Chapter-II

Overexpression of HABP1 regulates cell survival and drug resistance via hyaluronan induction
INTRODUCTION

We have observed in the previous chapter that upon stable overexpression of HABP1 in liver cancer cell line HepG2, there is an increase in cellular proliferation, transformation efficiency, tumorigenic potential, cell adhesion and a marked inhibition of cell migration.

A substantial part of the tissue volume is extracellular space, which is largely filled by an intricate network of macromolecules constituting the matrix which is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them. In the tumor, various cell types are known to interact with each other and their microenvironment by exchanging information through cell–cell and cell–extracellular matrix (ECM) interactions. Disruption of the cell-ECM interactions elevates abnormal inter-and/or intracellular signaling, leading to deregulation of cell proliferation, growth, cytoskeleton reorganization, and other abnormalities, including carcinogenesis. HA which is a major ECM component, is involved in promoting ‘transformed’ characteristics to cancer when it is present in elevated levels on cells. Although 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.

HA forms part of the ECM by linking HA-binding molecules into macromolecular aggregates and regulating a variety of cell behaviors, such as cell adhesion, motility, growth, and differentiation. HA has a very rapid rate of turnover in vertebrate tissues, and increased amounts of HA in human cancers reflect simultaneous enhanced synthetic and degradative enzyme levels. Early in the course of a malignancy, high molecular weight HA may be required to open up
tissue spaces, for the flow of nutrients at the primary site. But when simple diffusion no longer suffices, the action of hyaluronidase, particularly Hyal2, can provide the HA fragments that induce angiogenesis and the neo-vascularization to support the malignancy (West et al, 1985). Several studies have demonstrated that hyaluronidase levels correlate with tumor progression. This has been particularly documented in tumors of the male genito-urinary tract, in prostate (Kovar et al, 2006) and urinary bladder cancers (Lokeshwar et al, 2005). Aggressiveness of other human cancers also correlates with hyaluronidase, including breast and laryngeal cancer. Over-expression of Hyal2 in murine astrocytoma cells has been shown to accelerate tumor formation (Novak et al, 1999).

HA synthesis correlates with the level of HAS mRNA, suggesting that transcriptional regulation is an important determinant of the net HAS activity. HAS expression is often increased by growth factors like EGF, KGF, PDGF (Pienimaki et al, 2001; Karvinen et al, 2003; Pasonen-Seppanen et al, 2003) and both growth factors and their receptors are often overexpressed in cancers. It is now well established that excessive production of HA in cancer and increased HA serum levels and its deposition in tumor tissue are often associated with malignant progression in many cancers, including breast cancer and colorectal cancer (Kimata et al, 2008). The biosynthesis of HA, which is critical in establishing its biological form, is multiply regulated by three mammalian HA synthases; HAS1, HAS2, and HAS3. Previous studies have suggested that the three HAS isoforms differ from each other in the expression profiles during embryonic development and in the enzymatic characteristics. Furthermore, the respective HAS transfectants clearly differ in their ability to form the HA matrix and in the molecular masses of the HA produced by them. Because HA has a wide variety of biological and physiological roles, some of which are size-dependent, the existence of three different HAS isoforms implies
that HA functions are diversely regulated through control of the activities and expressions of the HAS isoforms. The malignant transformation of cells is known to impair regulation of HA synthesis. During this process, transcriptional regulation of hyaluronan synthase (HAS) genes at multiple steps, allow cells to optimize the extracellular environment for tumor growth and malignant progression. HAS isoforms are known to be involved in different stages of malignant tumor progression. Expression of HAS2 and HAS3 genes has been shown to result in excess HA production and enhanced tumorigenic ability of fibrosarcoma, melanoma, and mesothelioma cells. Moreover, induced expression of HAS1 has been shown to restore the metastatic potential of mouse mammary carcinoma mutants which previously had low levels of HA synthesis. Inversely, suppression of HAS2 or HAS3 decreases HA production and reduces the tumorigenic potential of various cell lines (Udabage et al, 2005; Nishida et al, 2005). Of the three HAS isoforms, only HAS2 gene expression was increased in the less malignant HR-3Y1 cells transformed with v-Ha-ras, demonstrating that expression of HAS2 gene was mainly regulated by Ras signaling pathway (Itano et al, 2004). HAS2 is probably the most important synthase as mice lacking the ability to express HAS2 (knock-out mice) die at mid-gestation. Whereas HAS1 or HAS3 knock-out mice showed no effect on fetal development (Camenisch et al, 2000). The proximal promoter region of all three HAS genes show constitutive activity, of which HAS2 has the lowest basal level, and therefore, the HAS2 gene seems to be the main candidate for modulating HA synthesis rate by external stimuli (Monslow et al, 2004).

HA can influence normal and abnormal cell behavior in different ways. Due to its biophysical properties, unbound HA has a profound effect on the biomechanical properties of extracellular and pericellular matrices in which cells reside. HA forms a multivalent template for
specific interactions with other pericellular and extracellular macromolecules, which contributes to the assembly, structural integrity, and physiological properties of these matrices, thus influencing cell behavior. HA on the cell surface binds to several receptors, e.g., CD44 and RHAMM that activate intracellular signaling pathways and influence cellular form and behavior directly. Several studies indicate that HA interaction with the HA receptor RHAMM regulates ERK activity. Induction of HA production also causes recruitment of stromal cells and deposition of HA enriched stromal matrix, increased angiogenesis and enhanced cell survival signals in the tumor cells. HA-rich tumor microenvironments, which may be favorable for cancer invasion, are likely generated from complex interactions between tumor cells and stromal cells infiltrating from adjacent host connective tissue. In vitro HA synthesis was synergistically increased in co-cultures of human lung tumor cells with fibroblasts (Knudson et al, 1984) and similar synergistic effects have been demonstrated with the combination of fibroblasts and other tumor cell types (Merrilees et al, 1985). It is believed that cancer epithelial cells may stimulate the adjacent stromal cells to produce a new HA rich tissue structure favorable for tumor growth (Edward et al, 2005) and stromal cells secrete factors that enhance cancer cell migration into the new matrix.

Multidrug resistance in cancer cells is often due to ATP-dependent efflux pumps, but is also linked to alterations in cell survival and apoptotic signaling pathways. Perturbation of HA-cell interactions in malignant cancer cells by treatment with HA oligosaccharides (1200–4000 Da) induces apoptosis under anchorage-independent conditions and reduces tumor growth in vivo in multidrug-resistant MCF-7/Adr cells. HA oligomers compete for endogenous polymeric HA, thus replacing high affinity, multivalent and cooperative interactions with low affinity, low valency receptor
interactions. HA oligomers suppress the PI 3-kinase/AKT cell survival pathway, leading to proapoptotic events such as decreased phosphorylation of BAD and FKHR (forkhead transcription factor), increased PTEN (tumor suppressor) expression, and increased caspase-3 activity. These effects are mainly due to disruption of hyaluronan-CD44 interactions, although it remains possible that interactions with other extracellular, cell surface or intracellular binding proteins are also involved (Misra et al, 2005).

In view of the above ground information, an attempt has been made to study the various cell survival pathways mediated by alteration in HA levels and distribution, which are responsible for enhanced survival and proliferation and increased transformation efficiency in HABP1 overexpressing stable cell line HepR21. As HA is known to be a modulator of chemoresistance, we have also explored whether overexpression of HABP1 has any effect on anti-cancer drug sensitivity and multi drug resistance.

**Results**

**Increased levels of HA are present in HepR21 cells**

HA levels are linked to cell proliferation and tumorigenic potential of cells with high HA levels present in tumor cells. HA is also reported as a scavenger of ROS. 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. We standardized a competitive ELISA method for the quantification of HA as given in 'Materials and Methods' using biotinylated HA binding protein (b-HABP). For the standard curve, varying concentrations of HA was used (ranging from 1-500ng) and the curve was found to be linear within 10-200ng of HA (Fig. 20A).
Fig. 20: Elevated levels of HA are present in cell lysate and culture supernatant of HepR21 cells. HA was quantified in the cell lysate and culture supernatant of HepG2 and HepR21 cells by competitive ELISA as described in 'Materials and Methods'. (A) A standard curve for HA which is linear in the range of 10-200ng of HA. (B) Histogram showing relative increase in levels of HA in HepR21 cells in culture supernatant and cell lysate as compared to HepG2 cells.
The value of the samples was calculated from the standard curve. 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. 20B].

**HA ‘cable-like’ structure formation in HepR21 cells**

Increase in HA levels are linked to cell survival and increased tumorigenicity of the cells. HepR21 cells were also shown to be more ‘adhesive’ than HepG2 cells. We therefore 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 while in HepG2 ‘unpolymerized’ type of HA was seen with very little or no ‘cable-like’ structure [Fig. 21]. Moreover, the immunofluorescence studies indicated that the levels of HA are higher in HepR21 cells as compared to HepG2 cells.

**‘HA cable’ like structures are disrupted by HA degrading enzyme**

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 became detached from each other [Fig. 22A]. As anticipated, upon treatment with SpnHL, all the ‘cable’ like structures were lost in HepR21 cells confirming that they were indeed ‘HA cables’. As anticipated, upon treatment with
Fig. 21: 'HA cable' like structures are present in HepR21 cells. HepG2 and HepR21 cells were cultured on coverslips, fixed and probed with biotinylated HA binding protein (b-HABP). HA was detected by 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, unpolymerized HA can be seen. All bars represent 10μm.
Fig. 22A: HA cable structure is disrupted on treatment of cells with bacterial hyaluronidase (from Streptococcus pneumoniae, SpnHL). HepG2 cells were treated with different concentrations of SpnHL for 30 min in SFM. The cells were then fixed and probed with b-HABP followed by streptavidin-cy3. Hoechst was used to visualize the nucleus. Figure A shows in HepG2 cells, upon SpnHL treatment, the cells become detached from each other.
Untreated

HepR21 cells were treated with different concentrations of SpnHL for 30 min in SFM. The cells were then fixed and probed with b-HABP followed by streptavidin-cy3. Hoechst was used to visualize the nucleus. Figure B shows the 'HA cable' structure was disrupted in HepR21 cells on treatment with SpnHL. All bars represent 10μm.
SpnHL, all the ‘cable’ like structures were lost in HepR21 cells confirming that they were indeed ‘HA cables’ (Fig. 22B). The cable structure was disrupted by treatment with 10ng-1000ng of the enzyme.

Enhanced expression of HAS2 mRNA in HepR21

The dynamic turnover of HA is tightly regulated by altering the expression profiles of HAS isoforms. Increased expression of HAS2 and Has3 has been shown to result in increased HA production leading to malignant tumor progression. HAS2 is probably the most important synthase as mice lacking the ability to express HAS2 (knock-out mice) die at mid-gestation. Whereas HAS1 or HAS3 knock-out mice showed no effect on foetal development (Camenisch et al, 2000). The proximal promoter region of all three HAS genes show constitutive activity, of which HAS2 has the lowest basal level, and therefore, the HAS2 gene seems to be the main candidate for modulating HA synthesis rate by external stimuli (Monslow et al, 2004).

To determine whether increase in HA levels in HepR21 is associated with increased levels/activity of HAS2 gene, we performed RT-PCR using HAS2 specific primers. Total RNA was subjected to cDNA synthesis and the cDNA concentrations were normalized by amplification of 323bp GAPDH fragment using gene specific GAPDH primers. Comparable amounts of amplified GAPDH transcripts obtained from equal quantities of RNA from the two cell lines confirmed the integrity and the quantitative nature of the RT-PCR reaction. The reaction conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were within the exponential phase of amplification. The sequence of forward and reverse primers for HAS2 used in the study is given below:
Fig. 23: Enhanced expression of Has2 mRNA in HepR21 cells. (A) Steady state levels of Has2 mRNA in HepG2 and HepR21 cells was determined by RT-PCR as described in 'Materials and Methods'. Elevated levels of Has2 mRNA were seen in HepR21 (Lane3) as compared to HepG2 (Lane2). GAPDH mRNA levels (Lane4) and HepR21 (Lane5) served as an internal control for the integrity and quantity of the RNA used (B) The graph shows the normalized 2.5 fold increase in the HAS2 levels in HepR21 as compared to HepG2.
HAS2 Forward: 5’TTTCTTTATGTGACTCATCTGTCTCAACGGG3’
HAS2 Reverse: 5’ATTGTTGGCTACCAGTTTATCCAAACGGG3’

2.5 fold increase in the HAS2 RNA transcripts was observed in HepR21 cells, confirming transcriptional upregulation of HAS2 in HepR21 (Fig. 23A and B).

**Enhanced activation of ERK in HepR21 cells**

The MAP kinase cascade and the cell cycle regulation pathways are closely related since the activation of the former often determines whether the cell shall follow normal growth and division or will undergo apoptosis. ERKs, members of the MAPK family, are known to be activated by receptor tyrosine kinases, cytokine receptors, and G-protein-coupled receptors. In particular, ERK phosphorylation of certain target proteins is directly involved in transcriptional activation by coordinating extracellular cues and intracellular signals (Bourguignon et al, 2005). HA-CD44 interactions are reported to activate ERK and ERK dependent cyclin D1 gene expression leading to increased cell proliferation (Kothapalli et al, 2008). As we have observed that HA levels are augmented in HepR21 cells, we wanted to further investigate whether MAP kinase pathway is activated in HepR21 cells. For this purpose, 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. 24). Thus results indicated an enhancement in activation of MAP kinase signaling pathway in HepR21 cells.

**Ras expression is upregulated in HepR21 cells**

*Ras* is a family of genes encoding small GTPases that are involved in cellular signal transduction. Activation of Ras signaling causes cell
Fig. 24: p-ERK levels are upregulated in HepR21 cell line. (A) Western blot analysis 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. Equal amounts of protein in the samples is confirmed by immunodetection with anti-tubulin antibody. (B) The graphs show the normalized fold increase in the p-ERK levels in HepR21 as compared to HepG2.
Fig. 25. Ras levels are upregulated in HepR21 cell line. (A) Western blot analysis with anti-Ras antibody shows an increase in Ras levels in HepR21 cells. Equal amounts of protein in the samples is confirmed by immunodetection with anti-GAPDH antibody. (B) The histogram shows the normalized 2.5 fold increase in the Ras levels in HepR21 as compared to HepG2 cells.
growth, differentiation and survival. It is known that HA, via activation of Ras activates MAP kinase signaling pathway which is responsible for HA-mediated cell survival (Serbulia et al, 2000). As we have observed upregulation of HA and enhanced activation of ERK, we subsequently checked Ras levels in HepG2 and HepR21 cells to ascertain whether HA mediates ERK signaling through Ras activation. 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. 25).

**Enhanced expression of AKT and phosphorylated AKT in the stable clone**

As we observed HepR21 cells to be more tumorigenic as evident from their growth in low serum conditions, enhanced anchorage independent growth, and increase in the levels of HA, we then attempted to examine the levels of AKT and p-AKT in the stable clone since AKT is known to be a prime candidate in cell survival pathway mediated by increased HA levels.

Immunoblot analysis of equal amounts of lysates of HepG2 and HepR21 showed increase in the levels of both AKT (4.5 folds) and p-AKT (2.4 folds) in HepR21 cells as compared to HepG2 cells (Fig. 26A) indicating the activation of AKT-mediated cell survival pathways in HepR21 cells.

Subsequently, we then examined the levels of AKT1 mRNA in both the cell lines by RT-PCR. Total RNA was isolated and subjected to cDNA synthesis and the cDNA concentrations were normalized by amplification of 323bp GAPDH fragment using gene specific GAPDH primers. The reaction conditions were chosen such that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were within the exponential phase of amplification.
Fig. 26: Akt and p-akt levels are upregulated in HepR21 cells. (A) Immunodetection with anti-Akt and anti-pAkt antibody shows an increase in Akt and p-Akt levels in HepR21 cells. Equal protein loading is confirmed by probing the blot with anti-actin antibody. The graphs (B) and (C) show the normalized fold increase in the p-Akt (2.4 folds) and Akt (4.5 folds) levels in HepR21 as compared to HepG2. (D) Increased expression of Akt mRNA in HepR21 cells by RT-PCR: Steady state levels of Akt mRNA in HepG2 and HepR21 cells was determined by RT-PCR as described in 'Materials and Methods'. Elevated levels of Akt mRNA were seen in HepR21 (Lane4) as compared to HepG2 (Lane5). GAPDH mRNA levels remain unaltered in HepG2 (Lane3) and HepR21 (Lane2) and it served as an internal control for the integrity and quantity of the RNA used. The graph (E) exhibits the normalized fold 1.8 fold increase in mRNA levels in HepR21.
The sequence of forward and reverse primers for AKT1 used in the study is given below:

AKT1 Forward: 5'GAGGTGCTGGAGGACAATCAC3'
AKT1 Reverse: 5'ACACACTCCATGCTGTCATCTT3'

Enhanced expression of AKT1 RNA transcripts (1.8 folds) was observed in HepR21 cells as compared to HepG2 cells, confirming transcriptional upregulation of AKT1 in HepR21 (Fig. 26 B and C).

**β-Catenin levels are augmented in HepR21 cells**

Increased levels of HA are reported to induce cell survival pathway via the activation of AKT and β-catenin. To see if the survival in HepR21 cells is mediated by β-catenin, we tested the expression levels of β-catenin in HepR21 and HepG2. Immunoblot using anti-β-catenin antibody shows an increased expression of β-catenin in HepR21 cells when compared to HepG2 cells (Fig 27). This indicates that β-catenin contributed in AKT mediated cell survival in HepR21 cells.

**Overexpression of HABP1, specifically enhances the promoter activity of cell cycle regulatory genes**

Since we observed increased cellular proliferation of HepR21 cells, we sought to determine the molecular mechanistic details underlying this phenomenon. We have already observed enhanced activation of ERK in HepR21 cells. Moreover, it has been recently reported that low molecular weight HA binding to CD44 selectively stimulates ERK activation and ERK-dependent cyclin D1 gene expression thereby stimulating cell cycle progression and mitogenesis (Kothapalli et al, 2008). Cellular proliferation is considered to be a direct consequence of enhanced functional activity of cell cycle regulatory genes. Here we determined promoter activity of several cell cycle regulatory genes and compared their activities in HepR21 cells.
Fig. 27: β-catenin levels are upregulated in HepR21 cells. (A) Western blot analysis with anti-β-catenin antibody shows an increase in the expression of β-catenin in HepR21 cells. Equal amounts of protein in the samples is confirmed by immunodetection with anti-tubulin antibody. (B) The graph shows the normalized 2.5 fold increase in the β-catenin levels in HepR21 as compared to HepG2.
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). Cyclin D1 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. E2F family members play a major role during the G1/S transition in the mammalian cell cycle. Phosphorylated retinoblastoma protein binds to the E2F transcription factor preventing it from interacting with the cells transcription machinery. In the absence of pRb, E2F mediates the trans-activation of E2F target genes that facilitate the G1/S transition and S-phase. E2F target genes encode proteins involved in DNA replication (for example, DNA polymerase, thymidine kinase (Tk), dihydrofolate reductase and cdc6), chromosomal replication (replication origin-binding protein HsOrc1 and MCM 5). When cells are not proliferating, E2F DNA binding sites contribute to transcriptional repression. Cdc25 is a dual-specificity phosphatase which removes inhibitory phosphate residues from target Cyclin-Dependent Kinases (Cdks). Cdc25 proteins control entry into and progression through various phases of the cell cycle, including mitosis and S phase. Cyclin B1 is known to form complexes with p34 (cdc2) to form the maturation-promoting factor (MPF), which is expressed predominantly during G2/M phase of the cell cycle.

For this purpose, control HepG2 cells and HepR21 cells were transiently transfected with CD1-Luc, E2F-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 promoters of CD1 and E2F were observed, where as no change was evident for Cdc25 and CB1 promoter-reporter constructs (Fig. 28). On the basis of these findings, we hypothesize that overexpression of HABP1 is attributing for the enhanced activity of cell cycle regulatory
Fig. 28: Promoter activity of cell cycle regulatory proteins cyclinD1 (CD1) and E2F are increased in HepR21 cells. HepG2 and HepR21 cells were transfected with promoter-reporter plasmids cyclin B1-Luc (CB1-Luc), cdc25-Luc, CD1-Luc and E2F-Luc for 24 hours. β-gal plasmid was used in transfection as an internal control. Following the period of treatment, the cells were used for assaying transcriptional response as described under 'Materials and Methods'. Fig A shows no significant change in promoter activity of cdc25 and cyclin B1. Figure B shows about 3-fold increase in promoter activity of E2F in HepR21. Figure C shows about 3-fold increase in promoter activity of cyclin D1 upon HABP1 overexpression.
genes CD1 and E2F and as a consequence increased cellular proliferation is observed in the stable clone HepR21.

**AKT modulates the CD1 promoter activity in HepR21 cells**

In our earlier findings 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. In our experimental conditions, the absolute promoter-reporter activity was higher for CD1-Luc promoter-reporter construct compared to E2F-Luc, hence we have used CD1-Luc reporter vector for further experiments. We co-transfected the vectors encoding dominant negative 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 HABP1 stably expressing cells, whereas in control HepG2 cells only a marginal reduction was evident (Fig. 29). 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.

**Cyclin-CDK inhibitor, p21 is downregulated in HepR21 cells**

Cell-cycle progression relies on the activation of cyclins and cyclin-dependent kinases (CDKs), which successively act together in G1 to initiate S phase and in G2 to initiate mitosis. To prevent abnormal proliferation, 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
Fig. 29: 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. 24hrs post-transfection, cells were assayed for transcriptional response as described in ‘Materials and Methods’. 50% reduction in CD1 promoter activity was observed when dominant negative AKT form was co-transfected into HepR21 cells whereas in HepG2 cells only marginal decrease in CD1 activity could be observed.
Fig. 30: p21 levels are downregulated in HepR21 cells. (A) Western blot analysis with anti-p21 antibody shows a marked decrease in the levels of p21 in HepR21 cells as compared to HepG2 cells. Equal amounts of protein in the samples is confirmed by immunodetection with anti-tubulin antibody. (B) The graphs show the normalized four fold decrease in the p21 levels in HepR21 as compared to HepG2.
activity and induce cell-cycle arrest. p21 thus functions as a regulator of cell cycle progression at G1 phase. As we have observed an increase in cyclin D1 promoter activity in HepR21 cells, we then wished to see the expression levels of cyclin-cdk inhibitor, p21 in both cell lines. Western blot analysis by using p21 antibody shows a significant decrease (4 folds) in the levels of p21 in HepR21 cells as compared to HepG2 cells (Fig. 30) indicating the activation of cyclin D1 mediated survival pathways in HepR21 cells.

**HepR21 cells exhibit high sensitivity to anti-cancer drugs**

HA is a known modulator of drug resistance in cancers. To study if increase in the HA levels in HepR21 has some effect on drug resistance, we checked the cytotoxicity profile of two anticancer drugs 5'-Fluorouracil (5FU) and doxorubicin (Dox) in HepG2 and HepR21 cells. Equal number cells were treated with varying concentration of drugs for 3 days followed by MTT assay. Interestingly, in case of both the drugs, HepR21 cells were found to be more sensitive to the killing by the drug as compared to HepG2 cells. The effective dose at which 50% and 30% of cells are killed (ED_{50} and ED_{30}) was less in HepR21 cells on treatment with both 5FU and Dox, when compared to HepG2 cells (Fig. 31). Thus, in HepR21 cells, more cells were killed at lower drug concentration implying that HepR21 cells are more sensitive to anti-proliferative effects of anti-cancer drugs doxorubicin and 5-FU when compared to the parent HepG2 cells.

**Overexpression of HABP1 leads to decrease in MDR1 mRNA levels**

It is known that multi-drug resistance (MDR) in cancer cells is regulated by ABC transporters family of genes and MDR1 is a prime candidate of this family. So next, we examined the expression of MDR1 mRNA in both the cell lines by RT-PCR. Total RNA retrieved from the two cell lines was subjected to cDNA synthesis. cDNA
Fig. 31: Cytotoxicity profile of 5’ Fluorouracil (5-FU) and Doxorubicin (DOX). Standard curve for the cytotoxicity profile of 5-FU (A) and Dox (B) in HepG2 and HepR21. HepG2 and HepR21 cells were trypsinized, counted and $2 \times 10^4$ cells of each cell line were seeded in 96 well plates. After overnight incubation at 37°C in CO$_2$ incubator, serial dilutions of drugs were added to the wells in triplicates. After 3 day incubation, MTT assay for cell proliferation was done. 15µl of MTT solution (5mg/ml stock in DDW) was added to each well followed by incubation for 4hrs. The media was then removed and the formazon crystals were dissolved in 150 µl of DMSO and the absorbance at 570nm was taken on ELISA plate reader. A curve of % cytotoxicity vs the concentration of the drug was plotted and effective dose50 ($ED_{50}$) and $ED_{30}$ values were calculated from the curve. In case of both the drugs, HepR21 cells were found to be more sensitive to the drug killing. The table (C) shows $ED_{50}$ and $ED_{30}$ values of Dox and 5-FU in HepG2 and HepR21.
Fig. 32: RT-PCR for MDR1 mRNA levels. (A) Steady state levels of MDR1 mRNA in HepG2 and HepR21 cells was determined by RT-PCR as described in Materials and Methods. Decreased levels of MDR1 transcript (238 bp) were seen in HepR21 as compared to HepG2. GAPDH mRNA levels were same in HepG2 and HepR21 and it served as an internal control for the integrity and quantity of the RNA used. (B) The graph shows the normalized fold decrease (2 folds) in MDR1 mRNA levels in HepR21 as compared to HepG2.
concentrations were normalized by amplification of 323bp GAPDH fragment using gene specific GAPDH primers. Comparable amounts of amplified GAPDH transcripts obtained from equal quantities of RNA from the two cell lines confirmed the integrity and the quantitative nature of the RT-PCR reaction. The reaction 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 an exponential phase of amplification. The sequence of forward and reverse primers for MDR1 used in the study is given below:

MDR1 Forward: 5'TGATGCTGCTCAAGTTAAAGG 3'
MDR1 Reverse: 5'CTTCAGTAGCGATCTTCCCA 3'

A 2 fold decrease in the MDR1 RNA transcripts (238bp) was observed in HepR21 cells as compared to HepG2 cells, confirming transcriptional down regulation of MDR1 in HepR21 (Fig. 32).

**Discussion**

In the present study, we have observed that upon stable HABP1 overexpression in HepG2 cells, there is a marked increase in total HA pool in the cell. HA-mediated signaling pathways were found to be activated in HepR21 cells, which lead to enhanced cellular proliferation. The MAP kinase pathway was shown to be activated in HepR21 cells as shown by increased levels of Ras and activated ERK (p-ERK). We observed that increased levels of AKT and activated AKT (p-AKT) are present in the stable clone HepR21 which is overexpressing HABP1 indicating the activation of PI-3kinase/AKT mediated cell survival pathways. Approximately 2.5 fold increase in the levels of β-catenin, a downstream mediator of AKT, was also observed in HepR21 cells. By luciferase assays we have shown an increase in the promoter activity of cyclin D1 and E2F upon HABP1 overexpression whereas there was no effect on the promoter activity of cyclin B1 and cdc25. There was a decrease in the cyclin D1 promoter
activity in HepR21 cells upon co-transfection with dominant negative AKT plasmid implying that AKT activation plays an important role in cyclin D1 mediated cell survival in HepR21 cells. Moreover, we have also observed a significant decrease in p21 levels, which is an inhibitor of cyclin-cdk complexes.

The increase in HA levels in HepR21 could be attributed to an increase in HAS2 levels as observed by RT-PCR. Interestingly, immunofluorescence studies showed that HA forms extensive 'cable' like structure in HepR21 cells while in HepG2 cells, HA was present in a depolymerized form with little or no 'HA cable' present. To confirm that these 'cable' structures were composed of HA, HepR21 cells were treated with varying concentrations of HA degrading enzyme, SpnHL. It was observed that on treatment with SpnHL, these 'cable-like' structures were disrupted confirming that these structures were actually enriched in or composed of HA.

In the present study we also observed that HABP1 overexpressing HepR21 cells were more sensitive to the anti-cancer drugs doxorubicin and 5'-FU, as compared to HepG2 cells. We subsequently checked the levels of MDR1 in both cell types and found that in HepR21 cells there is a transcriptional down regulation of MDR1 when compared to HepG2 cells.

HA synthesis is known to be controlled at the level of HAS transcription, with latter being influenced by growth factors, cytokines, and hormones. Of the three HAS isoforms, only HAS2 gene expression was shown to be increased in the less malignant HR-3Y1 cells transformed with v-Ha-ras, demonstrating that expression of HAS2 gene was mainly regulated by Ras signaling pathway (Itano et al, 2004). HAS2 is probably the most important synthase as mice lacking the ability to express HAS2 (knock-out mice) die at midgestation. Whereas HAS1 or HAS3 knock-out mice showed no effect on fetal development (Camenisch et al, 2000). The proximal promoter
region of all three HAS genes show constitutive activity, of which HAS2 has the lowest basal level, and therefore, the HAS2 gene seems to be the main candidate for modulating HA synthesis rate by external stimuli (Monslow et al, 2004).

Post-transcriptional factors like HAS protein phosphorylation may also contribute to the rate of HA synthesis (Karvinen et al, 2008). It has been reported that the Ras-ERK (extracellular-signal-regulated kinase), MAPK (mitogen-activated protein kinase) and PI3K (phosphoinositide 3-kinase) pathways are important for growth factor PDGF-BB-stimulated HA production in human dermal fibroblast cells, and that HA binding to its receptor CD44 is important for PDGF-BB-induced cell growth. HAS2 transcriptional activity and HA synthesis were shown to be inhibited by treatment with the inhibitors of MEK1/2 and PI3-kinase (Li et al, 2007). It has been reported that overexpression of osteopontin (a tumor associated, secreted phosphoprotein) in a tumorigenic human mammary epithelial cell line, contributes to increased HAS2 expression and HA production to promote anchorage-independent growth and adhesion to bone marrow endothelial cells. Antisense-mediated down-regulation of HAS2 in these cells resulted in decreased HAS2 expression, HA secretion, and pericellular matrix retention. Furthermore, antisense inhibition of HAS2 was shown to significantly decrease anchorage-independent growth (Cook et al, 2006). Recent findings on human intestinal mesenchymal cells has shown that interleukin-1β induces HAS2 transcription and increased HA synthesis via p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase pathways suggesting a role of HA in inflammation (Ducale et al, 2005). HA synthesis is shown to be stimulated by EGF in epidermal keratinocytes in monolayer and organotypic cultures and by all-trans-retinoic acid (RA) in human skin organ cultures (c et al, 2005). In silico/in vitro scanning of the first 10 kb of the human HAS2 promoter
has identified an active STAT-response element in the proximal region. ChIP assays on a number of RE-containing promoter regions of the human \textit{HAS2} promoter showed all-trans-RA-dependent histone 4 acetylation and binding of RAR, RXR, MED protein, and Pol II to a promoter region containing a complex retinoic acid response element (RARE) indicating that \textit{HAS2} gene is a potent EGF and RA responding gene with a complex regulation (Saavalainen \textit{et al}, 2005). \textit{In silico} analysis of the \textit{HAS2} proximal promoter region has revealed a range of putative upstream transcription factor-binding sites. These included Sp1, NF-Y, and CCAAT sites within the F3 region, and an NF-\kappa B motif further upstream. Electrophoretic mobility shift assays (EMSAs) has demonstrated binding of Sp1 and Sp3 to three oligonucleotide probes spanning 63 bp immediately upstream of the \textit{HAS2} transcription initiation site. Mutation of key residues in consensus Sp1/Sp3 recognition sites within these regions ablated the ability of the F3 promoter region to drive transcription of the \textit{HAS2} promoter-luciferase gene. Small interfering RNA (siRNA) knockdown of mRNAs for either Sp1 or Sp3 resulted in a significant decrease in \textit{HAS2} transcription levels (Monslow \textit{et al}, 2006). It has been reported that human \textit{HAS2} gene promoter is under the control of the inducible transcription factors NF-\kappa B and RAR and the constitutively active factor Sp1. These regulatory proteins share common cofactors, which provide numerous possibilities for functional interactions between the signaling pathways, of which they are the end point (Saavalainen \textit{et al}, 2007).

Appearance of HA on epithelial cells is a general feature of inflammation. During inflammation, HA adopts a special conformation 'cables', which immobilizes and deactivates monocytes (Evanko \textit{et al}, 2007). It has been shown that the hyaladherins inter-\alpha-inhibitor (I\alpha I) and versican incorporate into, and are part of the HA cables and CD44 receptors mediate the binding of mononuclear leukocytes to the cables (de La Motte \textit{et al}, 1999). Such deactivated macrophages
produce proteinases, growth factors and matrix molecules that clear the inflammation and initiate wound healing (Day and de La Motte, 2005). Tumor inflammatory cells are often deactivated, in an immunologically suppressed state where they are unable to destroy the cancer cells. HA oligosaccharides have been recently shown to promote the deactivation of tumor macrophages (Kuang et al, 2007). Tumor-associated HA may function in attracting, binding and deactivating leukocytes potentially lethal to the tumor cells, thus, leading the tissue into a state of chronic wound healing process, favorable for tumor growth. High molecular mass HA on tumor cells may also prevent firm attachment of leukocytes on cancer cell surface necessary for a proper immune reaction. It has been shown recently that HAS overexpressing cells form unique plasma membrane extensions, which form the skeleton of an extensive HA coat. Similar structures are also seen in tumor-derived fibroblasts which secrete large amounts of HA and bind monocytes. It is speculated that these HAS-induced extensions with their HA cover can also act as local immunological shields either via mechanical basis or via immunomodulatory actions (Tammi et al, 2008).

It has been recently reported that 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). The phosphoinositide 3-kinase/AKT signaling pathway, upregulated in most malignant cancer cells, is an anti-apoptotic pathway regulated by several receptor tyrosine kinases, e.g. EGFR, ERBB2 and IGF-1R. These receptor kinases are known to be important in malignant cell properties such as deregulated proliferation, anchorage-independent colony formation and invasiveness.
We have observed enhanced promoter activity of cyclin D1 and E2F and downregulation of cyclin dependent kinase inhibitor, p21 in HepR21 cells which can augment cellular proliferation by promoting G1/S transition. Cell cycle transition from G1 to S phase is regulated by distinct cyclin-dependent kinases (cdks) that are regulated by various cyclins, cdk inhibitors post-translational modifications like phosphorylation. In mammalian cells, key cyclins involved in G1 to S transition include cyclin D, E, and A. Cyclin D forms a complex with cdk4/6 in early G1 phase, cyclin E associates with cdk2 later in G1 phase, and cyclin A associates with cdk2 at the beginning of S phase (Draetta, 1994). The expression level of cyclin D1 has been shown to be rate-limiting in cellular proliferation induced by a variety of stimuli (Joyce et al., 1999). Accumulation of the cyclin D1/cdk4/6 complex in early to mid-G1 phase leads to activation of the kinases that phosphorylate and inactivate the tumor suppressor retinoblastoma (Rb), which is necessary for cell cycle progression through the G1 to S phases. pRb serves as a gatekeeper for G1 phase, and passage through the restriction point leads to DNA synthesis. Consistent with its critical role in cell cycle progression, increased expression of cyclin D1 has been observed in several tumors (Lee et al., 1999). Ectopic overexpression of cyclin D1 in transgenic mice has been shown to induce formation of tumors (Nakagawa et al., 1997). The cyclin D1 null mice have shown remarkably decreased development of tumors (Robles et al., 1998). It has been recently reported that low molecular weight HA binding to CD44 selectively stimulates ERK activation and ERK-dependent cyclin D1 gene expression thereby stimulating cell cycle progression and mitogenesis (Kothapalli et al., 2008). The cyclin D1 protein levels are largely controlled at the transcriptional level and by ubiquitin-mediated degradation. 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 genes are amplified in a subset of hepatocellular carcinomas and both these genes are downstream targets of β-catenin. It has been identified that the β-catenin/lymphoid enhancer facor-1(LEF-1) signaling pathway can activate the cyclin D1 promoter directly through an LEF-1 responsive element (Shtutman et al, 1999).

It has been reported that integrin-mediated cell adhesion controls cell cycle progression by regulating the expression and activities of cyclins, cdks, and cdk inhibitors (Assoian and Schwartz, 2001). Overexpression of cyclin D1 has been shown to induce anchorage-independent cell cycle progression of NIH3T3 and Rat1 cells (Resnitzky, 1997). Integrin-mediated cell adhesion is reported to be required for sustained ERK activation and induction of cyclin D1 expression by growth factors (Roovers et al, 1999). Cyclin D1 is an important downstream molecule involved in PI-3K/AKT-mediated cell transformation upon arsenite exposure. Inhibition of cyclin D1 expression by dominant negative mutants of PI-3K, and AKT, and the knockdown of the cyclin D1 expression by its specific siRNA in the HaCat cells (human keratinocyte cell line) has been shown to result in impairing of anchorage-independent growth of HaCat cells induced by arsenite (Ouyang et al, 2008). Therefore, enhanced activation of Ras, AKT, ERK and β-catenin mediate downstream signaling events which eventually lead to activation of cyclin D1 promoter thereby stimulating cell cycle progression and mitogenesis.

HA, phosphoinositide 3-kinase, and ErbB2 have been shown to form a positive feedback loop that strongly amplifies MDR1 expression and regulates drug resistance in MCF7/Adr cells (Misra et al, 2005). It is reported that overexpression of HA binding protein, which competes with endogenous receptors by binding to endogenous HA can perturb endogenous HA-cell interactions (Ward et al, 2003). Thus, overexpression of HABP1 might disturb the interactions between HA
and its receptors leading to decrease in MDR1 expression and increased drug sensitivity of cells.

Taken together we conclude that stable overexpression of HABP1 in HepG2 cells leads to increase in HAS2 levels because of which high levels of HA are synthesized. HA mediates downstream signaling events leading to activation of various cell survival pathways and increased drug sensitivity and decreased MDR1 expression.