Chapter-III

Analysis of molecular signaling profile in a human liver cancer Cell line stably overexpressing HABP1
INTRODUCTION

All living organisms carry precise instructions in their genome concerning how they grow and function. Genomics is the field of biological sciences that aims to study and decode this genetic information. Thousands of genes and their products (i.e., RNA and proteins) in a living organism function in a sophisticated and orchestrated way that sustains the mystery of life. Over the past decades, systemic approach to genetic studies has elucidated numerous genes and their functions. Making use of the vast amount of data produced by 'genomics' is the main task of the functional genomics. While genomics, proteomics, and structural biology focus on the static aspects of the molecules of life (e.g. sequences and structures of DNA or proteins), functional genomics attempts to study the dynamic aspects such as gene transcription and its regulation, as well as the interaction of genes and their products. Recent advances in genomics, most importantly the sequencing of human and other organisms' genomes, have provided revolutionary information that enables the characterization of diseases at the molecular level. This data is elaborately exploited by DNA microarrays, i.e., miniature measurement devices that contain probes for practically all genes of the study subject and enable genome wide studies of a given subject in a single assay. This technique is a result of the technological advancement in the field of micro-fluidics, robotics and computer technology. Human Genome Project (HGP), Polymerase Chain reaction (PCR) and the base pairing rule found by Watson and Crick are some of the important groundwork which led to the development of this gene technology. The concept of this technology appeared as an oligonucleotide array on a solid surface in the early 1990s (Fodor et al, 1991, 1993; Chetverin and Kramer, 1993; Pease et al, 1994). Subsequently, the complementary DNA (cDNA) microarray was
developed by Patrick O. Brown's group at Stanford University in 1995 (Schena et al, 1995).

Soon after the introduction of the first arrays for gene expression analysis several different modifications of the high throughput platform have been introduced, including array comparative genomic hybridization (array CGH) for DNA copy number (Solinas-Toldo et al, 1997), single nucleotide polymorphism (SNP) array for genotyping (Wang et al, 1998), and the methylation array for epigenetic (Hatada et al, 2002) assays.

DNA microarray hybridization applications are usually directed at gene expression analysis or screening samples for single nucleotide polymorphisms (SNPs). In addition to the molecular biologically related analyses and genomic research applications, such microarray systems are also being used for pharmacogenomic research, infectious and genetic disease, cancer diagnostics, and forensic and genetic identification purposes. Microarray technology continues to improve in performance aspects regarding sensitivity and selectivity and in becoming a more economical research tool. Additionally, microarray technology that has been developed for DNA analysis is now also being applied to new areas of proteomic and cellular analysis. There are two major application forms for the DNA microarray technology: 1) identification of sequence (gene / gene mutation); and 2) determination of expression level (abundance) of genes.

Currently, there are two different formats of microarray based technologies dependent on the target nucleic acid components, i.e., the oligonucleotide array and the cDNA microarray. The oligonucleotide type of array consists of oligonucleotide targets, generally less than 25 mer in length (Shoemaker et al, 1996; Lipshutz et al, 1999), which are generated in situ on a solid surface by light directed synthesis (GeneChip®, Affymetrix, Inc., Santa Clara, CA, USA) (Fodor et al, 1991; Hacia et al, 1996). Synthetic linkers modified
with photochemically removable protecting groups are attached to the glass substrate. Light is then directed through a photolithographic mask to specific areas on the surface to produce localized photodeprotection. Hydroxyl-protected deoxynucleotides are incubated with the surface so that chemical coupling occurs at the sites that have been illuminated in the preceding step. By repetition of these procedures with new masks, hundreds of thousands of oligonucleotides can be synthesized in a very small area (Fodor et al., 1991; Lipshutz et al., 1999). Alternatively, oligonucleotide arrays can be constructed by the spotting of pre-synthesized oligonucleotides onto the solid surface (Marshall and Hodgson, 1998; Ramsay, 1998). Because oligonucleotide arrays are designed and synthesized based on sequence information, physical intermediates such as cloning and polymerase chain-reaction (PCR) are not required. Specific sequences, which are non-overlapping or minimally overlapping, can be designed to increase the hybridization sensitivity, even through their shorter sequences (Lipshutz et al., 1999). In contrast, the cDNA microarray is fabricated by the printing of cloned and amplified cDNAs onto the solid surface. The advantages of the cDNA microarray compared with the oligonucleotide array have been thought to include less susceptibility and higher specificity due to the longer sequences of the targets (Bilban et al., 2000). However, cDNA may contain repetitive sequences that are often observed in various genes, or similar sequences that are found in family member genes. These non-specific sequences may affect the sensitivity of the cDNA microarray. There is a tendency that the cDNA microarray is used for the screening of steady-state mRNA expression levels and the oligonucleotide array is applied when more precise analysis, including the detection of single nucleotide polymorphisms, is required (Wang et al., 1998; Sapolsky et al., 1999; Lindblad-Toh et al., 2000).
Fig. 33: Principle of the cDNA microarray analysis system. Target cDNAs are cloned, and amplified by PCR. Purified PCR products are printed onto glass microscope slides with a robotic microarrayer. cDNA probes (test or reference) labeled with different fluorescent dyes (Cy3-dUTP and Cy5-dUTP) are synthesized from total RNA or mRNA derived from test and reference samples. Pooled probes are hybridized to the microarray. Hybridized fluorescent signals are detected with a dual-wavelength laser scanner. Separately scanned images are combined and pseudocolored by means of specialized computer software. Normalized ratios of Cy3/Cy5 are calculated for individual target genes.
A microarray experiment typically aims to identify the relative differences between first computational techniques utilized for inferring the differential expression relied on the simple assumption that the reliability and, consequently, the significance would increase together with the magnitude in the gene expression. Accordingly, the fold changes calculated between samples served also as a significance cut-off. More strict statistical evaluation has been established and the number of methodological papers introducing novel statistical approaches has been increasing as the biological papers presenting microarray results. Usually, in gene-wise analyses, p-values are calculated for each gene present on the microarray by using the t-test or some other analytical strategies such as the ANOVA, which helps to estimate the contribution of experimental factors to the distribution of the measured gene expression. Next, a cut-off is found to separate the differentially expressed genes from the genes whose expression is not changed. This cut-off is usually based on a multiple testing criterion such as the Bonferroni or the false discovery rate (Benjamini and Hochberg 1995). Post-hoc corrections are also recommended because the number of genes tested is much bigger than the amount of samples replicated across two or more biological conditions.

A typical microarray experiment results in lists of differentially expressed genes. Long gene lists, however, cannot be considered the end point of the analysis. Rather, they have to be regarded as the starting point of a more meaningful interpretation, whereby biological patterns are typically highlighted. By taking advantage of the increasing knowledge about the functions of the genes within the cells, it is also possible to infer the overall changes in terms of functions and processes. This essentially shifts the level of analysis from individual genes to sets of biologically related genes. The annotation terms are usually obtained from libraries such as Gene Ontology (Ashburner et al, 2000) or KEGG (Ogata et al, 1999).
Metabolic pathways, though, are controlled to a large extent by protein-based events, having no direct implication to the levels of mRNA measured by microarray assays. Similarly, one can test whether the expression of genes sitting in specific portions of chromatin (i.e. cytobands or entire chromosome) are involved in certain experimental conditions. For any of the annotations used for grouping the genes, the terms are defined \textit{a priori} and constructed independently from the experimental data. The most popular method starts from a list of differentially expressed genes and assesses whether a given gene set is over-represented by using a test for independence in a contingency matrix (Khatri and Draghici 2005). These methods imply the use of a strict significance cut-off for the differential expression of individual genes. Alternatively, one can test whether the ranked list of genes annotated in a given gene set differs from a uniform distribution by using the Kolmogorov-Smirnov test (Mootha \textit{et al}, 2003). Other approaches do not compute the p-values per each gene, but start the analysis directly from the raw expression data. It has been proposed to test whether samples with similar expression profiles have similar class labels. This can be achieved by using logistic regression models, ANOVA models, or a t-test after reducing the gene set to its first principal component (Tomfohr \textit{et al}, 2005).

Signaling processes manifest themselves not at the level of genes, but at the protein level too. Whilst aberrant gene activity may jeopardize cellular milieu, there is a poor correlation between level of gene expression and relative levels of active protein within the tissue (Petricoin and Liotta, 2003). Human protein HABP1/p32/gC1qR is a multi-compartmental and multi-functional cellular protein expressed in a wide range of tissues and cell types including lymphocytes, endothelial cells, dendritic cells, and platelets. Although the exclusive biological significance of this protein is yet to be elucidated, its
relevance as a modulator of intra- and extra-cellular ligands is becoming increasingly apparent. Physical and functional interactions of HABP1 with HA and a varied class of proteins from smARF (Reef et al, 2007), Hrk (Sunayama et al, 2004), CBF (Chattopadhyay et al, 2004), lamin B receptor (Simos and Georgatos, 1994), etc; and various viral, bacterial and parasitic proteins, describe its importance in cellular signaling and in infectious diseases. Over the years, information gained about this single molecule suggests that the multiplicity of functions associated with this protein may in fact be nature’s way of ensuring redundancy, especially if the molecule plays a vital role in disease manifestation.

In previous chapters, we have observed that stable overexpression of HABP1 in liver cancer cell line, HepG2 leads to phenotypic changes, enhancement in cell survival, cell adhesion and tumorigenic potential, and inhibition of cell migration. HA-mediated cell survival pathways were found to be activated in the stable clone. Therefore, in this chapter we have used microarray technology to understand the cellular pathways and the target genes which get modulated upon stable HABP1 overexpression in liver cancer cell line, HepG2, leading to enhanced cellular proliferation, adhesion and increased transformation efficiency.

Result and Discussion

Study of High throughput Analysis of HABP1 overexpression in HepG2 cells

Any alteration in mRNA population upon HABP1 overexpression in hepatic cancer cell line HepG2 was investigated by genome wide expression profiling using genomic DNA microarrays by Agilent's Custom GE Human 8x15k, AMADID No: 16332 array. The array used is a high resolution tool for genome-wide DNA copy number variation
profiling without amplification or complexity reduction. Probe coverage spans both coding and non-coding regions with emphasis on well characterized genes. Probe design and selection have been carefully optimized and validated for maximal sensitivity and specificity. This microarray has been designed to meet the unique and challenging demands of CGH-all on a single chip (Barrett et al., 2006).

Initially, the total RNA was taken out from the control (HepG2) and the experimental sample (HepR21) by Qiagen's RNeasy Minikit. The quality of eluted RNA was evaluated by Bioanalyzer (Table 4; Fig. 34).

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD 260/280</th>
<th>OD 260/230</th>
<th>RNA conc. ng/μl</th>
<th>Total Yield (ng)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT_271_L_HepR21</td>
<td>2.1</td>
<td>2.1</td>
<td>1160.0</td>
<td>34799.4</td>
<td>OP, OC</td>
</tr>
<tr>
<td>GT_271_L_HepG2</td>
<td>2.1</td>
<td>2.2</td>
<td>2125.4</td>
<td>63761.1</td>
<td>OP, OC</td>
</tr>
</tbody>
</table>

Table 4: RNA concentration and purity estimated using Nanodrop spectrophotometer. OP: Optimal purity (OD260/280 >1.8 and <2.2; OD260/230>1.0 and <2.4); OC: optimal concentration (>50 ng/microlitre).

The bioanalyzer profile confirms that the samples have optimal purity and optimal concentration and can be processed further for Microarray analysis. Agilent's 'Low RNA Input Linear Amplification Kit PLUS' generates fluorescent cRNA (complimentary RNA) with a sample
input RNA range between 200 ng and 1 μg of total RNA or a minimum of 10 ng of poly A+ RNA for one-color processing. The method uses T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3-labeled CTP. There is routinely at least a 100-fold RNA amplification with use of this kit. Samples were labeled using the Agilent Low Input RNA amplification Kit and the quality control was performed using NanoDrop analysis of labeled cRNA. The amplified and labeled cRNA is shown to be suitable for hybridization (Table 5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dye</th>
<th>pmol/μl</th>
<th>Conc: ng/μl</th>
<th>OD 260/280</th>
<th>Specific Activity (pmol dye/μg cRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepR21</td>
<td>Cy3</td>
<td>1.62</td>
<td>77.62</td>
<td>2.28</td>
<td>20.87</td>
</tr>
<tr>
<td>HepG2</td>
<td>Cy3</td>
<td>6.43</td>
<td>255.43</td>
<td>2.3</td>
<td>25.37</td>
</tr>
</tbody>
</table>

Table 5: Nanodrop analysis of labeled cRNA. Specific activity greater than 8.0 is considered to be good and the amplified and labeled cRNA can be further processed for hybridization.

The spot distribution in the images were manually verified and found to be devoid of uneven hybridization, streaks, blobs and other artifacts. Hybridization across the slide was good based on number of feature that were “g(r) is PosAndSignif” which indicates feature is positive and significantly above background (Fig. 35; Table 6).

The microarray images were overall clean, showing uniform intensity with very low background noise (Fig. 36). Data analysis was done using GeneSpring GX version 7.3 and Microsoft Excel. The normalization was done using GeneSpring GX using the recommended Per Chip and Per Gene Normalization: Normalize to specific samples.

<table>
<thead>
<tr>
<th>Slide Barcode</th>
<th>Samples hybridized</th>
</tr>
</thead>
<tbody>
<tr>
<td>251633210006_1_4</td>
<td>HepR21_cy3</td>
</tr>
<tr>
<td>251633210006_2_1</td>
<td>HepG2_cy3</td>
</tr>
</tbody>
</table>

Table 6: Design of hybridization

137
Data was normalized by the median per gene and per array by ANOVA test \((p>0.05)\) without assuming equal variances. Signal intensity must be a Present or Marginal call in all control samples when in HepR21 cells these are differentially expressed.

Fig. 35: Spatial Distribution of All Outliers on the Array 192 rows x 82 columns

The fold change i.e. calculated in \(\log_2\) is selected arbitrarily for the purpose of the present work as follows:

**Upregulated genes (ratio above 2)**

**Downregulated genes (ratio less than 0.5)**

**Fold change used for Upregulated \(\geq 1\) and Downregulated \(<-1\)**

**Fold change calculated is \(\log_2\)**
The genes are classified broadly in groups of upregulated and down regulated genes (Table 7; Fig. 38).

<table>
<thead>
<tr>
<th>Samples hybridized</th>
<th>Upregulated genes</th>
<th>Downregulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepR21 vs. HepG2</td>
<td>600</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 7: Differentially regulated genes

Fig. 37: Differentially expressed transcripts in HepR21 cell line
The significant functional classification of differentially regulated genes in experiment was obtained using GeneSpring GX software ontology browser. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways for each gene were retrieved using Bioconductor package. KEGG pathway is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks. A grand challenge in the post-genomic era is the complete computer representation of the cell, the organism and the biosphere, which will enable computational prediction of higher-level complexity of cellular processes and organism behaviors from genomic and molecular information. With this aim, a bioinformatics resource named KEGG is a part of the resource projects of the Kanehisa Laboratories in the Bioinformatics center of the Kyoto University and the Human Genome Center of the University of Tokyo. Utilizing the Bioconductor program (Gentleman et al, 2004), a detailed analysis of the differentially expressed genes was carried out in terms of their role in specific functional pathways utilizing KEGG pathways. Pathways with maximum significant differentially expressed genes are displayed in KEGG pathways (Fig. 38-48). It is interesting to note that the pathways related to cellular signaling which are responsible for cell adhesion and cellular metabolism and cell stabilization are highly expressed in HepR21 cells confirming that in HepR21 cells, cell survival pathways are activated. As evident from the Table 8, a cross-talk between different signaling cascades exists in HepR21 cells which ultimately lead to cell proliferation. Although the change in expression of different proteins studied previously as markers for cell growth and survival, does not translate into changes in their transcript levels, but their respective pathways are highly disturbed as revealed by high throughput microarray analysis. When the data was analyzed and functionally evaluated using specific KEGG pathways in detail, the profile of the differentially expressed genes generated great interest.
The perturbation in the functional pathway resulting in altered expression of genes varying in expression between HABP1 overexpressing stable clone, HepR21 and the parent cell line HepG2 are indicated in KEGG pathways, identifying the upregulated genes (red star) and the down regulated genes (blue star).

Table 8: Differentially expressed genes in HepR21 cells

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Differentially Expressed Genes</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upregulated</td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>gamma-glutamyl transferase</td>
<td></td>
</tr>
<tr>
<td>metabolism</td>
<td>(GGT)</td>
<td></td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>Cyclin-dependent kinase 6</td>
<td>DNA-damage-inducible</td>
</tr>
<tr>
<td></td>
<td>Cell division cycle 20 homolog (S. cerevisiae) (CDC20)</td>
<td>transcript 3 (DDIT3)</td>
</tr>
<tr>
<td></td>
<td>Cell division cycle (CDC2/CDK1)</td>
<td>Growth arrest and DNA-damage-inducible, alpha (GADD45A)</td>
</tr>
<tr>
<td></td>
<td>Cyclin E1 (CCNE1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAD2 mitotic arrest deficient-like 2 (MAD2L2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cell division cycle 16 homolog (CDC16/APC6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclin-dependent kinase 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(CDK3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ubiquitin-conjugating enzyme E2C (UBE2C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNF1-like kinase (SNF1L)</td>
<td></td>
</tr>
<tr>
<td>MAP Kinase</td>
<td>Mitogen-activated protein kinase 7 (MAPK7)</td>
<td>DNA-damage-inducible</td>
</tr>
<tr>
<td>Pathway</td>
<td>Calcium channel, voltage-dependent, R type, alpha 1E subunit (CACNA1E)</td>
<td>transcript 3 (DDIT3)</td>
</tr>
<tr>
<td></td>
<td>Mitogen activated protein kinase 8 (MAPK8/JNK1)</td>
<td>Growth arrest and DNA-damage-inducible, alpha (GADD45A)</td>
</tr>
<tr>
<td></td>
<td>Rho family, small GTP binding protein (RAC1)</td>
<td>Protein tyrosine phosphatase, receptor type, R (FTPRR)</td>
</tr>
<tr>
<td></td>
<td>Calcium channel, voltage-dependent, gamma subunit 8 (CACNG8)</td>
<td>Calcium binding</td>
</tr>
<tr>
<td></td>
<td>nuclear factor of kappa light</td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>p21/Cdc42/Rac1-activated kinase 1 (PAK1)</td>
<td>Tripartite motif-containing 35 (TRIM35)</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Nucleolar protein 3 (apoptosis repressor with CARD domain) (NOL3)</td>
<td>Calcium binding protein P22 (CHP)</td>
</tr>
<tr>
<td>Wnt Signaling Pathway</td>
<td>WNT1 inducible signaling pathway protein 1 (WISP1)</td>
<td>fem-1 homolog b (C. elegans) (FEM1B)</td>
</tr>
<tr>
<td></td>
<td>Rho family, small GTP binding protein (RAC1)</td>
<td>Programmed cell death 4 (neoplastic transformation inhibitor) (PDCD4)</td>
</tr>
<tr>
<td></td>
<td>Pygopus homolog 2 (Drosophila) (PYGO2)</td>
<td>Growth arrest and DNA damage-inducible, alpha (GADD45A)</td>
</tr>
<tr>
<td></td>
<td>Frizzled homolog (FZD1/FZD2)</td>
<td></td>
</tr>
<tr>
<td>Notch Signaling</td>
<td>LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase (LFNG)</td>
<td>Calcium binding protein P22 (CHP)</td>
</tr>
<tr>
<td></td>
<td>Tumor necrosis factor, alpha, converting enzyme (TACE)</td>
<td></td>
</tr>
<tr>
<td>Cell Adhesion Molecules</td>
<td>P-Cadherin (CDH3)</td>
<td>Neuroplin 2 (NRP2)</td>
</tr>
<tr>
<td></td>
<td>E-Cadherin</td>
<td>Protein tyrosine phosphatase, receptor type, F</td>
</tr>
<tr>
<td></td>
<td>Protocadherin gamma subfamily A, 8 (PCDHGA8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protocadherin alpha 8 (PCDHA8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protocadherin alpha 11 (PCDHA11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WNT1 inducible signaling pathway protein 1 (WISP1)</td>
<td></td>
</tr>
<tr>
<td>ECM-Receptor Interaction</td>
<td>Collagen, type V, alpha 2 (COL5A2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Collagen, type VI, alpha 3</td>
<td></td>
</tr>
<tr>
<td><strong>Adherens Junction</strong></td>
<td><strong>Caveolin 1</strong></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>beta-actin (ACTB)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinin alpha4 (ACTN4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ras-related C3 botulinum toxin substrate 1 (Rac1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Purine Metabolism</strong></th>
<th><strong>IMP (inosine monophosphate) dehydrogenase 2 (IMPDH2)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectonucleoside triphosphate diphosphohydrolase 6 (ENTPD6)</td>
<td></td>
</tr>
<tr>
<td>cGMP-stimulated phosphodiesterase 1: Homo sapiens phosphodiesterase 2A, cGMP-stimulated (PDE2A)</td>
<td></td>
</tr>
<tr>
<td>Purine-rich element binding protein A (PURA)</td>
<td></td>
</tr>
<tr>
<td>Fragile histidine triad gene (FHIT)</td>
<td></td>
</tr>
<tr>
<td>3'-phosphoadenosine 5'-phosphosulfate synthase 1 (PAPSS1)</td>
<td></td>
</tr>
<tr>
<td>Polymerasc (RNA) I polypeptide A (POLR1A)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Pyrimidine Metabolism</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectonucleoside triphosphate diphosphohydrolase 6 (ENTPD6)</td>
</tr>
</tbody>
</table>

Of all the genes upregulated or downregulated upon stable HABP1 overexpression in human hepatic cancer cell line, HepG2, several were of potential interest as mediators in many signaling events as well as for their recognized roles in cellular proliferation and cell-cell interactions. Overall upregulation in the transcripts of some of the types of fibronectin, laminin, collagen, cadherins, actinin along with Rac1, CdkS , cyclin E etc, validate strong cell-cell adhesion and activation of cell survival pathways as observed in HepR21 cells.

**Analysis of signaling networks in the light of transcriptomics**

We have observed that enhanced levels of glutathione are present in HepR21 cells as compared to HepG2 cells. HepG2 cell line, by itself is known to have higher levels of glutathione, which is an anti-oxidant...
and is required to protect the cells from toxins and free radicals. Upregulation of γ-glutamyl transferase (Ggt) (Fig. 38), a key enzyme in glutathione metabolism indicates increased glutathione synthesis which can be correlated with enhanced tumorigenicity in HepR21 cells. GGT is a plasma-membrane-combined heterodimeric glycoprotein which initiates the degradation of extracellular glutathione and its conjugates. The cleavage of glutathione by GGT into cysteinylglycine and a γ-glutamyl residues provides a mechanism for the recovery of cystein by the cell and thus for renewed glutathione synthesis. Elevated serum levels of GGT are highly correlated with biotransformation, nucleic acid metabolism and tumorigenesis. It is known to be a sensitive biomarker reflecting hepatocyte parenchymatous lesions (Yao and Dong, 2007).

The phenomenon of enhanced growth and proliferation in HepR21 is also evident with upregulation of key enzymes in purine and pyrimidine metabolism (Fig. 39 and 40).

**Activation of pathways related to cell proliferation**

**Cell cycle**

Our observation on enhanced cell growth and proliferation followed by increased promoter activity of cell cycle regulator protein cyclin D1 and transcription factor, E2F and downregulation of Cdk inhibitor p21, is validated by microarray data. Increased transcript levels of cell cycle proteins Cdk1, Cdk3, Cdk4, Cdk6, Cdc20, cyclin E, Apc6 etc, along with suppressed expression of cell cycle repressors Gadd45 and Ddit3 (Fig. 41) support our observation that enhanced activity of cell cycle proteins is responsible for increased cell proliferation as observed in HepR21 cells. Mammalian cells require the sequential activation of at least four different cyclin-dependent kinases, Cdk2, Cdk3, Cdk4 and Cdk6, to drive cells through
Fig. 38: Glutathione Metabolism
Fig. 39: Purine Metabolism
Fig. 40: Pyrimidine Metabolism

Pyrimidine Metabolism

- Pentose phosphate pathway
  - Glucose-6-phosphate
  - Fructose-6-phosphate
  - Ribulose-5-phosphate

- N-Carboxy-L-Aspartate
  - Urea cycle
  - O-L-Glutamine

- Uric acid metabolism

- Threonine
  - 3-Aminobutanal
  - Valine, leucine and isoleucine metabolism

- 5-Methyluracil
  - 5-Amino-4-hydroxy-2(3H)-furanone
Fig. 41: Cell Cycle

Orc1 Orc2
Orc3 Orc4
Orc5 Orc6

Mcm2 Mcm3
Mcm4 Mcm5
Mcm6 Mcm7

DNA → DNA biosynthesis

2-stage proteins

Orc (Origin Recognition Complex)
MCM (Mini-Chromosome Maintenance) complex

G1 S G2 M
interphase, as well as Cdk1 to proceed through mitosis. Progression through different phases of cell cycle is controlled at several checkpoints, where to continue or arrest the process is decided. Specifically, checkpoint at the G1/S transition is crucial, since there is no further extra-cellular control regarding cell duplication. The G1/S transition is controlled by the cyclin dependent kinases Cdk2, Cdk4 and Cdk6, being the two latter directly implicated in process carried out in the G1-S checkpoint. This process begins with the activation of the Cdk4/6 through external proliferative signals that provide them with kinase activity. In a second step, these proteins phosphorylate the retinoblastoma protein (pRb) with a subsequent release of the transcriptional factor E2F, which in turn activates the synthesis of necessary proteins for the S phase. Cdk4/6 is activated upon binding to cyclin D, as well as by phosphorylation at residue Thr177 in Cdk6 or at Thr172 in Cdk4, by a Cdk activating kinase (Cak). It has been recently shown that CDK1 activity alone can drive the mammalian cell cycle through cell division, as is the case in unicellular organisms such as yeast (Santamaria et al, 2007). Cyclin E binds to G1 phase Cdk2, which is required for the transition from G1 to S phase. The cyclin E/CDK2 complex phosphorylates p27Kip1, tagging it for degradation, thus promoting expression of Cyclin A, allowing progression to S phase. APC6 is a member of anaphase promoting complex (APC) which is an E3 ubiquitin ligase that marks target proteins for degradation by the 26S proteasome. Cdc20 binds to APC early in mitosis to activate it. Aneuploidy and genetic instability are a hallmark of colorectal cancer and other solid tumors, and they are thought to enhance tumor progression. The gene MAD2L2 (mitotic arrest deficient 2-like 2) encodes the spindle checkpoint protein MAD2L2, a key component of a surveillance system that delays anaphase until all chromosomes are correctly oriented. Defects in this mitotic checkpoint are known to contribute to genetic instability, i.e.,
numerical and structural aberrations of chromosomes. Gene expression profiling has shown MAD2L2 to be significantly upregulated in locally restricted colorectal tumors (Rimkus et al., 2006). Tumors with upregulated MAD2L2 expression had significantly higher numbers of aberrant mitotic Fig.s (anaphase bridges), an indication of chromosomal instability. Thus, overexpression of MAD2L2 correlates with bad prognosis in colorectal cancer. The ubiquitin-conjugating enzyme E2C (UBE2C), also known as UBCH10, along with the E3 ligase of the anaphase-promoting complex (APC), catalyzes the ubiquitination of mitotic cyclins A and B, as well as securin. UBE2C is essential for cell cycle progression, and mutation of its active site cysteine confers a dominant-negative phenotype. Overexpression of UBE2C at the mRNA level has been reported in a number of cancer cell lines and primary tumors, including lung, gastric, bladder, and uterine cancers, whereas only low levels were found in normal tissues (Lin et al., 2006). SNF1-like kinase (SNF1LK) also known as MSK (mitogen and stress activated protein kinase), has only one well-established function: regulation of gene expression by phosphorylation of transcription factors and chromatin-associated proteins. Evidence from studies using MSK1−/−MSK2−/− double-knockout mice and pharmacological inhibitors has established that MSK mediates ERK- or p38-induced phosphorylation of CREB at Ser133 and thereby stimulates transcription of CREB-target genes, including immediate early genes such as FOS, MKP1, NUR77 and JUNB. MSK mediates ERK- or p38-induced phosphorylation of histone H3 on Ser10 and Ser28 (Hauge and Frodin, 2006). These phosphorylation events occur in a limited set of genes and are thought to stimulate their transcription by altering chromatin structure locally by an unknown mechanism.

GADD45 (growth arrest and DNA-damage inducible) is known to be upregulated upon genotoxic and non-genotoxic stress to cells.
GADD45 proteins mediate cell cycle regulation through interactions with PCNA (Azam, 2001), the cyclin-dependent kinase inhibitor p21 (Kearsey, 1995), and the Cdk/cyclin B complex (Vairapandi, 2002). GADD45 expression is enhanced during apoptosis following induction by a variety of genotoxic agents. Several studies have shown that GADD45 proteins may play a role in apoptosis via activation of the c-Jun N-terminal kinase (JNK) and/or p38 mitogen-activated protein kinase (MAPK) signaling pathways (Takekawa and Saito, 1998; Harkin, 1999). We have observed down regulation of DDIT3 protein in HepR21 cells. DDIT3 is up-regulated by several stresses, such as amino acid or glucose starvation, endoplasmic reticulum (ER) stress, osmotic stress and hypoxia and is involved in ER stress-mediated apoptosis.

**Apoptosis**

We have observed enhanced cell proliferation and decrease in cell death in HepR21 which is supported by the microarray data wherein upregulation of anti-apoptotic proteins like ARC and Pak1 and down regulation of pro-apoptotic proteins like PDCD4, GADD45, FEM1B etc was found. Cancer cells suppress apoptosis through decreases in the abundance of pro-apoptotic effectors and increases in the levels of apoptosis inhibitors. Pak1 is a serine/threonine protein kinases whose activity is stimulated by the small GTPases Rac and Cdc42 and several protein kinases. Paks modulate many aspects of cellular biology, such as cellular proliferation, differentiation, transformation and survival, through multiple downstream signals. Pak1 protects cells from apoptosis and stimulates the phosphorylation of Bad, a pro-apoptotic Bcl-2 family member at serines 112 and 136, sites phosphorylated by protective signals (Jin et al, 2004). Stimulation of Pak1 and estradiol-estrogen receptor-α in mammary cancer cells is known to promote cell survival (Majumdar and Kumar, 2003). It was
found that estrogen rapidly activates Pak1 kinase activity in a phosphatidylinositol 3-kinase-insensitive manner. Furthermore, estrogen induced phosphorylation and perinuclear localization of the cell survival forkhead transcription factor FKHR (Forkhead homolog 1 rhabdomyosarcoma) in the cytoplasm in a Pak1-dependent manner. In addition, Pak1 directly interacted with FKHR and phosphorylated it and phosphorylation-dependent exclusion of FKHR from the nucleus impaires the ability of FKHR to activate its target Fas ligand promoter containing the FKHR binding motif (FRE) in cells treated with estrogen or expressing catalytically active Pak1. It is reported that elevated (alpha6) beta4 integrin-dependent Rac-Pak1 signaling supports resistance to apoptosis in mammary acini by permitting stress-dependent activation of the p65 subunit of NF-κB through Pak1 (Friedland et al, 2007). Apoptosis Repressor with CARD (caspase recruitment domain)) ARC, an apoptosis repressor is found to be abundant in primary human epithelial cancers of the colon, ovary and cervix but not corresponding benign controls suggest that induction of ARC may be a general feature of epithelial cancers (Mercier et al, 2008). Unlike most apoptosis inhibitors that oppose either the extrinsic or intrinsic pathways, ARC antagonizes both pathways through a variety of mechanisms. ARC inhibits the extrinsic pathway through direct binding of its CARD to death domains in the cytoplasmic tail of the death receptor and in FADD, an adaptor molecule. These interactions preclude assembly of the DISC and abrogate extrinsic signaling. ARC antagonism of the intrinsic pathway involves several mechanisms: (a) the direct binding of the ARC CARD to Bax, that inhibits Bax conformational activation and translocation to the mitochondria; and (b) direct binding of the proline-glutamic acid-rich region of ARC to the tetramerization domain of p53 that inhibits p53 tetramerization, thereby exposing a p53 nuclear export signal that relocates p53 to the cytoplasm. ARC can inhibit apoptosis
induced by death receptor activation, hypoxia, oxidative stress and serum deprivation. Programmed cell death 4 (Pdcd4) is a tumor suppressor protein that interacts with eukaryotic initiation factor 4A and inhibits protein synthesis. Pdcd4 also suppresses the transactivation of activator protein-1 (AP-1)-responsive promoters by c-Jun. Akt phosphorylation is known to regulate the function of Pdcd4 tumor suppressor protein. Akt phosphorylation at Ser67 and Ser457 residues of Pdcd4 causes nuclear translocation of Pdcd4. Phosphorylation of Pdcd4 by Akt also causes a significant decrease of the ability of Pdcd4 to interfere with the transactivation of AP-1-responsive promoter by c-Jun (Palamarchuk et al, 2005). Loss of PDCD4 expression in human lung cancer is shown to be a prognostic factor which correlates with tumour progression (Chen et al, 2003).

**TRIM35**, a member of the tripartite motif (TRIM) family also functions as a tumor repressor. **Fem1b**, a mammalian homolog of the C. elegans sex-determining gene Fem-1, induces caspase-dependent apoptosis in mammalian cells. Fem 1b was shown to induce apoptosis when overexpressed in breast carcinoma cells (Chan et al, 2000).

**MAP Kinase pathway**

The variations in mitogen activated protein kinase pathway (MAPKs) with upregulation of MAPK7, Rac1, Jnk1, Nfkb2 etc, and downregulation of Gadd45, CHP and Ddit3 (Fig. 42) might be contributing to enhanced cell proliferation, cell adhesion and change in cellular morphology upon stable HABP1 overexpression in HepR21 cells. The cyclin D1 gene is the target of many mitogenic signaling pathways, but the best studied pathway to cyclin D1 the ERK-MAPK pathway. In several cell types, a sustained ERK activity between 3 and 6 h after mitogen stimulation results in the induction of cyclin D1 mRNA in mid-G1 phase, at least when cells are re-entering G1 phase from quiescence (Welsh et al, 2001). Stimulation of MAP kinase
ERK5 regulates neuronal survival, muscle cell differentiation, and cellular proliferation and transformation. Hyperactivity of the ERK5 pathway is associated with highly aggressive forms of breast and prostate cancers. Mechanisms for ERK5 regulation of proliferation are not well understood. However, several targets of ERK5 have been identified, including nuclear factor κB (NFκB), c-myc, and cyclin D, all of which are potential regulators of proliferation (Cude et al., 2007). ERK5 plays a critical role in the G2/M progression, and NFκB-mediated transcription is identified as a key downstream mechanism by which ERK5 regulates the G2/M phase transition.

Rac1, a small GTP-binding protein, is known to have an essential role in cell proliferation and G2 to M progression (Moore et al., 1997). Rac is known to induce cyclin D1 mRNA. Rac signaling to cyclin D1 requires NF-κB and results in a somewhat earlier induction of cyclin D1 within the G1 phase. Once expressed, cyclin D1 binds to cdk4 or cdk6 and initiates the inactivating phosphorylation of Rb that, in turn, controls the transcription of E2F-dependent genes and progression into S phase (Kothapalli et al., 2008). Jnk1, a member of JNK family of kinases, regulates transcription through phosphorylation and activation of a diverse set of transcription factor targets, including the proto-oncogene c-Jun, in response to both pro- and anti-apoptotic stimuli. Phosphorylation on Ser63 of c-Jun by activated JNKs results in changes in transcriptional output and is associated with induction of cell cycle progression, upregulation of angiogenic survival and proliferation gene expression, and downregulation of death signaling through the Fas/Fas ligand extrinsic death receptor pathways. JNK activity is induced downstream of the MAPKKK, apoptosis signal regulating kinase1 (ASK1) and both JNK and c-Jun are implicated in the repression of differentiation of rhabdomyosarcoma and maintenance of a tumorigenic state (Durbin et al., 2009). Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (Nfκb2)
has been detected in numerous cell types that express cytokines, chemokines, growth factors, cell adhesion molecules, and some acute phase proteins in health and in various disease states. NFKB2 is activated by a wide variety of stimuli such as cytokines, oxidant-free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products. Inappropriate activation of NFKB2 has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, and AIDS. In contrast, complete and persistent inhibition of NFKB2 has been linked directly to apoptosis, inappropriate immune cell development, and delayed cell growth (Baldwin, 1996).

Upregulation of calcium gated channels CACNA1E and CACNG8 was also observed in HepR21 cells. There has been an increasing interest in calcium channels and cancer, as Ca^{2+}-mediated intracellular signaling pathways control a large range of cellular processes, including proliferation. The identification of up-regulation of calcium channel subunits in colon and prostate cancer suggests a potential generalized link with tumorigenesis, although the mechanisms by which this occur remain unclear (Wissenbach et al, 2004). CACNA1E encodes the ion-conducting α1 subunit of R-type voltage-dependent calcium channels widely expressed throughout the brain as well as in endocrine systems. Voltage-gated Ca^{2+} channels mediate the entry of Ca^{2+} into cells in response to membrane depolarization. The encoded protein Cav2, initiates rapid synaptic transmission and is regulated primarily by direct interaction with G proteins and SNARE proteins and secondarily by protein phosphorylation. Stable transfection of Cav2.3 into human embryonic kidney cells resulted in the specific activation of the MEK5/ERK5/Nur77 pathway, leading to up-regulation of the proliferation-associated early response genes EGR1/EGR2/EGR3 and FOS/FOSB (Natrajan et al, 2006). Interestingly, up-regulation of
various Frizzled genes was observed by Cav2.3 overexpression in HEK293 cells and Wilms' tumors, suggesting a role for Cav2.3 in activation of Wnt/Ca²⁺ pathways.

**CHP** (calcineurin homologous protein), an inhibitor of calcineurin signaling, is found to be downregulated in HepR21 cells. CHP is a Ca²⁺ binding protein, ubiquitously expressed in cells, which shares a high degree of similarity with calcineurin B (65%) and Calmodulin (59%). Although CHP was originally identified by screening a library to detect proteins that interacted with the Na⁺/H⁺ exchanger isoform NHE1, subsequent studies indicated that CHP has actions that are independent of its regulation of NHE1. Overexpression of CHP in mutant NHE-deficient fibroblasts was shown to inhibit cell proliferation. Overexpression of CHP in HeLa and Jurkat cells impairs the nuclear translocation and the transcriptional activity of NFAT. CHP effects on NFAT are likely mediated by its ability to inhibit calcineurin. CHP was found to inhibit calcineurin phosphatase activity in vivo and in a reconstituted assay with purified proteins by impairing the assembly of the heterotrimeric configuration. Additionally, activation of T-cell signaling led to a decrease in CHP abundance, suggesting a positive feedback mechanism of releasing an inhibitory action of CHP during T-cell receptor signaling (Lin et al, 1999).

**Wnt/β-catenin signaling**

Many genes of Wnt pathway like Wisp1, Pygo2, Fzd1/2, (Fig. 43) etc., were found to be upregulated and PP2A was downregulated in HepR21 cells indicating deranged Wnt signaling. The Wnt signaling pathway is essential in different biological processes. Misregulation of this pathway is a hallmark of many human cancers, including colorectal carcinomas. In the absence of a Wnt signal, cells degrade beta-catenin by a multiprotein complex consisting of the adenomatous
Fig. 43: Wnt Signaling Pathway
polyposis coli (APC) tumor suppressor protein, axin and the glycogen synthase kinase (GSK3β). This kinase phosphorylates beta-catenin marking it for subsequent ubiquitination and degradation. When the Wnt ligand docks to its Frizzled (Fz) receptor, the degradation complex is destabilized, and beta-catenin accumulates in the cytoplasm and translocates to the nucleus, where it functions as a cofactor for TCF/LEF (T-cell factor/lymphoid-enhancing factor). Pygo2 and Wisp1 are known to promote cell proliferation. Pygo2 promotes Wnt signaling in mammary epithelium through its synergistic interactions with di- and tri-methylated histones and the recruitment of β-catenin–BCL9 complexes to promoters of β-catenin target genes (Horsely, 2009).

WISP-1 (Wnt-1 induced secreted protein 1), a member of the CCN family of growth factors is a β-catenin related gene that can contribute to tumorigenesis. The promoter of WISP-1 was cloned and shown to be activated by both Wnt-1 and β-catenin expression and CREB site played an important role in this transcriptional activation. WISP-1 is known to demonstrate oncogenic activities; overexpression of WISP-1 in normal rat kidney fibroblast cells (NRK-49F) induced morphological transformation, accelerated cell growth, and enhanced saturation density (Xu et al, 2000). WISP-1 attenuates p53-mediated apoptosis in response to DNA damage through activation of the Akt kinase (Su et al, 2002). Fzd1 and 2 are the receptors for Wnt proteins and are known to be highly expressed in human esophageal cancers (Tanaka et al, 2001). In the absence of Wnt stimulation, cytosolic β-catenin is assembled into the so-called ‘Axin complex’ where it is sequentially phosphorylated by CK1 and GSK3 and targeted for proteasomal degradation. Activation of Wnt signaling leads to loss of this phosphorylation and stabilization of β-catenin. However it has been suggested that these previously known negative regulators of Wnt pathway also positively regulate this pathway and stimulate Wnt/β-catenin signaling (Truesdell and LaBonne, 2006). This supports our
observation that β-catenin and its downstream effectors like cyclin D are involved in cellular proliferation upon stable overexpression of HABP1.

**Notch Signaling**

The variation in notch signaling pathway with upregulation of *Tace* and *Lfng* (Fig. 44) was observed in HepR21 cells upon stable HABP1 overexpression. Notch, the highly conserved transmembrane calcium dependent receptor signaling pathway is activated by extracellular ligand binding to release intracellular domain which enters the cell nucleus to alter gene expression (Watt *et al.*, 2008). A recent study has revealed a role of Notch signaling in G1/S progression of cell cycle in T cells. Expression of the G1 proteins, cyclin D3, CDK4, and CDK6 was shown to be notch-dependent both *in vitro* and *in vivo* (Joshi *et al.*, 2009). Notch signaling also has a role in mechanism to control the actin cytoskeleton through the tyrosine kinase, Abl. Notch signaling is dysregulated in many cancers, and faulty Notch signaling is implicated in many diseases including T-ALL (T-cell acute lymphoblastic leukemia) (Sharma *et al.*, 2007). Tumor Necrosis Factor Alpha Converting Enzyme (TACE) cleaves the Notch protein just outside the membrane which releases the extra-cellular portion of Notch. The ligand plus the Notch extra-cellular domain is then endocytosed by the ligand expressing cell. After this first cleavage, an enzyme called γ-secretase cleaves the remaining part of the Notch protein just inside the inner leaflet of the cell membrane of the Notch-expressing cell. This releases the intracellular domain of the Notch protein, which then moves to the nucleus, where it can regulate gene expression by activating the transcription factor CSL (Lai, 2004). *Lfng* encodes a member of the glycosyltransferase superfamily which is a single-pass type II Golgi membrane protein that functions as a fucose-specific glycosyltransferase, adding an N-acetylglucosamine to the
Fig. 44: Notch Signaling pathway

**NOTCH SIGNALING PATHWAY**

- Delta
- Serrate
- Fringe

**Dvl**

**Numb**

**Dellex**

**S3**

**PSE2**

**PSEN**

**NCSTN**

**APH-1**

γ-Secretase complex

MAPK signaling pathway

- Co-repressor
- HDAC

- Hairless
- SMRT
- CIR

- CBP
- Groenho

- Co-activator
- MAML
- HATs

- SKIP

**Hes1/5**

**PreTo**

**NICD** (Notch intracellular domain)

Gene expression
fucose residue of a group of signaling receptors involved in regulating cell fate decisions during development (Moloney et al., 2000). The influence of Notch signaling, in crucial cell communication event is also related to the mechanical linkages to ECM proteins.

**Cell-cell communication**

Focal adhesion proteins **zyxin** and **actinin** (Fig. 45), which are required for dynamic assembly of focal adhesions, were found to be upregulated whereas transcript levels of **caveolin-1** (**CAV-1**) were found to be decreased upon HABP1 overexpression confirming that HepR21 cells display enhanced cell-ECM adhesion. Focal adhesions are actin-rich structures that enable cells to adhere to the extracellular matrix and at which, protein complexes involved in signal transduction assemble. Zyxin is a low abundance phosphoprotein that is localized at sites of cell-substratum adhesion in fibroblasts. Zyxin displays the architectural features of an intracellular signal transducer. The protein exhibits an extensive proline-rich domain, a nuclear export signal and three copies of the LIM motif, a double zinc-finger domain found in many proteins that play central roles in regulation of cell differentiation. Zyxin interacts with alpha-actinin, members of the cysteine-rich protein (CRP, family, proteins that display Src homology 3 (SH3) domains and Ena/VASP family members. Zyxin and its partners have been implicated in the spatial control of actin filament assembly as well as in pathways important for cell differentiation. Based on its repertoire of binding partners and its behavior, zyxin may serve as a scaffold for the assembly of multimeric protein machines that function in the nucleus and at sites of cell adhesion (Beckerle, 1997). Zyxin functions as a messenger in the signal transduction pathway that mediates adhesion-stimulated changes in gene expression and its higher expression can be correlated with the modulation of cytoskeletal
Fig. 45: FOCAL ADHESION

[Diagram showing the Focal Adhesion Kinase (FAK) signaling pathway and its interactions with various cellular components.]
organization of actin bundles (Reinhard et al, 1995). The close subcellular proximity of different actin filament crosslinking proteins suggests that these proteins may cooperate to organize F-actin structures to drive complex cellular functions during cell adhesion, motility and division. **Alpha-actinin** and filamin, two major F-actin crosslinking proteins that are both present in the lamella of adherent cells, display synergistic mechanical functions. Moreover, F-actin networks assembled in the presence of alpha-actinin and filamin strain-harden more readily than networks in the presence of either alpha-actinin or filamin (Esue et al, 2009). Expression of alpha-actinin is known to be upregulated in human hepatocellular carcinoma (Nishiyama et al, 1990).

**Cav1** has been identified as an independent predictor of decreased survival in breast and rectal cancer and is significantly associated with the presence of distant metastasis for colon cancer patients (Joshi et al, 2008). Rho/ROCK signaling promotes tumor cell migration by regulating focal adhesion dynamics through tyrosine (Y14) phosphorylation of Cav1. Phosphorylated Cav1 is localized to protrusive domains of tumor cells and Cav1 tyrosine phosphorylation is dependent on Src kinase and Rho/ROCK signaling. Stable expression and knockdown studies of Cav1 in tumor cells showed that phosphorylated Cav1 expression stimulates Rho activation, stabilizes FAK association with focal adhesions, and promotes cell migration and invasion in a ROCK-dependent and Src-dependent manner. Downregulation of Cav1 therefore supports our observation that cell migration is inhibited upon HABP1 overexpression.

We have observed that HepR21 cells enhanced adhesion and took longer time for trypsinization which is supported by higher expression of ECM constituents like **fibronectin, laminin**, and some types of collagen ([Fig. 46](#)). Enhanced fibronectin expression has been implicated in carcinoma development. In non-small cell lung
carcinoma, fibronectin expression is shown to be increased and adhesion of lung carcinoma cells to fibronectin enhances its tumorigenicity. Fibronectin has been shown to stimulate the gonadal steroids that interact with vertebrate androgen receptors, which are capable of controlling the expression of cyclin D and related genes involved in cell cycle control. Fibronectin has been shown to stimulate non-small cell lung carcinoma cell growth through activation of Akt/mammalian target of rapamycin/S6 kinase and inactivation of LKB1/AMP-activated protein kinase signal pathways (Han et al, 2006).

**Laminins** bind to cell membranes through integrin receptors and other plasma membrane molecules, such as the dystroglycan glycoprotein complex and Lutheran blood group glycoprotein. Through these interactions, laminins critically contribute to cell attachment and differentiation, cell shape and movement, maintenance of tissue phenotype, and promotion of tissue survival (Colognato et al, 2000). Elevated serum levels of laminin have been used as a tumor marker in breast cancer. Laminin-1 is speculated to enhance the malignant behavior of colon cancer cells by accelerating proliferation as well as by decreasing cell loss. (Kim et al, 1999). Down regulation of **NRP-2**, a receptor for the semaphorin and vascular endothelial growth factor families was seen in HepR21 cells, which is involved in tumor metastasis and angiogenesis.

Collagen is the major component of the interstitial ECM. ECM is known to play an active role in numerous biological processes such as cell shape, proliferation, migration, differentiation, apoptosis as well as carcinogenesis. A specific upregulation in transcript levels of **COL5A2** and **COL11A1** (which are not expressed in adult tissues) has been observed in stromal cells in colon carcinoma (Fisher et al, 2001). An increase of type V collagen expression has also been observed in mouse skin tumours (Marian and Danner, 1987) and human breast carcinoma (Barsky et al, 1982). It has been suggested that the specific
Fig. 46: ECM Receptor Interaction

ECM-RECEPTOR INTERACTION

ECM | Integrin | VLA proteins
---|---|---
Collagen | α1 | β1
Laminin | α2 | β1
Collagen | α3 | β1
Laminin | α4 | β1
Collagen | α5 | β1
Phactrin | α6 | β1
Laminin | α7 | β1

ECM | Integrin | VLA proteins
---|---|---
OPN | α1 | β1
Phactrin | α2 | β1
Tenascin | α3 | β1
Laminin | α4 | β1

ECM | Integrin | Cytoadhesin
---|---|---
OPN | αV | β3
Tenascin | αV | β3
Collagen | αV | β3
VWF | αV | β3

Other combination

Focal adhesion

Leukocytes proteins

Leukocytes proteins

ECM | Proteoglycan
---|---
HA | CD44
Collagen | Phactrin
Laminin | Thrombospondin
Collagen | Thrombospondin
Laminin | Syndecan

Glycoprotein

THBS1 | CD36
THBS2 | CD36
VWF | GPV
GPIbα | OPLL
GPIIX

Ig-SF

THBS2 | CD47
HA | RHAMM

(c) Kanehisa Laboratories
upregulation of mRNA expression of some collagens in stromal cells could be a downstream effect of tumorigenic changes in colonic epithelial cells in colorectal cancer. Alternatively, the expression of COL11A1 and COL5A2 could be the primary change giving rise to a tumorigenic response in epithelial cells (Fisher et al., 2001). **Type VI collagen**, a soluble extracellular matrix protein is known to be upregulated in adipocytes during tumorigenesis. It promotes GSK3beta phosphorylation, beta-catenin stabilization, and increased beta-catenin activity in breast cancer cells and may critically contribute towards tumorigenesis (Iyengar et al., 2003).

Increased expression levels of transcripts of several cadherins, (Fig. 47) was also observed in HepR21 cells. Cadherins (Calcium dependant adhesion molecules) are a class of type-1 transmembrane proteins. They play important roles in cell adhesion, ensuring that cells within tissues are bound together. They are dependent on calcium (Ca$^{2+}$) ions to function. It is known that CDH3 is calcium-dependent cell-cell adhesion molecule and is expressed in cells having high growth activity and/or high differentiation potential (Shimoyama and Hirohashi, 1991). Epithelial cells have more complex adhesion systems than fibroblasts. In addition to integrin-mediated adhesion to the ECM, epithelial cells rely on adherens junctions for tissue integrity and function, and cadherins plays a major role in mediating these adherens junctions in many epithelial cell types. E-cadherin is a transmembrane protein that mediates cell-cell adhesion by calcium-dependent homophilic binding through its extracellular domain. More than 85% of ovarian carcinomas have elevated E-cadherin levels, and suppression of E-cadherin function has been shown to decrease proliferation (Reddy et al., 2005). \(\beta\)-catenin binding to the cytoplasmic domain of E-cadherin acts as a link to the actin cytoskeleton. A current hypothesis suggests that cadherin-mediated binding of \(\beta\)-catenin may affect catenin-dependent transcription of LEF-regulated
Fig. 47: Cell Adhesion Molecules
genes. Interestingly, the cyclin D1 gene can be regulated by β-catenin and LEF, raising the possibility that the formation of E-cadherin-adherens junctions might control the expression of cyclin D1 by sequestering β-catenin. However, E-cadherin can also regulate Rac activity and therefore has the potential to regulate Rac-dependent induction of cyclin D1. E-cadherin stimulates Rac-GTP loading and promotes cell proliferation in a Rac-dependent manner in MCF10A cells (Liu et al, 2006).

**Profilin-1 (Pfn1)**, a ubiquitously expressed G-actin-binding protein, is found to be expressed at a significantly low level in both human breast cancer tissue and a variety of breast carcinoma cell, pancreatic and hepatic carcinoma cells compared to their normal counterparts. These observations suggest that loss of Pfn1 expression may have a general relevance in cancer progression. Pfn1-overexpressing CAL51 breast cancer cells failed to form tumors when xenografted subcutaneously in nude mice, which suggest that Pfn1 could also be a tumour-suppressor protein (Zou et al, 2007). Decreased transcript levels of Pfn-1 as observed in our study thus correlates with enhanced tumorigenicity as seen in HepR21 cells.

Enhancement in the transcript levels of adherens junction proteins like **cadherin**, **alpha-actinin**, **actin**, **Rac1** (Fig. 48) etc, account for the increased cell-cell and cell substratum adhesion as seen in HepR21 cells. Epithelial cells form cellular barriers that separate different tissues and body compartments. This requires that they polarize (i.e. develop distinct cell surface domains) and that they interact with one another through adhesive complexes, called junctions, between the cells. These intercellular cell-junction complexes are crucial for the biogenesis, maintenance and function of epithelia. They mediate adhesion and provide mechanical strength, they restrict diffusion across epithelia, and they regulate signaling pathways that control cell proliferation, polarisation and
Fig. 48: Adherens Junction
differentiation. In vertebrates, the epithelial junctional complex consists of 'tight-junctions' (also known as the zonula occludens), adherens junctions and desmosomes. In some tissues, gap-junctions, which form intercellular pores, can also be associated with the junctional complex and can be intercalated with tight junctions. Desmosomes and adherens junctions are adhesive junctions (Marschall et al, 2007). Both tight-junctions and adherens junctions are linked to the actin cytoskeleton and contain several actin-binding proteins. More recently, microtubule-binding proteins have also been found at both types of junction, which suggests that they also interact with microtubules. The main molecules of cell-cell adhesion are cadherins, transmembrane proteins of the classical cadherin family, which form in the presence of Ca$^{2+}$ cell-cell adherens junctions associated via the cytoplasmic plaque proteins with actin microfilaments. Cadherins provide for mechanical cell-cell adhesion and regulate cell shape, segregation, migration, proliferation, and differentiation. Cadherin/catenin complexes are linked to the actin cytoskeleton via a direct association between alpha actinin and alpha catenin. The cytoplasmic domain of cadherins is highly conserved and includes the membrane-adjacent site for cadherin binding to p120-catenin (p120) and C-terminal site for binding β-catenin, which regulate cell-cell adhesion. Cadherins bind via β-catenin, plakoglobin, and α-catenin to actin filaments, which stabilize the structure of adherens junction. The interaction of E-cadherin-β-catenin-α-catenin-actin filaments in the region of cell-cell contacts is dynamic. There are now numerous data concerning the involvement in adherens junction formation of small GTPases of Rho family (Rho, Rac, and Cdc42), regulating intracellular dynamics of actin cytoskeleton. It was shown that expression of dominantly negative mutants of RhoA, Rac1, and Cdc42 genes as well as injection into cells of negatively dominant forms of their proteins disturbed formation of E-cadherin-containing
contacts of MDCK epitheliocytes and keratinocytes. It was found that GTPases Rac1 and Cdc42 were recruited into zones of N- and E-cadherin-dependent cell-cell adhesion. GTPase Rac1, activated via phosphatidylinositol-3-kinase (PI3K), nectins, or via IQGAP1 and regulating the Arp2/3-mediated actin polymerization, is of essential significance for adherens junction formation. Rac1 is activated via the lipid product of PI3K-phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 interacts directly with GEFs (P-Rex1, SWAP-70, Vav1, Sos1, and evidently Tiam1) activating Rac1. Rac1, in turn, is able to stimulate via proteins of the WASP family (N-WASP and WAVE2) the Arp2/3-mediated actin assembly in the zone of cell-cell contact (Gloushankova, 2007). Since WAVE2 can form a supramolecular complex with Rac via IRSp53, it is assumed that the Rac-WAVE2-Arp2/3 pathway plays the leading role in the assembly of actin structures that stabilize cell-cell contacts.

Control over cytoskeleton with upregulation of a few genes in glycolysis pathway, carbon fixation, pyruvate metabolism, β-alanine and inositol biosynthesis again states basic metabolic and biosynthesis activation due to stable HABP1 overexpression.

**Rac1 is activated in HepR21 cells**

To validate our microarray data that the upregulation of Rac1 is important for augmented cell proliferation in HepR21 cells, we checked the levels of Rac1 in both the cell lines by Western blot. Immunoblot analysis of equal amounts of lysates from HepG2 and HepR21 showed approximately 3 fold increase in the levels of Rac1 in HepR21 cells as compared to HepG2 cells (Fig. 49).
Fig. 49: Rac1 levels are upregulated in HepR21 cell line. Western blot analysis with Rac1 antibody shows an increase in Rac1 levels in HepR21 cells. Equal amounts of protein in the samples is confirmed by immunodetection with anti-tubulin antibody. A three fold increase in Rac1 levels was observed in HepR21 cells.

Therefore our observation that stable overexpression of HABP1 in HepG2 cells leads to enhanced cellular proliferation, cell adhesion and tumorigenic potential is supported by the microarray analysis where we have found that various cell survival pathways and expression of cell adhesion molecules are upregulated in HepR21 cells. The transcript levels of several cell cycle regulatory proteins and anti-apoptotic proteins are upregulated and the levels of various cell cycle repressors and pro-apoptotic proteins are downregulated in HepR21 cells leading to augmented cell proliferation. Enhanced growth and proliferation in HepR21 was also evident with upregulation of key enzymes in purine and pyrimidine metabolism. Wnt/β-catenin signaling pathway was activated which supports our observation that β-catenin and its downstream effectors like cyclin D are involved in cellular proliferation upon stable overexpression of HABP1. Enhanced cell adhesion was supported by upregulation of cell adhesion molecules like cadherins, fibronectin, laminins etc. Enhancement in the transcript levels of proteins of adherens junction also support increased cell-cell and cell substratum adhesion as seen in HepR21 cells.