 clear band on the position of HABP1 can be seen in HepR21 but not in HepG2 cells (Fig. 7A and 7B). The clone was confirmed by transfecting the HepR21 cells with psil 570 (siRNA against HABP1 which has been used in our laboratory earlier). A decrease in the expression levels of myc-tagged HABP1 was seen in HepR21 cells as compared to mock transfected cells (Fig. 7C) confirming the enhanced expression of HABP1 in the clone due to genomic integration of full-length HABP1.

**Morphological changes in the stable clone**

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. We could see the change in morphology in HepR21 cells under phase contrast microscope itself, while they were being cultured. Another observation which we made while culturing the cells was that HepR21
Fig. 8: Morphological changes in the stable clones upon HABP1 overexpression. (A) Hematoxylin eosin staining of HepG2, HepG2 vector Control (HepVec) HepR21 and HepR6 cells show a considerable difference in their morphology. HepR21 and HepR6 cells have a bigger cytoplasm and nucleus as compared to HepG2 cells and HepVec cells thus giving a swollen appearance. (B) Differential Interface Contrast microscopy images of HepG2, HepVec, HepR6 and HepR21 cells show the same result. Bar represents 10μm for all.
Fig. 9A: Morphological changes in the stable clone HepR21 as seen by electron microscopy. Transmission and scanning electron microscopy images of HepG2 and HepR21 cells after 60 hrs of growth. Transmission electron micrographs of HepG2 and HepR21 cell show that both the cells are of similar characteristics, having 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 1200X).
Fig. 9B: Morphological changes in the stable clone HepR21 by electron microscopy. HepR21 cells were observed to be bigger in size as compared to HepG2 cells (magnification 2200X).
cells took longer time to get trypsinized as compared to HepG2 cells. Previous studies from our laboratory have shown ultrastructural changes like abnormal mitochondria with ruptured membranes and partially disrupted outer membrane of the nucleus, upon HABP1 overexpression in fibroblast cell line F111. 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. 9, 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. 9, Panel C). The only morphological difference seen was that HepR21 cells were bigger in size as compared to HepG2 cells (Fig. 9B). No significant change in nuclear to cytoplasmic ratio was observed in the two cell lines.

**Growth properties of the stable clone**

To explore whether overexpression of HABP1 has some effect 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 from 0 to 144 hours. HepG2 and Hep-Vec cells had similar growth characteristics in complete media, while HepR21 and HepR6 cells had better survival rates (Fig. 10) 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
Fig. 10: HepR21 cells exhibit better survival rates over a long period of growth. HepG2, HepR21, HepR6 and HepG2 vector integrated (HepVec) 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.
gets saturated after 120 hours and only about 20% of cells die beyond this time period.

**Subcellular localization of HABP1 in HepG2 and HepR21 cells**

In subsequent studies, we wanted to explore where the overexpressed protein localizes in HepR21 cells and whether the overexpression leads to some abnormal localization and expression of protein in the cells. The localization of HABP1 in both the cell types was studied by immunofluorescence using anti-HABP1 and anti-myc antibody. Our studies revealed that HABP1 expression pattern was similar in both the cell types, with HABP1 being localized mainly to the cytoplasm though some amount of protein was visible inside the nucleus when probed with anti-HABP1 (HepG2) and anti-myc antibodies (HepR21) (Fig. 11A).

To confirm whether the endogenous HABP1 and the stably transfected HABP1 have similar distribution pattern, co-localization studies were carried out in HepR21 cells by fixing the cells and immunostaining with anti-HABP1 and anti-myc antibody simultaneously. Endogenous HABP1 was found to co-localize with the myc-tagged stably transfected HABP1 showing that they have similar intra-cellular distribution pattern (Fig. 11B). To show whether the stably transfected HABP1 localizes to mitochondria as the endogenous protein, HepR21 cells were fixed, permeabilized and double stained with both anti-myc and anti-cytochrome C (a mitochondrial marker protein). Stably transfected myc-tagged HABP1 (alexa green) was observed to be colocalized with the mitochondrial protein cytochrome C (cy3) giving out a yellow colour (Fig. 11C) indicating that the myc-tagged HABP1 is localized to mitochondria.
Fig. 11: Similar localization of HABP1 in HepG2 and HepR21 cells. (A) To examine the localization of HABP1 in HepG2 and HepR21 cells, the cells were fixed with 2% paraformaldehyde for 15 min followed by 0.1M glycine for 5 min. Cells were permeabilized by treating with 0.1% Triton X-100 for 1 min. HABP1 is shown to have primarily cytoplasmic localization with some amounts present in the nucleus also in HepG2 cells (probed with anti-HABP1 antibody) and HepR21 cells (probed with anti-HABP1 anti-myc antibody). Hoechst was used for visualizing the nucleus. Bar represents 10μm for all.(B) To determine the localization of endogenous HABP1 and stably expressed myc-tagged HABP1 in HepR21, cells were fixed and double stained with anti-HABP1 (rabbit polyclonal) and anti-myc antibody (mouse monoclonal) followed by cy3-conjugated anti-rabbit antibody raised in goat and anti-mouse Alexa Green. The co-localized distribution of HABP1 inside the cell is observable as yellow colour. Endogenous HABP1 co-localizes with the stably transfected myc tagged HABP1 in HepR21 cells. (C) The figure shows colocalization of HABP1 (alexa green) probed with anti-myc antibody, with mitochondrial marker protein cytochrome C (cy3). The yellow colour indicates the colocalization of the two proteins.
Cell surface localization of HABP1

To determine whether HABP1 is present on the surface of HepG2 and HepR21 cells, we performed impermeabilized 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 clearly localized more on the specific areas on the cell rather than being present uniformly on the entire cell surface. This observation was clearer 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. 12A). The cell surface localization of HABP1 in both cell lines was confirmed by cell surface biotinylation. A higher expression of HABP1 could be seen on the cell surface when probed with HABP1 antibody in the biotinylated cell surface extracts. As expected, a faint band of myc-tagged HABP1 was observable when probed with anti-myc antibody (Fig. 12B).

HABP1 associates with condensed chromosomes during mitosis

During the course of our subcellular localization studies for HABP1 using anti-HABP1 and anti-myc antibodies, we made an unanticipated observation in the cells that were undergoing mitosis. In these dividing cells, we observed the localization of HABP1 along with the Hoechst stained mitotic chromosomes. HABP1 was seen to be localized with chromosomes in dividing cells, in various stages of mitosis, from prophase to telophase (Fig. 13A, 13B and 13C).

To confirm this observation, we made mitotic spreads of HepG2 and HepR21 by arresting the cells at metaphase (by treatment with colchicine for 2 hrs), followed by immunofluorescence analysis by
Fig. 12: Cell surface localization of HABP1 in HepG2 and HepR21. (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.
Fig. 13: **HABP1 associates with condensed chromosomes in different stages of mitosis.** Cells were fixed and probed with specific primary antibody followed by cy3-conjugated secondary antibody as described under 'Materials and Methods'. Hoechst was used to stain the nucleus. In Fig (A) HepG2 cells have been probed with anti-HABP1 antibody and in Fig (B) HepR21 cells have been probed with anti-HABP1 antibody and in Fig (C), HepR21 cells have been detected with anti-myc antibody. In both the cell types HABP1 localizes with chromosomes in different mitotic stages. (D) Mitotic spreads of HepG2 and HepR21 cells were prepared by treating the cells with colchicine for 2 hours followed by immunostaining with anti-HABP1 for HepG2 cells and anti-HABP1 and anti-myc antibody for HepR21 cells. In both types of cells HABP1 can be seen to be co-localized with the chromosomes.
Fig. 13: HABP1 associates with condensed chromosomes in different stages of mitosis. (D) Mitotic spreads of HepG2 and HepR21 cells were prepared by treating the cells with colchicine for 2 hours followed by immunostaining with anti-HABP1 for HepG2 cells and anti-HABP1 and anti-myc antibody for HepR21 cells. In both types of cells HABP1 can be seen to be co-localized with the chromosomes.
probing with anti-HABP1 and anti-myc antibodies. In chromosome spreads too, HABP1 was seen to be distributed along with the chromosomes (Fig. 13D). HA is known to accumulate at spindle in mitotic cells (Evanko et al., 2004). HA-HABP1 interaction during mitosis may facilitate the process of cell division by maintaining spindle pole stability.

**Stable overexpression of HABP1 in HepG2 cells leads to an increase in tumorigenicity potential of the cells**

Tumor cells adapt themselves to survive in low nutrient conditions. So, growth of cells in low serum conditions can be indicative of the tumorigenicity 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. 14A). 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. 14B). These results suggest that upon overexpression of HABP1, the cells are becoming more tumorigenic as indicated by better survival rates in low serum conditions with concomitant increase in the anchorage-independent growth.

**HepR21 cells exhibit enhanced adherent properties**

While culturing HepG2 and HepR21 cells we observed that cell-cell adhesion resulting in more proximal cell cultures was apparent in the latter. Also HepR21, cells appeared to adhere better onto the surface of the culture plates and took longer time to get trypsinized as
Fig. 14: Stable overexpression of HABP1 in HepG2 cells enhances 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 total number of colonies of HepG2 and HepR21 grown on 35mm dishes on soft agar for a period of two weeks. The error bars represent the column means of a set of quadruplicate for each sample.
Fig. 15: HepR21 cells display enhanced adhesion properties. (A) 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 2×10⁵ 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 in DDW for 25 minutes. The cells were washed and solubilized in 1% Triton×100 for overnight and the absorbance was taken at 570nm. HepR21 cells were shown to be more adherent to the culture plates in both CM and SFM. (B) Adhesion of the cells to immobilized HA. Flat bottom 96 well Tissue culture plates were coated with 5mg/ml HA for overnight. Cells were trypsinized and 2×10⁵ 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 570nm. The histogram shows that HepR21 cells are twice more adherent to HA as compared to the normal HepG2 cells.
compared to HepG2 cells. To check 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. 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 suspended in 1% Triton×100 for overnight and the absorbance of the released dye was taken at 570nm. The absorbance reading at 570nm is directly proportional to the number of cells. 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. 15A) 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. 15B).

**HABP1 overexpression leads to inhibition of cell migration**

We subsequently explored whether stable HABP1 overexpression in HepR21 cells has any effect on cell migration. To study the same, we did Transwell cell migration assay, to examine the migration of cells across 8.0μm Transwells (Nunc) in response to the chemoattractant (DMEM+5%FBS). By counting the migratory cells under phase contrast microscope, we found that in HepR21 cells, there was a marked reduction in the number of cells which migrated to the lower chamber as compared to HepG2 cells (Fig. 16A).

To confirm the above observation, wound healing assay was also performed to compare the cell migration in HepG2 and HepR21 cells. The cell monolayer was wounded with a pipette tip and the cells were kept in serum-free media and observed at different time points to see the extent of wound healing. DIC images were captured at 0 hours and 48 hours post wounding. As expected, the number of cells which
Fig 16: HABP1 overexpression leads to inhibition of cell migration in HepR21 cells. (A) Cell migration assay across transwells. HepG2 and HepR21 cells were trypsinized and resuspended in SFM. Transwells (8.0µm) were placed in a 6 well plate and 2x10^6 cells in 1ml of SFM were added to the upper chamber. 1ml of DMEM containing 5% FBS was added to the lower chamber of each well which acts as a chemoattractant. The plate was covered and incubated at 37°C in 5% CO2 incubator for atleast 16 hours. The cells were fixed with 2% paraformaldehyde for 10 minutes followed by washing with PBS and staining with 0.1% Crystal violet for 1hr. The plate was viewed under microscope and the number of migrating cells were counted in five different fields. The histogram shows the average number of cells migrated in HepG2 and HepR21 cells. A marked inhibition in cell migration was observed in HepR21 cells as compared to HepG2 cells. (B) Wound healing assay after 48 hours shows decrease in cell migration in HepR21 cells as compared to HepG2 cells.
migrated to the wounded area in HepR21 cells were significantly less than in HepG2 cells (Fig. 16B) indicating an inhibition of cell migration upon overexpression of HABP1.

**Overexpression of HABP1 in HepG2 cells does not induce cellular stress**

It has been previously reported from our laboratory, that upon HABP1 overexpression in normal fibroblast cell line, the protein accumulates in the mitochondria which leads to generation of ROS, mitochondrial dysfunction and ultimately apoptosis. To determine whether overexpression of HABP1 induces cellular stress in HepG2 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. 17). To investigate if the overexpression of HABP1 leads to the generation of reactive oxygen species, we assayed the intracellular H$_2$O$_2$ generated in HepG2 and HepR21 cells by fluorescence of H$_2$DCFDA (10µM) incubated in the dark under various conditions for 10 minutes. The fluorescence in the supernatant was measured by spectrofluorimeter at an excitation wavelength of 488nm and emission at 530nm. 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. 18A). The cells were treated with different concentrations of H$_2$O$_2$ (10, 20 and 50µM) and then the ROS formation was assayed by measuring the fluorescence of H$_2$DCFDA. There was no significant increase in ROS formation even after treatment with 50 µM of H$_2$O$_2$ (Fig. 18B) suggesting that this cell line is resistant to oxidative stress. Increase in ROS formation leads to induction of heat shock proteins like Hsp70.
Fig. 17: Overexpression of HABP1 does not change F-actin polymerization profile. 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.
Fig. 18: Overexpression of HABP1 in HepG2 cells does not induce ROS formation and cellular stress. (A) 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. (B) HepR21 and HepG2 cells were treated with different concentrations of H2O2 and ROS formation was assayed as described above. (C) Lysates of HepG2 and HepR21 were made in RIPA buffer. Equal amounts of protein were subjected to Western Blotting. Proteins were immunodetected using anti-Hsp70 and anti-GRP78 antibodies. Equal amounts of protein was confirmed by probing the blot with anti-actin antibody.
Fig. 19: Overexpression of HABP1 leads to increase in glutathione levels. Glutathione and SOD levels were quantitated in the lysates of HepG2, HepR21 and HepVec according to manufacturer's guidelines. Increased levels of Glutathione were seen in HepR21 cells while SOD levels remained same in all the cell lines.
and ER stress marker GRP78. So next we examined the levels of Hsp 70 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. 18C). 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. So, next we tested the levels of glutathione and oxidant guardians of intracellular systems like superoxide dismutase (SOD). In HepR21 cells, more than two fold increase in glutathione levels were seen (Fig. 19A) whereas the activity levels of superoxide dismutase were almost similar in all the three cell lines tested (Fig. 19B).

**Discussion**

We have developed a model cell line in HepG2 cells to explore the subcellular dynamics and organelle retention of HABP1. We have stably expressed myc-tagged full-length HABP1 in HepG2 cells and studied the phenotype of the stable clone. A full-length HABP1 gene tagged to myc-epitope at C-terminus was stably transfected into liver cancer cell line HepG2. We were thus able to get two stable clones HepR21 and HepR6 after selection with geneticin. The tagged HABP1 thus expressed was detectable with anti-myc antibody and was distinguishable from the endogenous HABP1. When compared to parental HepG2 cells, HepR21 and HepR6 cells had better survival rates in complete medium over a period of time; however, the cells manifested an overall enlarged cytoplasmic and nuclear compartments. No significant change in nucleus to cytoplasm ratio was observed between the two cell lines. We selected HepR21 cells for further studies as it showed uniform expression of HABP1 as compared to HepR6 cells which showed lower and non-uniform
expression of myc-tagged HABP1. The intracellular localization of HABP1 was similar in both HepG2 cells and HepR21 cells. HABP1 was localized mainly in the cytoplasm though some amounts could also be seen in the nucleus in some population of cells. HABP1 was also observed on the surface of both the cell types as seen by impermeable immunofluorescence and cell surface biotinylation studies but the amount of HABP1 which was detected on the surface of HepR21 cells was more as compared to HepG2 cells. Moreover, when probed with anti-myc antibody in HepR21 cells, HABP1 was seen to be concentrated on cell periphery. Interestingly, HABP1 was found to co-localize with condensed chromosomes during different stages of mitosis, both, in parental HepG2 cells and in HepR21 clone when probed with anti-HABP1 and anti-myc antibodies respectively. Arresting the cell cycle at metaphase by colchicine treatment also showed the association of HABP1 with the condensed chromosomes. HepR21 cells were shown to have increased tumorigenic potential as compared to HepG2 cells as indicated by their increased survival rates in low serum conditions. This was confirmed by increase in the anchorage independent growth of the stable clones overexpressing HABP1. The numbers of colonies formed by HepR21 on soft agar were significantly higher than that formed by HepG2 cells. HepR21 cells were also observed to be more adherent to each other, to the uncoated culture plates and to HA-coated plates. In another study, we observed that upon overexpression of full-length HABP1 in HepG2 cells, there is a complete inhibition of cell migration. The cytoskeletal integrity of the stable transfectants was checked by examining the expression profile of f-actin which was observed to be normal in HepR21 cells. No actin depolymerization was seen on overexpression of HABP1. We also found that HABP1 overexpression does not lead to the generation of cellular and oxidative stress as indicated by the levels of heat shock protein, Hsp 70 and Grp78 (a marker for ER stress). No significant
increase in ROS formation was observed in HepR21 cells upon treatment with H$_2$O$_2$. The enzyme activity of SOD were similar in HepR21 and HepG2 cells while significantly high levels of glutathione were seen in HepR21 cells.

In previous studies from our laboratory, we have demonstrated that constitutive overexpression of HABP1 leads to induction of apoptosis and formation autophagic vacuoles in normal fibroblast, HeLa and COS1 cells. We have earlier reported that the overexpression of mature form of HABP1 in normal fibroblast cells causes perturbed cell growth, extensive vacuolation and restricted entry to the S-phase, finally leading to apoptosis (Meenakshi et al, 2002). Ectopic expression of HABP1 in simple eukaryote *Schizosaccharomyces pombe* has been shown to induce morphological aberrations and inhibition in cell growth, with the interaction of cell cycle regulatory protein cdc25 (Mallick and Datta; 2005). Transient expression of HABP1 and its N and C-terminal truncated variants in COS1 cells were also found to induce cytoplasmic vacuolation along with disruption of f-actin network. The vacuoles were found to be autophagic in nature, indicating a stress condition (Sengupta et al, 2004). It has been reported from our laboratory that upon constitutive overexpression of mature form of HABP1 in fibroblast cell line F111, the protein accumulates in the mitochondria which leads to generation of reactive oxygen species (ROS), mitochondrial dysfunction and ultimately apoptosis (Roy Chowdhury et al, 2008). Overexpression of mature and proprotein forms of HABP1 in HeLa cells have been shown to induce growth inhibition, morphological changes, and apoptosis induction as shown by increase in percentage of subdiploid population. The number of apoptotic cells was shown to reduce significantly if the expression of HABP1 was knocked down using short interfering RNA specific to the HABP1 transcript (Anupama, 2005).
Glutathione (GSH) is the main non-protein thiol in the mammalian cells which participates in many critical cellular functions, including antioxidant defense storage of cysteine, maintenance of intracellular redox state, and modulation of cell growth. Many studies involving lymphocytes and fibroblasts have established that increased GSH level was associated with an early proliferative response and was essential for cell to enter S phase (Shaw and Chou, 1986; Hamilos et al, 1989). Increased glutathione levels have been reported during proliferation of rat hepatocytes, which stimulates hepatocytes to shift from G₀ to G₁ phase of the cell cycle (Lu and Ge, 1992). The increased GSH levels have been attributed to increased availability of cysteine and induction of γ-glutamylcysteine synthetase heavy subunit (GCS-HS). It is known that glutathione (GSH) levels increase in hepatocytes during active proliferation (Huang et al, 2000). It has been shown that GSH levels is increased in human liver cancers as a result of increased expression of GCS-HS and GSH synthetase at the transcriptional level (Huang et al, 2000). Moreover, in liver, HA is primarily metabolized by sinusoidal endothelial cells but the role of liver epithelial cells in this process is elusive. Therefore, we have chosen an alternative human liver epithelial cell line (HepG2), as a model system to examine whether the phenotypical cellular effects (like apoptosis, vacuolation) are specific to cell types of the same organ. We chose C-terminus tagged construct because it has been reported that HABP1 tagged at N-terminus by Flag, changes the conformation and localization of the protein whereas tag at C-terminus by myc did not change the conformation and localization of HABP1 (van Leeuwen and O hare, 2001). We have used the full-length HABP1 (proprotein) for our study as the mature form of HABP1 introduced into the cells may not undergo proper processing and folding leading to abnormal phenotype and cellular stress.
We observed a higher expression of HABP1 on the cell surface in HepR21 cells which is recently reported to be a marker of tumor cells (Ruoslhti et al, 2008). No cell surface expression of HABP1 was observed when it was overexpressed in F111 and HeLa cells earlier in our laboratory. Significantly higher HABP1/p32 expression is common in human cancers and the HABP1/p32 levels are often greatly elevated compared with the corresponding normal tissue. In an extensive study using combinatorial immunoglobulin libraries and phage display, it was shown that HABP1 is preferentially overexpressed in adenocarcinoma cells and it contributes to the malignant phenotype (Rubinstein et al, 2004). It has been recently reported that HABP1/p32, 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. 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 (Ruoshalti et al, 2008). Thus, increased cell surface expression of HABP1 as seen in HepR21 cells may be responsible for its increased tumorigenicity.

HA has been shown to accumulate intracellularly in the perinuclear region of aortic smooth muscle cells during premitotic and mitotic stages (Evanko et al 2004). Elevated synthesis of HA is known to occur during G2/M stages and HA tends to accumulate at the mitotic spindle suggesting associations with intracellular structures (Brecht et al, 1986). RHAMM, an intracellular hyaladherin, has been reported to help in maintaining spindle pole stability. HA within mitotic cells is reported to co-localize with microtubules and RHAMM at the spindle. Intracellular HA may provide a more compliant milieu
to facilitate the process of nuclei separation and subsequent cell division. It may also influence RHAMM function in maintaining spindle pole stability (Hascall et al, 2004). Thus association of HABP1 with mitotic chromosomes along with HA may have a significant role in cell division. Moreover, the crucial observations that HABP1 interacts with several vital nuclear proteins like lamin B receptor, CCAAT binding factor (CBF), TFIIB implies that such interactions may have important physiological significance and warrant further studies to decipher the molecular details of this multifunctional protein.

Increased generation of ROS has been observed in cancers, degenerative diseases and several other pathological conditions. Low levels of ROS can stimulate cell proliferation, promote genetic instability, and induce adaptive responses that enable cancer cells to maintain their malignant phenotypes. However, when cellular redox balance is damaged, high levels of ROS may cause various damages leading to cell death (Lu et al, 2007). HA is reported to have antioxidant properties and it is known to act mainly as a chemical reactive oxygen intermediate (ROI) scavenger in the extracellular space (Krasinski et al, 2009). Overexpression of HABP1 in F111 cells leads to generation of ROS and ultimately apoptosis while overexpression of HABP1 in HepG2 cells does not induce ROS formation. High intracellular antioxidant levels and alterations in HA levels could be a mechanism by which HepR21 cell line becomes resistant to oxidative stress.

HA is distributed ubiquitously in vertebrate tissues, both in the embryo and in the adult, its organization and function with respect to cells and tissues are variable. It is now widely recognized that HA is dramatically increased in most malignancies and is responsible for cell proliferation. These increases in HA correlates with tumor virulence, and is often used as a prognostic indicator. It has been recently reported that carcinomas arising from colon epithelia develop
or progress in a stromal microenvironment that is elevated in HA; interactions between elevated HA and the CD44 receptors on epithelial tumor cells activate an 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 (Misra et al, 2008). Alterations in the intracellular levels of HABP1 may serve as a signal to activate the signaling cascades which leads to induction of HA and small increments in ROS production which activate various cell survival pathways and increase in tumorigenicity as seen in HepR21 cells.

Work from a number of laboratories has shown that HA in the pericellular matrix can have both adhesive and anti-adhesive properties, which are regulated on several levels. It has been reported that extended HA chains in the pericellular coat of chondrocytes mediate the early, long-range adhesive interactions that precede the formation of firm integrin-mediated adhesion complexes when cells are plated on tissue culture substrates (Cohen et al, 2004; Cohen et al, 2006). The pro- or anti-adhesive character of the pericellular matrix may be regulated by the amount, size, cellular location, and malleability of the HA and would therefore be more complex in three-dimensional conditions. 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 (de la Motte et al, 2003). The high degree of crosslinking of HA within the cables would create a more stable structure and more ordered presentation of HA and/or its associated proteins, which can influence receptor clustering and intracellular signaling. The apparent dual role of HA in adhesion and de-adhesion, therefore, is based on a complex interplay between HA synthesis, degradation and internalization, local
concentrations of HA and binding and/or cross-linking proteins which would dictate the viscosity and physical form presented to cell surface receptors and the consequent 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 (Evanko et al, 2007). It has been recently reported that cyclin D3 mediates synthesis of a HA matrix that is adhesive for monocytes in mesangial cells stimulated to divide in hyperglycemic medium (Ren et al, 2009). Enhanced cell adhesion as seen in HepR21 cells may be attributed to alterations in HA levels and distribution and upregulation of cell adhesion molecules like cadherins, laminins and fibronectin.

The AKT/PKB family of kinases, AKT1, -2 and -3 play critical roles in regulating growth, proliferation, survival, metabolism, and other cellular activities. AKT kinases control these activities by phosphorylation-mediated regulation of multiple substrates (Brazil et al, 2004). Deregulated or enhanced AKT signaling has also been implicated in a variety of human cancers, and may promote tumorigenesis. It has been reported that while AKT can promote tumor progression through increased cell survival mechanisms, it can block breast cancer cell motility and invasion by a mechanism that depends, at least in part, on the NFAT transcription factor (Yoeli-Lerner et al, 2005). Signaling through AKT has been shown to reduce NFAT expression levels due to ubiquitination and proteasomal degradation, mediated by the E3 ubiquitin ligase HDM2. Activation of AKT, a downstream effector in HA signaling leading to cell proliferation, may be the reason for inhibition of cell migration. These possibilities will be addressed in the next chapter.

In conclusion, we have found that unlike in fibroblast cell line F111, and the cancerous cell line HeLa, where mature form of HABP1
was overexpressed, upon stable overexpression of HABP1 in liver epithelial cell line HepG2, there is an increase in cellular proliferation, transformation efficiency, tumorigenic potential, cell adhesion and a marked inhibition of cell migration. HepR21 cells have high levels of GSH which might be responsible for the cell survival under adverse conditions. In view of the earlier observations that HABP1 overexpression induces apoptosis and ROS formation in different cell types, we assume that the cell survival pathways are supposedly activated in HepR21 cells. Enroute to such studies, the cell line HepR21 is expected to serve as a potent tool.