AIM & OBJECTIVES
Hyaluronan (HA), a biochemically simple polymer of n-acetyl glucosamine and glucuronic acid, but a functionally complex and space filling component of ECM has drawn a considerable attention in the last decade. Previously thought of as a passive space filling component of ECM, it has been considered as magic glue in the recent perspective. HA is likely to be the common thread of many biological events e.g. embryonic development, tumorogenesis and other pathological disorders. As established, tumors are supported by a distinct type of stroma, crucial for their survival and progression. HA present in the stroma of many common tumors and on the surface can either suppress or enhance the development of tumors, depending on the context (Csoka, 1997). Even recent reports suggest that HA promotes anchorage-independent growth and invasiveness, which are the hallmarks of malignant phenotype (Toole, 2002). It also protects cancer cells against immune cell attack. Melanoma, a highly metastatic tumor, is caused by malignant transformation of normal melanocytes. To invade, cells must detach and reattach efficiently to ECM (Albelda et al, 1990). Serum HA level is often elevated in metastatic patients. Also, the functional roles of HA have been reported in B16F10 melanoma growth and metastasis (Mummert, et al., 2003). High levels of HA in many cancer cells as well as in embryonic tissues correlate with their rates of cell proliferation and migration.

Cell migration and ECM invasion are some of the major steps in embryonic development, wound healing and cancer cell metastasis. ECM, composed of collagens, proteoglycans and GAGs, forming a highly fibrillar network, serves as substratum for cell adhesion and migration. It also constitutes barriers that maintain tissue integrity, impede cell migration and regulate molecular diffusion and transfer of stimuli. Thus ECM remodeling is an essential event that occurs in morphogenesis, growth, inflammation, tumor development and metastasis. Proteinases and hydrolytic enzymes e.g. hyaluronidase are important in regulation of this process. Amongst the proteolytic
enzymes, matrix metalloproteinases (MMPs) and the tissue inhibitor of matrix metalloproteinases (TIMP) play a major role in the regulation of cancer cell migration, ECM invasion and metastasis by degrading the ECM proteins. When cells migrate in the tissue, ECM located at the migratory direction has to be degraded. MMPs are a group of enzymes that degrades the ECM. MMPs are regulated by different cytokines, ECM proteins and other factors (Nagase et al., 1999). MMPs gene products can be sub grouped into soluble type MMPs and the membrane-type MMPs (MT-MMPs). Since MT-MMPs are tethered to the plasma membrane, they are well placed for pericellular proteolysis that associates with cell growth, migration and morphological change of cells in tissue (Nagase and Woessner, 1999; Seiki, 1999). MT1-MMP was discovered as the first membrane type MMP and was identified as a cell surface proMMP-2 activator (Sato et al., 1994). MMP-2 (also called as type IV collagenase or gelatinase A) degrades several ECM proteins (Nagase and Woessner, 1999). It has been reported that CD44, a major receptor for HA and frequently expressed by invasive tumor cells, is cleaved by MT1-MMP, promoting cell migration (Kajita et al., 2001).

On the other hand, melanoma progression is regulated by a complex network of autocrine/paracrine positive and negative growth factors. Various transcription factors are important in these processes. Amongst them, the transcription factor NFκB plays a crucial role in regulating cell proliferation, migration and survival leading to neoplastic transformation. Integrin mediated cell migration pathway is through nuclear factor inducing kinase (NIK), a member of MAP kinase family. NIK interacts with IKKs via its C-terminal end and this interaction is required for the activation of NFκB, following phosphorylation and degradation of IκB. Such activation is reported to have regulatory role in gene expression during oncogenesis. One of the downstream signaling of NFκB is activation of mt1-mmp gene. The gene product MT1-MMP finally cleaves pro-MMP-2, thereby activating it.
The linkage of ECM to the cell requires transmembrane cell adhesion proteins that act as matrix receptors and tie the matrix to the cell's cytoskeleton. The principal receptors on animal cell for binding most ECM proteins— including collagens, fibronectin, and laminins are the integrins. Most integrins are connected to bundles of actin filament. After the binding of a typical integrin to its ligand in the matrix, the cytoplasmic tail of the β subunit binds to several intracellular anchor proteins. These anchor proteins can bind directly to actin or other anchor proteins, thereby linking the integrin to actin filaments in the cell cortex. Also, integrins have been found to have an important role in intracellular signaling pathways. Integrins being the primary receptors for ECM proteins, contribute in multiple ways to the process of cell migration. The bridge that integrins form between the intracellular actin cytoskeleton and the ECM allows cells to exert force on their environment. This force generation is critical for many cellular processes, including efficient cell movement (Horwitz and Parsons, 1999).

The integrin αvβ3 was first identified as the "vitronectin receptor" but will adhere to a host of other ECM proteins, including fibronectin, laminin, collagen, and osteopontin (Smith and Cheresh, 1990; Horton, 1997). Integrin αvβ3 is known to promote cell attachment and spreading, as well as cell locomotion (Seftor et al., 1992; Danen et al., 1994) and is shown to mediate K1735 murine melanoma cell motility (Li et al., 2001). Also, integrin αvβ3 mediates chemotactic motility in human melanoma cells through different signaling pathways (Aznavoorian, S., 1996). Studies have shown that integrin receptors can initiate a signaling cascade that can affect many aspects of cell growth (Danen et al., 1995).

Many other intracellular signaling proteins bind to and associate with this integrin-adaptor protein-cytoskeleton complex, and this forms the basis of a focal adhesion. The components of focal adhesions are diverse and include scaffolding molecules, GTPases, and enzymes such
as kinases, phosphatases, proteases, and lipases. Multiple protein-protein interactions have been defined at focal adhesions. Focal adhesions are large, dynamic protein complexes through which the cytoskeleton of a cell connects to the ECM. Because most proteins have several potential interacting partners, this allows the cell an opportunity to construct various signaling complexes leading to diverse behaviors. Molecules at focal adhesion like, vinculin, paxillin, src, FAK etc. regulate cell migration. FAK is a protein tyrosine kinase, expressed in many tissues and is involved in a wide range of pathological processes. The major role of FAK, which acts as downstream of integrin is that it regulates cell migration. Tyrosine phosphorylation is one of the key signaling events occurring at focal adhesions. Recent work (Kirchner et al. 2003), using YFP-Src-SH2 domains as a live cell probe for tyrosine phosphorylation events indicates, that recruitment of proteins such as FAK, vinculin, and paxillin at focal adhesions precedes significant tyrosine phosphorylation.

One enzyme that has also been implicated in integrin-induced signal transduction along with FAK is phosphatidylinositol 3-kinase (PI 3-kinase) (Howe et al, 1998). PI3 Kinase has been implicated as a key signaling molecule for integrin activation and regulation of actin reorganization and cell adhesion (Vanhaesebroeck et al, 2001) and also having implications in cell migration. The dynamic assembly and disassembly of focal adhesions play a central role in cell migration. It is at focal adhesions, where integrin and proteoglycan mediated adhesion links to the actin cytoskeleton.

The actin cytoskeleton is also essential for tyrosine phosphorylation of FAK. Members of the Rho family of GTP-binding proteins are key regulators of the actin cytoskeleton, and Rho itself promotes contractility and the formation of stress fibres and focal adhesions. Several lines of evidence suggest that activation of Rho induces tyrosine phosphorylation of FAK, whereas inhibition of Rho blocks this event in response to certain stimuli. Oncogenic forms of the non-receptor
tyrosine kinase Src alter cell structure, in particular the actin cytoskeleton and the adhesion networks that control cell migration, and also transmit signals that regulate proliferation and cell survival. These studies also shed light on the role of focal adhesion kinase (FAK) downstream of v-Src and other signaling pathways in controlling migration, invasion and survival of transformed cells. The mechanism has also been proposed by which FAK activates the signal transducer and activator of transcription (STAT) pathway, regulating cell adhesion and migration (B. Xie et al. 2001). Also, a loss of expression of transcription factor AP-2 in metastatic melanoma cells has been observed (Menashe Bar-Eli, 1999).

After coming across a molecule like HA, that has a list of diverse functions, not only in ECM, but also on the cell surface and inside the cell; attention was diverted to identify a new family of proteins, which have specific affinity for HA. A number of these proteins have been purified and characterized. These proteins may originate from the ECM, the cell surface or inside the cell. The interaction of HA with HA binding proteins regulate many aspects of cell behavior such as migration, cell-cell adhesion, differentiation - a phenomena, well documented in developing, regenerating, remodeling tissues and in tissues undergoing malignant tumor cell invasion (Knudson and Knudson, 1993). HA serves either as a hydrated matrix through which cells migrate or as a surface on which cells roll, using the HABPs to grip HA. The family of HA binding proteins was termed as hyaladherins (Toole, 1990). Among hyaladherins, CD44 and the receptor for HA mediated motility (RHAMM) have been identified in aortic smooth muscle cells (ASMC) (Turley et al, 2002). Also, HA-CD44 complexes have been reported to trigger signaling cascades that modulate inflammation and tumor progression (Heldin et al. 2008). High molecular weight HA independently activates RhoA and Rac through CD44 and RHAMM, respectively. HA-induced migration depends exclusively on RHAMM-mediated PI3K-dependent Rac activation (Gouëffic et al., 2006).
D'Souza and Datta (1986) for the first time reported the presence of a novel naturally occurring HA binding protein in rat liver, purified through HA affinity chromatography. Amino acid analysis showed that the protein is rich in glutamic acid and glycine and it is a sialic acid containing glycoprotein. Gupta et al. (1991a) purified this protein from rat kidney and showed that this native 68kDa glycoprotein is composed of 34 kDa subunits. Monospecific anti-HABP1 antibodies confirm its novel nature, as it does not cross react with other ECM proteins like laminin, fibronectin or collagen type IV. Using rat histocytoma, Gupta and Datta (1991) demonstrated for the first time, the involvement of this protein in cell adhesion and solid tumor formation. This protein is localized on the cell surface of human fetus lung fibroblast and is secretory by nature (Gupta et al. 1991). Babu et al. (1991) further showed that 34 kDa HABP1 is phosphoprotein in nature. This 34 kDa HA binding protein 1 (HABP1) is one such protein which has been identified from human fibroblast cDNA, sequenced and overexpressed from our laboratory (Deb and Datta, 1996). It is a multifunctional protein, and sequence search analysis of cDNA showed its complete homology with cDNA sequence of p32, a protein co purified with pre-mRNA splicing factor SF2 (Krainer et al., 1991) and gC1qR, a receptor of globular head of C1q, the first component of classical complement pathway (Ghebrehiwet et al., 1994), represented as synonym in human genome. This 34 kDa HA binding protein is present in fibroblasts, macrophages, lymphocytes, epithelial cells and plasma cells and is present ubiquitously in all types of tissues. This protein is more phosphorylated in transformed cells than in normal ones and mediates HA-induced cellular signaling. The phosphorylation of this protein is enhanced with PMA, calreticulin A, and Ca\textsuperscript{2+} ionophore, confirming its role in the cellular signaling cascade. The role of this novel HABP was established in reproduction, by Ranganathan et al., (1994). The differential localization of this protein on the sperm head, mid piece and tail of different organisms has been observed. It is involved in sperm maturation, motility and fertilization process.
The crystal structure of trimeric HABP1/p32 shows asymmetric charge distribution on the surface of HABP1, suggesting its possible role in maintaining the quaternary structure through protein-protein interactions (Jiang et al., 1999). Apart from HA, different laboratories have reported different proteins interacting with this protein. Starting from interacting with splicing machinery, it has been reported to interact with different proteins involved in immunological pathways, different types of viral proteins as well as signaling molecules. Given the wide array of plasma, viral and nuclear proteins that interact with p32/HABP1, it clearly represents a multi-functional binding protein. HABP1 has a substantial no. of ligands like C1q (Ghebrehiwet et al., 1994), HK(Dedio and Muller-Ester, 1996; Herwald et al., 1996; Joseph et al., 1996; Lim et al., 1998), Vitronection (Lim et al., 1996; Lim et al., 1998), HA (Deb and Datta, 1996; Gupta et al., 1991), Fibrinogen (Lu et al., 1999) and Factor XII (Joseph et al., 1996; Lim et al., 1998), that are present in the ECM. For HABP1 to interact with these ligands, it should be either localized on the extra cellular side of the plasma membrane or in the ECM.

Gupta and Datta (1991) and Deb and Datta (1996) have shown that HABP1 interacts with HA and plays an important role in tumor cell adhesion. Furthermore, Gupta et al. (1991a) have shown the surface localization of HABP1 in fibroblast cells using immunoflourescence techniques. They have also shown the secretory nature of this protein. Rao et al. (1997) demonstrated that HABP1 specifically transduces the HA signal and that leads to PLC-γ phosphorylation and IP₃ formation. Apart from gC1q, HABP1/gC1qR binds to several other serum proteins.

The involvement of HA in different stages of carcinoma is well documented. The differential expression of HABP1 during progression of epidermal carcinoma has already been reported from our lab (Ghosh et al., 2004). Also, the ability of C1q to interact with various types of vascular endothelial cells leading to the production of biologically active proteins or expression of adhesive molecules has also been well
documented (Peerschke et al., 1996). Including gC1qR as a novel vitronectin-binding protein, integrins α1β3 and α5β5 expressed on endothelial and other cells have been implicated in cell adhesion and migration on vitronectin substrata (Preissner, K. T., 1991). Also, transmigration of human ovarian adenocarcinoma cells through endothelial extracellular matrix involves αv integrin and the participation of MMP2 (Leroy-Dudal J. et al., 2005).

It has been reported that the MT1-MMP cleavage sites were located within the N-terminal part of the mature gC1qR molecule. The N-terminal sequencing of the 17 kDa fragment indicated that MT1-MMP cleaved the mature gC1qR protein at the PSQG79Q80KVE site, providing the 17 kDa fragment with the N-terminal QKVEEQE sequence. In the known crystal structure of the doughnut-shaped gC1qR (Jiang et al., 1999), the PSQG79Q80KVE segment represents a highly disordered 68PTFDGEEEPSQGQKVEEQEPE88 part of the loop connecting the β3 strand to β4 strand. The surface location of this structurally disordered loop explains its sensitivity to proteolysis (Razanov, D. V., et al., 2002). Since cleavage site of MT1-MMP resides in HABP1 as PSQG79Q80KVE sequence, reduction in HABP1 level in metastasis in carcinoma was related to its degradation by MT1-MMP (Ghosh et al., 2004). Therefore, HABP1, present both as an ECM and cell surface protein may have some regulatory role to play in cell adhesion, growth and chemotaxis, in association with integrins and MMPs.

It has been shown that interaction of endothelial cells with C1q induces a diversity of functions, including adhesion and spreading (Peerschke et al., 1996). It has also been shown that C1q mediated cell adhesion and spreading requires the cooperation between C1q receptors and integrins (Feng et al., 2002). Also, the functional roles of HA have been reported in B16F10 melanoma growth and metastasis (Mummert et al., 2003).
Having diverse cellular activities and being one of the members of WE family, HABP1 can interact with several cellular proteins and regulate different biological activities. Recently, the report confirms HABP1 as an interacting protein of lethal giant larvae (Lgl) protein, regulating cell polarity (Bialucha et al, 2007). Both overexpression and knockdown of HABP1 disturb normal polarization suggesting, that precisely controlled HABP1 level is required for normal cell polarization, which is in turn crucial for migration and epithelial morphogenesis. Defective cell polarity induces more invasive phenotype in malignant tissues.

The very basis for initiation of our investigation was the report, indicating HA as a regulator of growth and metastasis in B16F10 melanoma cells (Mummert et al, 2003). Therefore, knowing HA as an enhancer of cell migration in melanoma and also the involvement of its ligand HABP1 in regulation of cell polarity and its overexpression in carcinoma, the aim of the present study lied in unraveling the regulatory role of HABP1 in cell migration and the downstream signaling and hence its role in metastasis and tumorogenesis.

**In the present study the objectives were:**

- Initially to study the effect of HABP1 supplementation on cell migration and comparing the morphology of migrating melanoma cells with the normal cells. Also, in order to study the mechanism of action, the endogenous HABP1 localization profile in B16F10 cells was also to be studied and assess its involvement in the process of cell migration.

- To investigate the cell surface interactions of exogenous HABP1 and the downstream signaling pathways involved, including the activation of transcription factors and MMPs and actin polymerization at lamellipodia.

- Finally, to validate the *in vitro* data by *in vivo* studies.