FREBP - an anchorage dependent transcription factor

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To my Family...

Husband, Mom, Dad, Sister and Brother...

for all their love and support...

Always...

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INTRODUCTION

Thesis Organization

My work involves characterizing the properties of FREBP (basic Fibroblast Growth Factor Response Element Binding Protein) and identifying the signal transduction pathways that are involved in its regulation. In the introduction I have first outlined the role of extracellular matrix (ECM) in various tissue remodeling events. Then I have discussed about the ECM receptors, like integrins, and the various pathways that may be involved in the regulation of the FREBP. I have limited the scope of my discussion of specific molecules to their involvement in the signal transduction pathways relating to the ECM. A brief discussion follows this section that describes the ECM enzymes along with the important roles that they play in tissue remodeling events and cancer. The next section offers a brief introduction to mitogen regulated protein3 (MRP3), a bFGF regulated gene containing the basic-fibroblast growth factor response element (FRE) in its proximal promoter, regulation of gene expression by Fibroblast growth factor's (FGF's) and the AP-1 transcriptional element. MRP3 is a member of the MRP/PLF family of glycoproteins. Subsequently the dissertation consists of background information on FRE, the transcriptional element that binds to our transcription factor FRE-binding protein (FREBP). A brief summary of the work done on the FREBP to date follows. Currently purification of FREBP is underway in our lab with the results looking very promising. Finally the document describes in relative detail the experimental methods, results and a brief discussion of the study done in order to characterize this transcription factor in the laboratory.

ECM and tissue remodeling

Our understanding of the importance of the extracellular matrix (ECM) has grown over the past 15 years from it being viewed as primarily a structural support mechanism for the cell to now being demonstrated to play a major role in other processes like growth factor regulation. The ECM has also been shown to modulate growth factor binding proteins and the initiation of signal transduction pathways that control gene expression by interacting with cell surface receptors. The combined activities of the molecules within the cell and the molecules in the ECM have a major effect on the behavior and functioning of the cell. The different ECM components like collagens, fibronectin, fibrinogen, laminin and plasminogen interact with the various cell surface receptors within the cell like integrins, cadherins, Axl, ephrins, immunoglobulins and selectins. These interactions could lead to upregulation or downregulation of various signal transduction pathways like the MAP kinase pathway, PI-3 kinase pathway and FAK pathway resulting in different outcomes from apoptosis to cell differentiation [1]. ECM remodeling is an integral part of many physiological phenomenon like wound healing, differentiation during development, neovascularisation, mammary gland and uterine changes during the menstrual cycle and trophoblastic changes during pregnancy. Some pathological conditions where the ECM plays a major role are inflammation, degradation of bone and joints, autoimmune diseases and cellular proliferation in cancer. Clinical and basic research on the development of cancer, its metastatic behavior and the subsequent activation of the metastatic cascade at other places have established that there is an active involvement of the ECM and its various adhesion molecules in cancer [2]. Due to their varied impact on numerous cellular functions and their ability to influence the

intracellular milieu, it is extremely important to define methods to control the functioning of ECM proteins within the cellular environment.

ECM receptors: integrins

Some of the cell surface receptors that interact with the ECM are the integrins, cadherins, Axl, ephrins, immunoglobulins, selectins and the Notch family of proteins [3]. The main receptors that are associated with the ECM are from the integrin family, which are transmembrane glycoproteins that influence intrinsic and extrinsic events of cellular behavior [4]. All integrin receptors consist of heterodimers. The alpha and beta subunits consist of an amino-terminal extracellular domain, a transmembrane domain and a short cytoplasmic domain. These subunits can associate in multiple combinations [5]. Interestingly, these receptors can act in both directions and are able to transmit signals from the inside to outside of the cell and vice versa [6] [7]. Integrins are expressed under different physiological conditions but this expression varies considerably with different cell types [8].

A variety of proteins are identified as integrin ligands namely the collagens, fibronectin, fibrinogen, laminin and plasminogen [9]. Each subunit of integrin receptors can bind to different ligands. These ligands have been categorized on the basis of their binding specificity with respect to different integrin receptor subunits and their respective functions. Beta subunit cytoplasmic domain binding ligands include the actin-binding proteins like talin, filamin and myosin; signaling proteins like FAK and ILK; as well as other adaptor proteins like Grb2, paxillin, Shc and caveolin-1. A second group of ligands binding to the

alpha subunit cytoplasmic domain consist of calreticulin, caveolin-1 and F-actin [10]. Previously, certain cell surface receptor types were believed to respond exclusively to particular ligands. For example tyrosine kinase receptors respond to growth factors. Now there is evidence that ECM molecules, like collagens, are also ligands for certain tyrosine kinase receptors [11]. There also exists considerable evidence of interaction and cross-talk between growth factor and ECM regulated pathways.

There are a variety of cell responses that ECM receptors regulate including cell spreading, proliferation, migration, differentiation and progression through the G1 phase of the cell cycle [12], wound repair, angiogenesis, the inflammatory response, apoptosis, and gene expression [13]. Interaction of integrins with the ECM is known to have an effect on their half lives and the adhesion-dependent signals that they transmit [14]. Integrins associate with their ECM ligands and some other adaptor proteins, bringing about a change in the actin cytoskeleton to produce large protein complexes called focal adhesions, so named because they form and accumulate as a result of cell adhesion. These focal adhesion complexes are aggregations of many proteins that have signaling initiation properties [15]. Formation of these focal adhesion complexes initiates phosphorylation of tyrosine residues on certain signal transduction molecules like Src kinase, phosphatidyl inositol-3 Kinase (PI3K), focal adhesion kinase (FAK) [16] as well as ERK1/2 and MEK from the MAP kinase pathway [17]. Recent studies have shown that the cell can also regulate focal adhesion assembly following integrin mediated cell adhesion [18]. In the later part of my discussion, I will elaborate in detail the mechanism and various methods involved in the activation of these molecules and also the different pathways in which they participate.

The integrins closely interact with the ECM to play a significant role in tissue remodeling processes like wound healing and development [19]. During these processes integrins not only facilitate attachment of the cells to the matrix but, under stressful conditions, they compensate for the negative effects of low level of growth factors. This interaction plays a very important role in defining the behavior of normal cells and provides valuable discriminatory criteria between the normal cells and the cancerous/neoplastic cells [20]. The ability of integrins to act in this way is mostly attributed to their ability to activate upstream molecular pathways via activation of nuclear receptors and second messenger systems that initiate downstream signaling mechanisms, which influence transcription, gene expression and protein synthesis [21]. These cascades establish links between the cell's outer environment and its inner milieu [22]. Furthermore it is evident that among all adhesion molecules the role of certain integrins has been specifically identified in cell spreading, proliferation, migration, differentiation [12] as well as oncogenesis [23], whereas the expression of integrins in cell lines and tumor tissues correlates directly with the development of cancerous pathology [24]. For example, in Chinese hamster ovary cells there is an inverse relation of the expression of integrin proteins like β and $\alpha5/\beta1$ [25] and the development of the tumorigenic phenotype [26]. Studies have also shown an association between lower expression of other integrins like $\beta1$, $\alpha V\beta3$ and $\alpha6\beta1$ and cancerous pathologies of ovarian carcinoma [27] and colorectal cancer [28]. Osteosarcoma cell lines over expressing $\alpha5/\beta1$ showed increased cell adhesion to type I collagen and increased cell migration and invasion rate compared to the wild type cells [29]. In two-dimensional cell migration assays using mouse mammary carcinoma cells, antibodies against $\alpha1$, $\alpha2$, $\alpha6$ and $\beta1$ decreased cell invasion on a reconstituted basement membrane, against $\alpha6$ and $\beta1$

decreased cell locomotion and against $\beta 1$ interfered with cell adhesion to basement membrane [30]. There have also been studies showing a direct effect of extracellular matrix attachment on gene expression. For example, cultured primary mammary epithelial cells show an increase in expression of the genes encoding alpha-casein, beta casein, gamma-casein and transferrin in response to ECM attachment [31] [32]. Together these data indicate that it is of utmost importance to outline the different pathways that are stimulated under these conditions especially focusing on identifying key players that could be used as a site of inhibition for cell processes like movement, proliferation and angiogenesis during cancer treatment and could aid in a better therapeutic control of numerous cancer forms and their metastasis.

The hallmark of cancerous cells in-vivo is their ability to survive and proliferate and move to different sites within the body mainly by overcoming the need of integrin mediated adhesion or anchorage dependence [33]. These intercellular and cell to ECM attachments and their combined interactions are well known to play a very important role in making cells invasive and malignant [34]. The role of integrins and the various signal transduction pathways activated by them like the PI-3 kinase pathway, play a role in the regulation of cellular functions like tumor development and progression. The integrins through their interaction with the cytoskeletal components like laminin and type I collagen can initiate signals via tyrosine kinases that effect several other growth regulatory cascades. The activation of the Rho-family of small GTPases acts on members of the MAP kinase pathway like Ras and regulate cell signals like cell growth, motility, survival and invasiveness [35-37]. More deaths are caused by the secondary metastasis of a particular type of cancer rather

than the primary itself and generally by the time the primary tumor is detected it is too late for any curative measures. Cancer metastasis is facilitated by a joint effort between cell adhesion by integrins and matrix degradation by different proteases [38]. This important phenomenon of metastasis is of most concern in cancer and now, with more recent and detailed data, integrins have been established as playing a important role in metastasis [39].

ECM regulated pathways

Integrins are vital in the different growth factor signaling pathways [40]. Integrins are known to affect biological processes similar to those affected by growth factors like cell migration and programmed cell death. [41]. Oncogenic transformation of cell lines like rat1, NRK and NIL8 by Rous sarcoma virus or murine sarcoma virus leads to lower expression of the $\alpha 5\beta 1$ integrins and reduced ability to establish fibronectin assembly. These transformed cells demonstrate remarkable preponderance to the development of cancerous changes indicating a close co-relation between the protein expression and disease development [42]. There is extensive cross talk between integrin and growth factor receptors that is accomplished either by their clustering together on the cell membrane or by interaction of proteins or signals at different points downstream in the signal transduction pathways initiated by these receptors [43]. This cross-talk is further established when the same signal transduction pathways are initiated by both integrins and growth factors like the MAP kinase, PI-3 kinase and Focal adhesion kinase [44] [45]. The discussion that follows elaborates on these pathways in more detail.

The main pathways that will be discussed herein are the mitogen activated protein kinase (MAPK), focal adhesion kinase (FAK) and phosphatidyl inositol-3 kinase (PI-3 K) pathway [46]. All these pathways are activated as part of the cellular processes and biological phenomenon that I am studying and some of their molecules are known to be very important players in these processes. From research already done we know that there are common molecules like Src kinase, PI-3 kinase, MEK, FAK, Ras and Rho family of GTPases that participate in several of these pathways and regulate various cell functions like growth, spreading, migration, survival, proliferation, apoptosis and invasiveness [47]. Most of these molecules are associated with, and activated in, different cancers and some of them are also being used clinically as inhibitors along with other therapeutic inhibitors.

The mitogen-activated kinases are cytosolic serine/threonine protein kinases that participate in several signal transduction pathways that function in the same cell and regulate different cellular events like cell proliferation, survival, motility, death and gene expression [48]. Our focus is the prominent RAS-RAF-MEK-ERK pathway or MAP Kinase pathway that is stimulated by growth factors [49, 50]. Activity of the MAPK pathway can also respond to integrin mediated cell adhesion to the ECM under specific conditions, specifically enhancing ERK activity [51]. Hence, integrins function together with growth factor receptors to activate the ERK-1/2 MAPK pathway [52].

The events of the conventional MAP kinase pathway, when activated in response to mitogens or the ECM, begin with the activation of Ras followed by the GTP bound Ras binding to Raf Kinase further leading to its cellular membrane translocation and finally phosphorylation and activation of Raf (MAPKKKinases). Active Raf then phosphorylates

and activates MEK (MAPKKinase) [17], which in turn activates extracellular regulated kinase {ERK1/2 (MAP Kinase)}. Active ERK plays a important role in adhesion dependent cell cycle regulation in response to activated integrins [53]. ERK1/2 lack a nuclear localization signal but can further translocate to the nucleus and phosphorylate transcription factors like Elk and CREB and thereby affect gene expression [54, 55].

There are different mechanisms by which the MAP kinase pathway can be affected and regulated by the integrins. We will see how these different mechanisms involve molecules participating in the MAP kinase pathway that are capable of being activated by integrins. Raf, unique from the other members, can be directly activated as a result of cell adhesion by integrins, which bypasses the need for Ras activation of this protein kinase [56]. Integrin-dependent adhesion affects both the members downstream of Raf, ERK and MEK. ERK, a key player in cell division, migration, and survival is affected at several checkpoints by integrin mediated anchorage-dependence [57] The different mechanisms proposed to explain the direct activation of ERK by integrins are tyrosine phosphorylation of FAK, activation of p21-activated kinase by its two regulators Rho-family GTPase Rac and SH2/SH3-adaptor protein Nck [58]. MEK seems to be a focal point in the cell's decision to grow and migrate and can also be regulated by integrin attachment. This activation does not involve the activation of Ras and Raf and can be initiated directly by integrins [15]. Regulation of MEK is important for cell adhesion as constitutively activated mutants of MEK in NIH3T3 cells can induce anchorage independent growth [59]. However, the exact molecular pathways by which MEK regulates these events have yet not been elucidated [60].

The integrins and their cytoskeletal ligands also play a very important role in the nucleocytoplasmic distribution and trafficking of proteins that have a signaling potential. It is now being established that this control by cell adhesion molecules has a broad impact on the cell's ability to make decisions and also effects gene expression, thus establishing a communication between the ECM and the nucleus. For example the Jun activation domain binding protein (JAB)-1 binds to the $\beta 2$ cytoplasmic domain of the lymphocyte function-associated antigen (LFA-1), a member of the $\beta 2$ integrin family. JAB-1 is a co-activator of the transcription factor C-Jun and can enhance activation of AP-1 driven promoters [61]. Cell adhesion also especially effects the nucleocytoplasmic distribution of the MAP kinases [62]. Translocation of ERK from the cytoplasm to the nucleus requires its phosphorylation and homodimerization [63] and is also cell adhesion dependent [55]. The factors aiding this translocation in addition to the GTPase Ran, which is a inherent requirement [64], are still under study. However, once translocated to the nucleus, ERK is known to phosphorylate ELK-1, which in turn regulates the serum response factor (SRF) [65].

The MAPK pathway is activated in prostate cancer [66] and is believed to be highly involved in the development of breast cancer. Increased expression of activated MAP kinases has been demonstrated in various breast cancer tissues by using immunohistochemical techniques and enzymatic assays. Hormones like estradiol, progesterone and testosterone can bind to their membrane associated receptors and increase the activity of MAP kinases [67]. This pathway and its molecular components have been well established as playing a central role in regulating different aspects of tumor development. When activated, some members of the MAPK pathway individually have cell transforming potential [68]. Ras is a known

oncogene, implicated in the development of tumors like multiple myelomas [69]. Studies done with mutated Raf, have shown its correlation with tumor development experimentally and clinically [70]. In addition, active MEK has also been shown to cause cellular transformation [59]. Finally ERK1/2 were found to be constitutively active in many tumor cell lines. For example from cell lines derived from epithelial and granulose cell ovarian tumors. The expression of MAPK phosphatases-1 (MPK-1) was down regulated in these cell lines. Silencing ERK 1/2 protein expression by using RNA interference completely suppressed tumor cell proliferation [71]. ERK 1/2 have also been shown to mediate the increased invasiveness in prostate cancer cell invasiveness via the G-protein-coupled P2Y purinoceptor [72].

It is now well-established that the MAP Kinases play a role in regulating transcription of genes by acting on different transcription factors such as Elk-1, CREB, MEF2A, GATA-2, Rsk1, AP-1 etc. [73]. For example, once activated, Rsks, a MAPK activated kinase, phosphorylates BAD (a proapoptotic protein) at serine 112 and CREB (cAMP response element binding protein) at serine 133. Phosphorylation of BAD by Rsks suppressed BAD-mediated apoptosis in neurons and phosphorylation of CREB promotes cell survival [74]. These transcription factor-dependent mechanisms mediated by the MAP kinase pathway play a very important role in the expression of several genes and thus regulate many physiological processes that involve cell decisions like apoptosis or survival. Because of its central role in regulating integrin signaling and gene expression, the MAP Kinase pathway is a focus of research for developing gene therapy interventions that target specific molecules such as Ras, Raf and MEK to accompany drug therapy [75].

The FAK pathway is the second protein kinase signal transduction pathway under focus in our discussion. FAK is a 125 kDa protein tyrosine kinase containing a central catalytic domain flanked by N-terminal and C-terminal non-catalytic domains [76]. The 150 residue C-terminal domain also called the "activation loop" contains multiple binding sites for SH3 domain containing signaling proteins like $p130^{cas}$, GTPase activating protein GRAF for RhoA and Cdc42, HEF-1 and PI-3 kinase. The site of phosphorylation in this C-terminal regulatory region is the $Y^{576/577}$ [77]. The C-terminal domain also contains the focal adhesion targeting (FAT) sequence, a stretch of 159 amino acids essential for the sub-cellular localization of FAK with integrins [78]. FAK is activated in many biological responses like cell motility, cell survival and FAK is known to play an active role in cell cycle progression [79-81]. Its activity is increased in many different human cancers like oral [82] and ovarian cancer [80]. Prostate carcinoma cell lines having overexpressed FAK show increased migration [83]. FAK is proposed to be involved in tumor invasion and metastasis [84].

The exact mechanism of FAK activation is still not well understood although several types of interactions and associations between certain adaptor proteins and FAK in response to integrins have been proposed [85]. The role of adaptor proteins is already well established in ECM regulated pathways and FAK plays a very important role in this ECM pathway regulation as we will see in the discussion that follows [86]. Adaptor molecules like Grb-2, c-Src bind to FAK in response to integrins that resulting in FAK activation [87]. A popular proposed mechanism for the activation of FAK is the autophosphorylation of FAK at tyrosine-397 in response to integrin activation. The tyrosine-397 is the site of association of two Src family kinases, the pp60src and the pp59fyn with tyrosine-phosphorylated forms of

FAK. In Src-transformed chicken embryo cells most of the pp125FAK was in the form of a complex with pp60src [88]. Once activated as a result of phosphorylation by FAK, Src can phosphorylate various signaling molecules such as Tensin, Paxillin, Cas, ERK, Ras, PI-3 kinase etc. [89, 90]. Another hypothesis suggests that Grb2 is directly activated by FAK and Src through phosphorylation of Shc, an adaptor protein that gets activated once it binds to phosphorylated Tyrosine-397 of FAK [91]. Grb2 binds phosphor Shc [92, 93]. FAK can also colocalize with integrins at cell-ECM contact regions resulting in changes in its tyrosine kinase and phosphorylation state [94] and can also indirectly connect to beta1 integrins via molecules like paxillin and talin [95]. Activated FAK can also associate with other cytoskeletal molecules like $p130^{Cas}$ (Crk associated substrate), paxillin, Tensin, Rho, Cdc42, Ras and ERK from the MAP kinase pathway [96] [97]. FAK gets dephosphorylated by protein tyrosine phosphatases like PTEN [98] and SHP-2 [99], which effect the cells behavior directly by reducing its motility [100].

The relationship of FAK with the MAP kinase pathway is well established. FAK associates with adaptor proteins like Grb2 and Sos and activate members of the MAP kinase pathway like ERK1/2 in response to stress activation [101]. This interaction between FAK and ERK regulates the MAPK pathway in response to integrins [102]. For example over expression of FAK resulted in stimulation of ERK in response to integrin attachment to fibronectin and dominant-negative constructs of FAK in the same study inhibited ERK stimulation [103]. The relationship between ERK and FAK was further established when it was shown that FAK deficient cells were deficient in integrin mediated activation of ERK [104].

The association of FAK with cancer has been attributed to is ability to control certain cancer phenotypes [105]. FAK is required for certain tumor cells to adhere to the substratum, to proliferate [106] and its overexpression is observed in oral [107], ovarian [80], breast, thyroid [108] squamous cell carcinoma of larynx [109] and prostate cancer [81]. FAK has a special predisposition for expression in certain invasive tumors [110, 111]. The direct effect of FAK over-expression is stimulation of cell spreading and migration [112], which is demonstrated in the cells of FAK-deficient mice that show reduced cell motility and enhanced cellular adhesions [113].

Finally we discuss the third pathway under our focus, the PI-3 kinase pathway. The PI3-kinase is a heterodimeric lipid kinase comprising of two subunits: an 85 kDa regulatory subunit containing SH2 and SH3 domains (p85) and a 110 kDa catalytic subunit (p110). This pathway plays a major role in cell survival processes in response to growth factor and extracellular attachment to the substratum like cell cycle progression, apoptosis and neoplastic transformation [114]. PI-3 kinase is expressed in invasive tumors [115] and its involvement is shown in the development of mammary carcinogenesis [116]. In cells expressing oncogenic variants of the *abl* proto-oncogene the activity of PI-3 kinase was increased [117]. PI-3 kinase is activated in response to ECM receptor activities like integrin-mediated attachment [118]. It associates with FAK in response to integrin attachment and phosphorylates FAK at Y397 [119]. Thereby, it regulates the ability of FAK to regulate cell migration. This interaction was demonstrated by doing site-directed mutagenesis studies. In these studies it was shown that creating a FAK point mutation proximal to Y397 abolishes the ability of PI-3 kinase but not Src kinase to bind to FAK. When these mutants were

overexpressed in CHO cells, FAK was unable to promote cell migration. They also further confirmed this finding by showing that the PI-3 kinase inhibitors LY294002 and Wortmennin inhibited FAK promoted migration in a dose dependent manner [120]. PI-3 kinase has also been known to be activated in response to E-cadherin receptors that are known to play a role in many biological processes similar to the ones in which integrins are involved like cell growth, development and differentiation [121].

Activated PI-3 kinase phosphorylates the membrane lipid, phosphatidylinositol-4, 5-bisphosphate (PtdIns-4, 5-P2), to form phosphatidylinositol-3, 4, 5-tri-phosphate (PtdIns-3, 4, 5-P3) [122]. Inositol phosphates activates other enzymes [123]. For example p70S6 kinase is activated by PDK1, which in turn gets activated by IP3,4 [124]. PKB/AKT is directly activated by IP3,4 and other IP phosphorylated derivatives [125]. Another important molecule that is phosphorylated by PI-3 kinase is the ERK1/2 from the MAP kinase pathway [126, 127]. In addition to its activation of ERK1/2, PI-3 kinase also plays an important role in activation of Ras, another member of the MAP kinase pathway and can affect the ability of Ras to mediate transformation of cells [128, 129]. Hence the PI-3 kinase pathway, once activated, can have its effects independently or work by regulating other pathways like the MAP kinase pathway.

ECM enzymes: matrix metalloproteinases

The matrix metalloproteinases (MMPs or matrixins) are the most important enzymes involved in the ECM degrading events during all tissue remodeling processes [130]. These are multidomain, zinc and calcium dependent proteases whose substrates are certain ECM

molecules and the inactive MMP zymogens [131]. The known members of the MMP protein family include the Collagenases: {Collagenase 1 (*MMP-1*), Collagenase 2 (*MMP-8*), Collagenase 3 (*MMP-13*), Collagenase 4 (*MMP-18*)}; Gelatinases: {Gelatinase A (*MMP-2*), Gelatinase B (*MMP-9*)}; Stromelysins: {Stromelysin 1 (*MMP-3*), Stromelysin 2 (*MMP-10*), Stromelysin 3 (*MMP-11*)}; Membrane Type Metalloproteinases (MT-MMPs): {MT1-MMP (*MMP-14*), MT2-MMP (*MMP-15*), MT3-MMP (*MMP-16*), MT4-MMP (*MMP-17*), MT5-MMP (*MMP-24*)}; Others: {Matrilysin (*MMP-7*), Metalloelastase (*MMP-12),* Enamelysin *(MMP-20*); Not named (*MMP-19*), and not named *(MMP-23*)}[132]. The MMPs are produced by a variety of cell types and their production is regulated by growth factors and cytokines that activate the genes *via* downstream transcription factors like AP1, NFkB etc. All MMPs are secreted as latent zymogens that are inactive because of the binding of the conserved cysteine (cys-73) to a zinc molecule [133]. The mechanism of activation, which is considered universal for most of these enzymes, is termed the "cysteine switch" and, as demonstrated with HFC (human fibroblast collagenase), involves the dissociation and replacement of the cys-73 ligand of the zinc molecule with water, thus turning the inactive zinc to an active form [134]. Generally pro-MMPs and tissue inhibitors for MMPs (TIMP's) are secreted together and this maintains the delicate balance of the cell in its physiological state [135]. The pro-MMP's can be activated *in vitro* by various means like action of different proteases like trypsin, chymase and plasmin or of other MMPs like MMP-2, MMP-7, MMP-13 and MMP-3 and also by the action of different proteolytic mechanisms functional at cell-surface, intracellular or extracellular level. *In-vivo* their regulation occurs at different levels ranging from gene transcription to enzyme activation and inhibition.

The MMPs play an active role in tissue remodeling events such as wound healing [136], trophoblastic implantation [137], embryogenesis [138], bone resorption and morphogenesis [139]. MMPs have also been further implicated in pathological processes [140] like arthritis [141], multiple sclerosis , osteoporosis, Alzheimer's [142] and tumor metastasis during cancer [143]. The physiological roles of the MMPs will be discussed in detail in the following discussion. Almost all the events during wound healing are directly or indirectly interdependent and related to the proper functioning of the MMPs like cell migration, angiogenesis, degradation of provisional matrix consisting of fibrin and plasma proteins and remodeling of newly formed granulation tissue [144-146]. During angiogenesis MMPs help endothelial cells penetrate several components of the ECM like the subendothelial basement membrane, fibronectin containing interstitial matrix and laminins that form a integral part of the extracellular matrix [147]. MMP's like MMP-9 and MMP-13 play a important role in skeletal tissue remodeling by recruiting cells like osteoclasts, chondroclasts, osteoprogenitors and endothelial cells necessary for bone morphogenesis [148]. The MMPs have also been shown to play a role during reproductive events such as ovulation, corpus luteum formation/regression, implantation, lactation [149] and mammary and uterine involution [138, 150]. Maintaining the correct balance between ECM production and degradation during tissue remodeling is very important because excessive production can lead to formation of keloids (hypertrophic scars) and excessive degradation can lead to formation of chronic ulcers [151].

MMPs are expressed in numerous pathological conditions including collagen and bone disorders like osteoporosis, osteoartheritis, chondrodysplasias, osteogenesis imperfecta,

certain renal disorders, cancers and other diseases [152] [153]. During cancer MMP's in addition to playing a role during invasion and metastasis, now MMP's are known to play a role in cancer initiation and promotion [154]. Expression of collagenase in transgenic mice increases its susceptibility to treatments with initiator (7,12-dimethyl-benzaanthracene) and promoter (12-O-tetradecanoylphorbol-13-acetate) and subsequent development of skin carcinogenesis [155]. Upregulation of various growth factor signaling pathways during cancer promotion can lead inturn to the activation of different mmp genes. For example, in colon cancer cell lines EFG upregulates the expression of MMP-7 via the activation of PEA3 transcription factor [156]. Helicobactor pylori strains that posses the cag pathogenicity island (cag+) have a greater risk of distal gastric carcinoma formation. Differential expression of mmp-7 was seen from H. pylori cag (+) isolates from human gastric mucosa and this was suggested to cause the preponderance in H. pylori cag (+) persons to the development of gastric carcinomas [157]. They are secreted by cancer cells and their surrounding normal stromal cells and can degrade ECM and inactivate proteinase inhibitors. All these abilities have established them as playing a key role in cancer development [158]. The roles attributed to MMPs include degrading ECM, digesting basement membranes and invading blood vessels, when combined together results in leading to rapid tumor metastasis [159, 160].

Generally, MMPs are expressed in cells that are differentiating [161] or that demonstrate an invasive phenotype [162, 163]. Although their patterns of expression must be very strictly regulated, we do not know much about their expression patterns and how expression is regulated *in vivo* [164]. Stromelysin-2 is upregulated in normal wound healing in response to

growth factors [165]. MMP-12 is expressed by tumor cells in skin cancer [166], and MMP-3, 7,10, 11 and 14 and TIMPs-1, 2, 3 and 4 were expressed in head and neck squamous cell carcinoma and not the surrounding tissue [167]. MMP expression can directly affect the development of a pathological condition or could indirectly contribute towards it. For example MMP-11 (stromelysin-3) even though not directly associated with tumorogenicity studies has been shown to increase the availability of growth factors to the cells [168]. The expression of certain MMPs like stromelysins and gelatinases is increased in tumor metastasis and progression [169].

The MMPs play multiple roles in tumor invasion and metastasis and numerous MMPs are involved in ECM remodeling events with the pattern of expression being different for different MMPs [170]. For example, MMP-9 is expressed during bone development, the hair follicle growth cycle, neurogenesis, and tissue repair [171] [172] [173]. It is also expressed in gliomas, especially the malignant and invasive types [174]. MMP9 activation also enhances tumor cell intravasation [175] and tumor progression and invasion [176, 177]. The 94-kDa type IV collagenase is expressed by eosinophils during basal cell carcinoma development [178] and in wound healing by basal keratinocytes [179, 180]. Expression of matrilysin, collagenase and stromelysin1 is increased in gastrointestinal tumors [181]. MMP-7 expression is increased during colon cancer [182], MMP-13 expression was seen in carcinoma of the urinary bladder [183] and MMP-3 expression is associated with development of mammary carcinoma [184]. Stromelysin-1 acts as a natural tumor promoter [185] and expression of the rat homologue of stromelysin, transin, is correlated with tumor progression [186]. All MMP's were found activated and expressed in samples from breast

cancer [187] . MMPs are also known to play a important role in recruiting osteoclasts and thus making the bone the most common site for secondary carcinoma development [188].

The integrins and their ligands, like fibronectin, regulate the expression of several MMP genes [189]. Alpha1 and alpha2 integrins are involved in the development of invasive behavior of mouse mammary carcinoma cells through the regulation of stromelysin-1 [30]. MMP-2 has been localized to the surface of tumor cells and shown to be associated with the integrin alphaVbeta3 [190]. Integrin alpha2beta1, through its interaction with collagen 1, regulates functions like cell adhesion and invasion in osteosarcoma [29] cell line. Alpha5beta1 and alpha4beta1 integrins regulate MMP expression cooperatively in fibroblasts [191]. Type IV collagenase expression was induced by using anti-integrin antibodies in keratinocytes [192]. Integrin alpha2beta1 regulate MMP-1 gene expression [193].

The MAPK pathway is believed to regulate MMP gene expression through activation by the integrins [194, 195]. For example in human skin fibroblasts induction of MMP-13 along with activation of MAP Kinase pathway was seen in response to collagen [196]. Membrane type 1 MMP regulates activation of MAP kinase pathway in response to collagen [197]. Activation of MAP kinase pathway, especially MEK, was shown to be the focal point in the activation of MMP-2 [198] and MMP-9 genes [199]. In ovarian cancer cells MMP-9 was activated via the MAP kinase pathway [200].

From the studies above we can say that the role of MMPs and their inhibitors in relation to normal and abnormal functioning of our body is very important and identifying molecules that could be participating in this regulation will be very helpful to broaden our

understanding [201]. Now, therapeutically and clinically, the focus on MMPs has increased with the development of new MMP inhibitors. Some inhibitors are already being used as drugs in different diseases like certain connective tissue disorders [202], while others are still in different phases of clinical trials [203]. Some of them are being used as drugs in cancer as supplemental therapy and still others are undergoing development [204] [205].

Anchorage dependence

Cells respond to growth factors and extracellular matrix (ECM) individually or in combination with each other through their receptors. Integrins play a very important role in this cell-ECM attachment along with a specific condition called anchorage-dependence of growth [206]. Normal cells are anchorage dependent and when these normal cells are detached, they undergo anoikis, which is apoptosis caused by loss of adhesion to the substratum [207]. Various ECM receptors like integrins play a very important role in the maintenance of anchorage dependence [208]. Under normal conditions the important mitogenic signal transduction cascades like MAP Kinase, PI-3 Kinase, Src Kinase and Focal Adhesion Kinase require integrin-mediated cell adhesion to the substratum in order to be regulated and be functional [209]. Some molecules from these pathways have the capability of overcoming the need for substrate attachment under certain conditions and are capable of inducing anchorage-independent growth [210]. This property becomes especially important in tissue remodeling processes because the cells involved in these events show increased deviation from their normal anchorage requirement and by becoming anchorage independent they then acquire the ability to move from their original position to different areas. This is also very important in relation to conditions like cancer.

Tumor cells have the ability to be fully functional in suspension, overcome anchorage dependence or become anchorage-independent. Hence tumor cells remain viable and initiate various signal transduction pathways even while detached from the ECM [211]. Unraveling the complexities of this phenomenon will give us a better idea about the role it plays in tissue remodeling processes like wound healing and diseases like cancer and will give the clinician a better tool to treat these diseases. Anchorage can also effect the distribution of transcription factors between the cytoplasm and the nucleus. Outlining the key players like the receptors, the signal transduction pathways, transcription factors and genes that are regulated in response to anchorage dependence would be a big contribution towards conceptualizing and developing new therapies [212]. It would outline and establish certain molecular markers that could be used for new clinical and therapeutic intervention strategies in order to control and cure diseases like cancer.

Mitogen regulated protein/proliferin's

Mitogen regulated proteins/Proliferins (MRP/PLF's) are a family of glycoproteins that are secreted by cells in response to mitogen/growth factor stimulation [213]. MRP/PLFs can be produced by the following mouse cell lines: BALB/c 3T3 [214] [215], BNL [216], Krebs ascites carcinoma cells [217], 10T1/2 cells [218] and Ehrlich ascites carcinoma cells [219]. Swiss 3T3 cells make mainly PLF1 [220]. These cell lines secrete PLF1 in response to the following mitogens/growth factors: serum [213] [216], phorbol esters [221], basic fibroblast growth factor (bFGF) [213], epidermal growth factor (EGF) [222], transforming growth factor-alpha (TGF-alpha) [223]. The apparent molecular weight of MRP/PLF when unglycosylated is ~22kDa and when glycosylated is ~34 kDa [213]. The MRP/PLF's are

members of the Prolactin-Growth hormone superfamily [217]. There are four to six *mrp/plf* genes [224] [225] [226] and four *mrp/plf* cDNAs have been cloned. These are *plf1* [214] [217], *plf2* [227], *mrp3* [216] and *mrp4* [220]. The amino acid sequences of the proteins encoded by these genes are 91-98% identical and similar to each other [225].

Three promoters have been cloned for the three *mrp/plf* genes; *mrp3, plf1* and *mrp4*. The promoters of these three genes are very similar to each other between positions –576 to +65 with *plf1* promoter and *mrp4* promoter being 97% identical to each other [226] and *mrp3* being 98.4% identical to *plf42* and 97.4% identical to plf149 [225]. These promoters contain many active basal as well as regulatory transcriptional elements that may have a potential effect on *mrp/plf* gene expression *in vivo* like the TATA box, AP1 transcription factor consensus binding site [225], Sph elements [228], composite glucocorticoid response element (cGRE) [229] and several GATA recognition motifs like GATAG, GATT and GATAA [230].

Despite the similarities in promoter sequences, different *mrp/plf's* are expressed during various physiological events. All *mrp/plfs* (*plf1*, *mrp3* and *mrp4*) are expressed *in vivo* in the mouse placenta in the trophoblastic giant cells [231], with the expression being maximum at 9-13 day of gestation. This period of expression is also the period of rapid placental growth [232]. *Plf1* and *mrp3* are also expressed in the embryo and PLF1 and/or MRP3 proteins are found in the amniotic fluid and some parts of the embryo during development [233]. PLF1 and/or MRP3 but not MRP4 are found in the maternal blood. This may be because MRP4 is rapidly degraded or because it cannot cross into the maternal blood stream from the placenta [232]. *Plf1* is expressed in the stomach and small intestine. *Mrp3* is expressed during wound

healing in adults and the protein is found in the suprabasal keratinocytes at the wound site, in the outer root sheath of the hair follicle during the hair follicle cycle (late anagen phase) and a small amount is expressed in the small intestine [234]. *Mrp4* is expressed mainly in the hair follicle in late anagen phase of the adult mouse tail and ear [220]. *Mrp/plf's* have also been found in fetal heart and vertebral column [235].

Mrp/plf's bind to two cell surface receptors: The mannose-6-phosphate receptor in maternal and fetal liver as well as placental membranes [236, 237] and a second receptor from murine mammary and uterine preparations that has not been cloned [238]. The two proposed physiological functions for the MRP/PLFs are as growth factors and angiogenesis factors. MRP/PLFs are proposed to stimulate uterine and placental growth and, being produced by the placenta, thereby be a means of signaling between the placenta and uterus [238]. PLF1 has also been shown to stimulate endothelial angiogenesis and neovascularisation [239, 240].

Regulation of gene expression by the FGF's

Growth factors regulate cellular functions like cellular differentiation, proliferation and growth during embryogenesis. Their coordinated regulation in vivo plays a very important role in the development of the normal adult human being. Growth factors achieve their effects by activating signal transduction pathways that ultimately result in the changed expression of downstream genes and the proteins that they encode. Hence, to understand the physiological roles of the various growth factors it is important to identify the signal

transduction pathways and the key players involved in the regulation of their expression [241].

The fibroblast growth factor family is comprised of the 26 members identified to date. Some of them are as follows: acidic FGF (FGF-1), basic FGF (FGF-2), int-2 (FGF-3), k-FGF (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (FGF-7), FGF-8, FGF-9 etc. These growth factors are conserved among species and are encoded by distinct genes. Several members of the FGF family play a role in regulation of embryonic development [242, 243], differentiation of cellular phenotypes during muscle and limb development [244] and transcription of myogenic genes [245, 246]. FGF's are also expressed in the developing [247] and adult central nervous system [248] and have been described as being involved in angiogenesis during wound healing [249]. Several FGF's like FGF-3, FGF-4 and FGF-5 can behave as oncogenes and have transforming potential *in vitro* [250]. The expression pattern of each FGF differs during mammalian development, for example while FGF-4 is expressed during the early 4 cell stage, blastocyst stage and also during later stages of development [244], the other isoforms like FGF-3 and FGF-5 are expressed primarily in parietal and visceral endoderm before gastrulation until mesoderm formation [251] [252].

Evidence from numerous studies indicates the presence of several regulatory fragments that transcriptionally regulate FGF expression in the cells. FGF-4 is known to be regulated by a putative octamer-binding site along with other cis-regulatory elements [253], FGF-3 is regulated as a result of transcription from three possible promoter regions [254] while FGF-2 has 5 potential SP-1 binding sites consisting of the functional core of the promoter and one potential AP1 site [255].

The FGF's execute their biological and physiological functions through interactions with multiple FGF receptors [256] of which 5 different receptor genes have been cloned and sequenced including FGFR-1 (Flg, Cek-1) [257], FGFR-2 (Bek, K-SAM, TK-14, Cek-3) [258] [259], FGFR-3 (Cek-2) [260, 261], FGFR-4 [262] and FGFR-5 (Flg-2) [263] [264]. Alternative splicing has been known to generate several isoforms among these five receptors [265]. These receptors are heavily glycosylated and consist of an extracellular domain, short transmembrane domain and a cytoplasmic domain. Several of these receptors and their splice variants bind to the following ligands like FGF-1, FGF-2, FGF-4, FGF-5, FGF-6, FGF-7 with variable specificity [266] but receptors for FGF-3 have not yet been identified. The importance of FGF receptors to fetal development is evidenced by the observation that many human birth defects have been attributed to mutations in these receptors like craniocynostosis (premature fusion of the cranial sutures) [267] and other skeletal abnormalities like achondroplasia [268], hypochondroplasia and thanatophoric dysplasia [269].

FGF binding to its respective receptor leads to dimerisation, activation of the receptor's tyrosine kinase activity [270] and receptor autophosphorylation [271]. The main sites for FGF receptor autophosphorylation are two tyrosine residues, Ty-766 and Ty-653 [272, 273]. Activated FGF receptors can phosphorylate downstream substrates like PLC-γ1 and PI-3 kinase. Upon activation PLC-γ1 hydrolyses phosphotidylinositol that produces two-second messengers; inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) [274]. PI-3 kinase (p85) phosphorylates the D-3 position of inositol resulting in the production of a novel phospholipid, the phosphotidyl-inositol-3-phosphate (PI3P) [275]. The FGF receptors can phosphorylate other cellular proteins like Raf-1 [276], interact with membrane-associated

proteins like heterotrimeric G-protein [277] and increase transcription of genes like *c-fos* [278].

There are different forms of bFGF (also called FGF-2) with molecular weight ranging from ~18.5 to ~24kDa due to the presence of different translation start sites [279-281]. bFGF can be found in almost all cell types, organs, tissues [250]. bFGF regulates a variety of functions at different stages of development ranging from induction of ventral mesoderm in embryos, neovascularisation during fetal development to adult processes such as various tissue remodeling events like wound healing [282]. bFGF also regulates basic cell functions like differentiation, proliferation, motility and migration [281, 283].

The effects of bFGF are mediated by way of the transmembrane FGF receptors and the heparin-sulphate proteogycans receptors [284]. There are several genes that are regulated by bFGF and the mechanism adopted differs greatly depending on promoter and cell type [285]. The rat osteocalcin gene promoter is activated by bFGF in MC3T3-E1 osteoblast cells by a segment spanning from –144 to –138 (GCATCA) and called the osteocalcin fibroblast growth factor response element (OCFRE). This activation is very specific for bFGF and other growth factors like EGF, PDGF, TGF-beta and IGF-1 do not activate the OCRE. In order to confer bFGF responsiveness two or more copies of the OC promoter fragment –154 to –113 that allow 42 bp between each of the three tandem repeats of OCFRE upstream of a minimal OC promoter are required. The proteins binding to OCFRE from nuclear extracts prepared from bFGF stimulated MC3T3-E1 cells are very specific for this sequence and AP-1 is not part of this complex. This element is regulated by phosphorylation as suggested by its activation by okadaic acid, an inhibitor of phosphatases [286] [287].

The AP1 element is downstream from FGF in many signal transduction pathways. For example, the human interstitial collagenase (MMP-1) is modulated by bFGF in many different cell types like the MC3T3-E1 osteoblast cells [288], in human smooth muscle cells [289] and in NIH 3T3 cells [290]. The human collagenase promoter consists of a bFGF response element containing an AP-1 element (TGAGTCA) as well as an Ets element (AGGATG). Their locations are between –100 to –61 bp upstream of the start site and both are proposed to act together in regulation of transcription. This element is regulated via a Ras and the MKP2 regulated MAP kinase pathway in MC3T3-E1 cells [288].

The rat prolactin gene is also regulated by bFGF in GH4 rat pituitary cells through two Ets elements located between –212 and -96. The bFGF site on the prolactin gene is overlapping with two other elements; a Ras response element and also an insulin response element but each is known to bind to different proteins and act through different signal transduction pathways. The MAP kinase pathway is involved in the regulation of rat prolactin by bFGF but the p21ras and Raf-1 kinase are not involved. The transcription factors involved in this regulation are yet to be identified [291].

The proenkephalin gene promoter is regulated by bFGF and c-AMP together by a Ras dependent pathway through the CRE-like DNA regulatory element (CRE-2). The CRE-2 binds to CREB/ATF proteins, which regulate transcription by a pathway that depolarizes cAMP and Ca++ in the process [292]. The syndecan-1 gene is regulated by bFGF via the FGF-inducible response element (FiRE). This element is 170 bp long and contains five DNA motifs that can bind to different proteins including an AP2 related protein, two FGF-inducible Fos-Jun heterodimers, an upstream stimulatory factor and an 46 kDa transcription

factor [293]. Certain other bFGF-responsive elements have also been identified. For example in cardiac muscles the skeletal alpha-actin promoter is regulated by bFGF via an SRE-1 element. [294].

The structure, receptors and functions of different FGF's are well documented but the role of different FGF's, especially bFGF, in regulating the expression of many genes is still not fully established [295]. One area that merits more investigation is the mechanism by which the various FGF's regulate gene expression through the activation of signal transduction pathways. The key players that are involved in the transcriptional regulation genes downstream from FGF's and the mechanism adopted by them is till now not clearly outlined. Our study is expected to contribute to identifying the specific signal transduction pathways involved in the transcriptional regulation of genes by FGF.

The AP-1 transcriptional element

Activator protein-1 (AP-1) is a very well established transcription factor that was first identified as a group of proteins binding to the TPA-responsive elements (TRE) in phorbol 12-o-tetradecanoate-13-acetate inducible (TPA) genes [296-298]. It contains several bZIP (basic region-leucine zipper) domain protein dimer complexes comprising of Jun (cJun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2). These subunits interact with other proteins like the Jun dimerisation partners (JDP1 and JDP2) and the activating transcription factors (ATF's: ATF2, LRF/ATF3 and B-ATF. The AP-1 subunits can form homodimers or heterodimers and bind to the AP-1 binding site, the TPA-response element (TRE) and also the cAMP response element (CRE) from promoters of many cellular genes [299]. AP-1 can

also regulate basal as well as inducible transcription from various genes including collagenase, *IL-2*, *c-jun*, *fos*, *Bcl-3* [298, 300, 301].

AP-1 constituent proteins are differentially expressed and activated in response to physiological and pathological stimuli like growth factors, inflammatory cytokines, antigen binding, environmental signals like cellular stress, UV and ionizing radiation, certain oncoproteins like v-src and Ha-Ras [302, 303]. Reports have shown that AP-1 activation plays a very important role in many biological and pathological processes like cell proliferation, survival, apoptosis, neoplastic transformation and tumor formation [304, 305]. AP-1 plays a vital role in the above processes by regulating the expression of certain cell cycle regulators like cyclin D1, glucocorticoid receptor, p53, $p21^{cip/waf1}$, $p19^{ARF}$ and p16 [305]. AP-1 gets activated by various mechanisms like autoregulation by its different subunits [306], through interactions with other proteins like maf proteins (v-Maf, c-Maf, MafB, MafF, MafG, MafK) [307], co-activators like Jun activation domain binding protein (JAB1) [308, 309] and also by phosphorylation by protein kinases like the MAP kinases [310, 311].

The AP1 binding site (TRE) is also found to be an integral part of the *mrp3* promoter region located at -231 to -225 [225]. Phorbol esters can stimulate transcription of the *mrp3* gene in cultured cells (CHO, L, 10T1/2 cells) and in placenta [221]. A 31 bp region that includes an AP-1 binding site, a glucocorticoid receptor-binding site and a SV40 SphI element is involved in this activation. Mutation of any of these two elements completely attenuated the phorbol ester induction. Furthermore 3 copies of the 31 bp region introduced upstream of a minimal thymidine kinase promoter was capable of inducing transcription in

response to phorbol esters [229]. This sequence was termed as the "composite" glucocorticoid response element (cGRE). In response to glucocorticoids in L cells, CHO cells and HeLa cells, this element can have a positive as well as negative regulatory effect. This element can be selectively bound *in vitro* to the glucocorticoid receptor as well as the AP-1 transcription factor. In the presence of cJun-cJun homodimers at the AP-1 site this element stimulates transcription from a minimal promoter in response to glucocorticoids. But if cJun-cFos heterodimers are present instead of cJun-cJun homodimers then a negative response is elicited in response to glucocorticoids [312]. PKC's induce cFos and cJun and it was shown that inhibiting PKC's did not have any effect on the bFGF regulation of mrp3 [313]. These experiments suggested that AP-1 is not involved in regulating mrp3 in response to bFGF stimulation. But AP-1 does regulate the *plf1* promoter in response to serum hence the AP-1 activity was also analyzed in our studies described here and its role established.

Basic-fibroblast growth factor response element (FRE)

bFGF also regulates the expression of the mitogen regulated protein/proliferin (*mrp/plf's)* gene, *mrp3*. Expression of the mitogen-regulated proteins (MRP/PLF's) is regulated by bFGF in cultured cells and bFGF receptors are shown to be present in tissues where these genes are expressed. *Mrp/plf* mRNA's begin to increase around 8-10h and peaks at 12-16h in response to bFGF in 3T3 cells [314]. Interestingly only *mrp3* is transcriptionaly activated by bFGF while the *plf1* and *mrp4* promoters are not, although all three promoters share 97% homology in sequence [315]. This identity was utilized to further our understanding of the mechanism of bFGF regulation of gene expression. Comparisons of the three promoters for *mrp/plf* were carried out to determine what part of the *mrp3* promoter

was responsible for regulation of *mrp3* transcription by bFGF. Truncated promoters were analyzed using a chloramphenicol acetyl transferase reporter assay. After a series of truncation experiments a region within the first 353 bp between –186 and –167 bp in this promoter was identified that binds to nuclear factors from different cell lines. This identified region was named as the "bFGF-responsive element" (FRE). This FRE sequence was found to be unique and not similar to any other reported elements. The FRE was shown to be transcriptionally active in a TK fusion promoter and to respond to bFGF. Therefore it was established as a possible new transcriptional element [313] [315].

The FRE sequence was also found in the promoters of many bFGF- regulated genes. These are as follows: MMP family members; stromelysin-1 (*mmp-3*), gelatinase A (*mmp-2*), collagenase type 1, gelatinase B (92 kDa type IV collagenase, *mmp-9*) and other proteases; cathepsinL, cathepsin B, tissue plasminogen activator. Furthermore the protease genes not regulated by bFGF like elastase, metalloelastase, trypsin, and chymotrypsin did not contain FRE or FRE-like sequences [313]. This was a significant finding because not only are *mmp's* expressed in the same tissues as *mrp/plf*'s but also the proteins expressed by these *mmp* genes are involved in similar physiological tissue remodeling events like wound healing.

mrp3 is regulated transcriptionaly in response to bFGF. It was proposed that FRE could be the new transcriptional element that takes an active part or is responsible for the regulation of genes like the *mrp3* and certain *mmp's* in response to the growth factor, bFGF. Hence this finding was an important step towards elucidating the mechanism by which bFGF regulates transcription.

FRE-binding Protein (FREBP)

FRE, the response element from the proximal promoter of the *mrp3* gene binds to one or more nuclear factors derived not only from cell lines like NIH 3T3 cells (mouse), CHO cells (hamster) and HeLa cells (human) but also from tissues like the placenta (day 11) and fetus, regions where *mrp3* is seen to be highly expressed. Nuclear extracts from maternal liver do not bind the FRE. These nuclear factor(s) preferentially bind to the FRE over the other similar sequences from the *plf42* and *plf149* promoters. Interestingly substitution of just one base pair in the FRE at positions –173 and –181 markedly changes the FRE's ability to compete for binding to these nuclear factor(s). The results from EMSA studies showed a single band with slower mobility in nuclear extracts. This binding of FRE with its nuclear proteins was not bFGF dependent and was the same from both bFGF stimulated and the unstimulated cells. In order to establish the segment from FRE oligonucleotide that specifically binds proteins, competition assays were performed using nuclear extracts from 3T3 cells. The results of the competition assay showed that an oligomer (-179 to –162bp) that contained the 3' 2/3 of the FRE oligonucleotide competed successfully over the oligomer (-191 to –174) containing the 5' 2/3 of the FRE oligonucleotide for the nuclear proteins. This outlines the region from the FRE that the nuclear proteins were binding [313].

Certain transcription factors that were potential candidates for binding to the FRE and for forming a complex were ruled out as the FRE-binding transcription factor by the following experiments. Expression from the *plf42* promoter in L cells and the expression of MRP/PLF protein and mRNA in 3T3 cells increase in response to phorbol myristate acetate. Since the serum induced expression from *plf42* in L cells is regulated through the AP-1

transcription factor [228] and bFGF is known to increase the expression of *cFos* and *cJun* [316], the FRE-nuclear protein complex was examined to exclude the role, if any, of other transcription factors in the formation of this complex. The AP-1 recognition element was not found in the FRE oligonucleotide sequence and it was also tested whether Fos and Jun were a part of the of FRE-protein(s) complex. The AP-1 oligonucleotide did not compete for the nuclear factors binding to the FRE oligonucleotide sequence and neither did the addition of anti-Fos/ anti-Jun antibodies alter the relative mobility of the FRE-protein complex. But the addition of anti-Fos and anti-Jun antibodies did produce a supershift of the AP-1-protein complex. These studies established that Fos and Jun are not components of the complex formed by the FRE with nuclear proteins. The FRE also contains a potential GATA site and GATA transcription factors, known to be important for the expression of the *mrp/plf* genes in the placenta [317]. Hence though these transcription factors could possibly bind to the FRE. But it was seen that these GATA factors were part of the 5'2/3 of the FRE that did not compete for the binding of the FRE-protein complex thus ruling these transcription factors out for playing any role in the regulation of *mrp3* expression via the FRE [315].

To determine if the binding of protein(s) to the FRE is regulated by bFGF, one and three tandem repeats were linked upstream of the minimal *mrp3* promoter –81 to +65-CATbasic reporter gene and were stably transfected into Swiss 3T3 cells. The addition of three tandem copies of FRE upstream of a basal TK promoter resulted in activation in response to bFGF [313]. The apparent native molecular weight of this FRE-protein complex was determined by the method of Hendrick and Smith [318] in 3T3 cells. To do this, the FREBP-FRE complex was resolved through nondenaturing polyacrylamide gels made of

different concentrations of acrylamide. The relative mobility of the standard proteins in the complex and the shifted bands was plotted versus percent acrylamide on a log scale. The slope of the lines from the standard proteins was further plotted against their MW and this standard curve was used to estimate the MW of the FRE-protein complex. The apparent MW of FRE-FREBP complex was estimated at ~60 kDa. Then the protein(s) bound to FRE were covalently linked by UV cross-linking to FRE (-186 to –167 oligonucleotides of *mrp3* promoter) and run on SDS-PAGE where 2-3 bands were detected. Proteins ranging from 32 to 37 kDa were identified after subtracting the MW of the oligonucleotide (~23-28 kDa) [313].

Further characterization of the FRE-protein complexes cross-linked by UV-irradiation was done. These experiments resulted in the conclusion that the average apparent molecular weight of FREBP is ~ 32-34 kDa. The FRE element linked in three tandem repeats upstream of the minimal thymidine kinase CAT reporter gene was shown to be bFGF responsive, hence it was proposed that the protein binding to this FRE is a bFGF activated protein. This nuclear factor that binds the FRE was termed as the Fibroblast growth factor response element binding protein (FREBP) [313]. It was hypothesized that this transcription factor that binds to the FRE element from the *mrp3* promoter regulates *mrp3* gene expression in response to bFGF.

Transcription factors in general can either be activated in response to certain stimuli or may be constitutively expressed in the cell [319]. Expression of the FREBP is tissue- specific because it was not expressed in the maternal liver but was detected only in the placenta and fetus, and was especially prevalent in proliferating tissues corresponding to the

developmental period of expression of the *mrp3* gene. Furthermore, FREBP activity was found in cells of several species including 3T3 cells from mouse; CHO cells from hamster and Hela cells from humans. FRE-like sequences from different MMP family members like stromelysin (mmp3: human and rat), and collagenase (mmp1: mouse) were capable of binding proteins in the nuclear extracts from above cell lines and of creating the same mobility shifted band as with the FRE. Also, successful competitive binding was observed from the previously mentioned MMP family member sequences for the proteins binding to the FRE and also for proteins binding to the FRE like sequences [313]. Hence, it was hypothesized that the same FREBP protein(s) could be binding to the FRE-like elements from the matrix metalloproteinase genes as binds to the FRE in the *mrp3*.

Based on the observation that the proteins binding the FRE and the FRE-like sequences from the *mmp* family members were competed off in a similar way by the FRE oligonucleotides it was suggested that it could be the FREBP that is binding to these FRE-like sequences from the *mmp* family members. It was also proposed that this FREBP might be the transcription factor that coordinately regulates the expression of *mrp3* and certain other FRE-containing genes from the *mmp* family members in response to bFGF. Thus, identifying and establishing the mechanism by which the FREBP is regulating these genes and identifying the pathway that is involved in the regulation of *mrp3* by FREBP in response to bFGF will be very important. The key molecules that participate in these pathways will provide us information on the mechanism(s) that play an important role in helping the cell become anchorage independent or sometimes also mitogen independent. This would be a

very important step in characterizing the regulation of certain transcription factors that could play a very important role in the ability of the cell to become cancerous [320].

The current study

In a preliminary experiment during purification of the FREBP, HeLa cells were grown in suspension in order to get sufficient amount of cells for protein purification. Nuclear extracts were prepared from these suspended cells and EMSA's run with radiolabeled FRE oligonucleotide. The FREBP activity was decreased in these suspended cells as compared to the monolayer cells. The results from these experiments done with HeLa cells suggested that the FREBP is an anchorage dependent protein (By Lee Bendickson and Christian Paxton, unpublished data). The current study was undertaken to first verify and then extend this finding in terms of its application to other fields of study. The focus of this study was mainly to identify key signal transduction molecules involved in regulation of the FREBP and to outline the pathway that regulates the FREBP in an anchorage dependent manner. The FRE is the transcriptional element that regulates the expression of *mrp3* gene in response to bFGF and this FRE/FRE-like element (sequence with a difference of 1-2bp with the FRE) is also present in many *mmp* genes like stromelysin-1, collagenase type 1, gelatinase B. FRE-like sequences from the MMP family members like stromelysin-1 (human and rat) and collagenase type 1 (mouse) were capable of binding proteins in nuclear extracts from various cell lines and depicted a mobility shift in the band at a position similar to the one seen with FRE with FREBP. Also, successful competition binding was observed with the

previously mentioned MMP family member sequences for the proteins binding to the FRE as well as for proteins binding to the FRE like sequences [313]. It was then hypothesized, taking the above data into consideration, that this FRE/FRE-like element from the *mmp* genes could be binding to the same protein (s) that binds the FRE from the *mrp3* gene [306].

Because the FREBP is proposed to be the transcription factor that regulates the expression of the FRE-containing *mrp3* gene as well as the *mmp* genes in response to bFGF, this study would provide us an in depth insight into the signaling pathway and the key molecule that regulates the FREBP and in turn give us information on the pathway that regulates expression of the *mrp3* and *mmp* genes. Furthermore, in the long term, this study would help establish a bFGF-regulated pathway that could be involved in the regulation of several important genes during physiological and pathological conditions that have the transcriptional element FRE in their promoters. It would enable us to understand and establish the mechanism by which bFGF regulates gene expression during conditions like tissue remodeling and cancer. The *mmp* genes that we propose are regulated by the FREBP in response to bFGF are activated during various physiological and pathological processes including various tissue remodeling events and several different types of cancer. Hence with this study we would have more information on how these genes are being regulated in these events. We would also have a better understanding of the mechanism of action adopted by bFGF and perhaps of other members of the FGF family of growth factors in regulating the *mmp* genes.

Integrins themselves, along with molecules from the previously mentioned pathways like MEK and FAK, are now established as playing an active role in cancer

development as well as being effective targets for cancer therapy [321]. Thus, identifying the specific molecules that regulate the FREBP in an anchorage dependent manner would be very useful in defining the role, if any, that the FREBP plays during tissue remodeling and/or cancer. This pathway could be new or one that has been previously described and is already well established.

Once it was determined that the FREBP is an anchorage dependent protein, it was pertinent to know whether it plays any role in the cell's ability to become anchorage independent. The property of FREBP being anchorage dependent gave us a very important clue pertaining to the involvement of the ECM and also the nature of the pathways that could be involved in its regulation. This finding was important as certain *mmp's* are also regulated by interactions with the cell surface like *mmp-2*, *mmp-3*, *mmp-7* and *mmp-9* [191, 322].To identify and map out the signal transduction pathway by which the FREBP is regulated, pathways that are already known to be regulated by the extracellular matrix like the MAP Kinase, FAK and PI-3 Kinase, were identified for investigation. Protein kinases and other proteins in these pathways like FAK, Src kinase, PI-3 kinase, Ras and MEK were identified as key molecules that are activated or participate in the signal transduction pathways that are regulated directly or indirectly by the ECM. Inhibitors of these molecules were used in studies with cell cultures either in a monolayer or in suspension cultures.

The results from these studies showed that a MEK inhibitor PD 98059 inhibits FREBP activity in cells that were adherent to the ECM and thus our interpretation was that MEK might be involved in regulating the FREBP activity in monolayer HeLa cells. PD 98059 binds to inactive MEK1 and prevents its phosphorylation and activation by Raf. It

does not inhibit all protein kinases but specifically inhibits activation of MEK protein kinase by Raf protein kinase. It also has an inhibitory effect on ERK5 and cyclo-oxygenases 1 and 2 at a higher concentration [323] [324]. A key question was whether MEK activity was anchorage dependent. This question was tested in two ways; first, the activity of MEK was established in suspension cultures of HeLa cells over the same time course as decreasing FREBP activity and second, we determined the effect of inhibiting MEK activity over the same time course in suspension cultures as the time course established for the declining FREBP activity. MEK activity was assessed by Western blot. Antibodies for its direct substrate, ERK (totalERK and phosphoERK) were used. Results from this study showed us that the activity of MEK decreases more slowly than the FREBP activity in HeLa cells placed in suspension cultures and inhibiting MEK in suspension cultures did not have an effect on the FREBP activity in suspended cells. Hence based on our studies we propose that MEK could be involved in the regulation of FREBP in monolayer cells but not in suspension cultures of HeLa cells.

MATERIALS AND METHODS

Promoter Analysis: The FRE sequence was compared with the proximal promoters of the MMP family members known to be regulated by bFGF using the following searches: BLASTN from Pub Med (Nucleotide-nucleotide blast and search for short nearly exact matches).

Materials: MEK inhibitor PD 98059, Src Kinase inhibitor PP2 and negative control PP3, PI-3 Kinase inhibitor LY 294002 and negative control LY 304002, p38 MAP Kinase inhibitor SB203580 were purchased from Calbiochem (La Jolla, CA). Antibodies to phospho-ERK (pERK) and total-ERK (tERK) (BD Biosciences) were a gift from Dr. Janice Buss (Iowa State University, Ames, IA). Secondary antibody horseradish peroxidase-conjugated anti-mouse was from Santa Cruz Biotechnology. The ECL Western blot analysis detection reagent was from Amersham Biosciences (Buckinghamshire, UK). The purified double stranded AP1 consensus Oligonucleotide (5'-CGCTTGATGAGTCAGCCGGAA-3') was purchased from Promega, Corp. (Madison, WI) and the FRE Oligonucleotide (5'-CTATATCAGTGAATCTAAAA-3') was purchased from the DNA sequencing and synthesis Facility (Iowa State University, Ames, IA). poly (dI-dC): poly (dI-dC) was from Pharmacia Biotech, Inc. (Piscataway, NJ). T4 polynucleotide Kinase was from Promega Facility at Iowa State University, Ames, IA.

Cell Culture and Suspension: HeLa cells were grown in Dulbecco-Vogt's modified Eagle's medium (DMEG) with addition of 10% bovine calf serum and 10 U/ml each of penicillin and streptomycin (GIBCO/BRL, Grand Island, NY). The cells were maintained on a incubator supplied with 10% CO2 and 90% air at 37^0 C. Cells were suspended in 50 ml spinner flask on Bell-stir multi-stir magnetic stirrer (Bellco glass Inc., USA) at speed 4 for

different time periods of suspension. Cells were detached using 0.25% trypsin/EDTA either from monolayer or from suspension and were then collected by spinning at 5000 rpm in model HN-S centrifuge and used for preparing nuclear extracts or whole cell extracts. Samples were normalized to the amount of protein determined by the Bradford assay using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL).

Nuclear Extract: Nuclei were isolated from Hela cells (monolayer, suspension, with/without treatment with inhibitors) using 1.2M KCL. The cells were washed with cold TBS and resuspended in cold hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM PMSF and 0.5 mM DTT). The cells were then transferred to a Dounce homogenizer embedded in ice and homogenized using a type B pestle. Cells were centrifuged at 3300xg for 5 min (Model 5415 centrifuge). Nuclei were resuspended in low salt buffer (20 mM HEPES, 25% Glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) and high salt buffer (20 mM HEPES, 25% Glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT) and these buffers were added drop wise equal to ½ packed nuclear volume each. This mixture was extracted for 30 min in tube rotator at 4^0 C. The extracted nuclei were centrifuged at 16000xg for 5 min (Model 5415 centrifuge) and the extract collected. It was then dialyzed for 12 h in dialysis buffer (20 mM HEPES, 20% Glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) and stored at -80^0 until further analysis. The nuclear extracts were analyzed by electrophoretic mobility shift assay (EMSA) to asses the FRE-FREBP binding.

Whole Cell Extract Preparation: HeLa cells grown as described above were taken from monolayer or suspension time points and were lysed with 1X SDS electrophoresis sample buffer (61.5 mM Tris, 10.5% Glycerol, 0.0015% Bromophenol Blue, 72 mM SDS)

using a tuberculin syringe (the whole cells extract was triturated a few times to break DNA) and stored at -80^0 until further analyzed by performing a Western blot as described below.

Oligonucleotides and Probes: Double-stranded ^{32}P-labeled oligonucleotides were end-labeled using T4 polynucleotide kinase (Promega) in the presence of [γ-^{32}P] ATP (5000 Ci/mmol; ICN Pharmaceuticals, Inc., Costa Mesa, CA), 0.1 mM EDTA, 10 mM NaCl, 10 mM $MgCl_2$ and 5 mM DTT. Unincorporated radiolabel was removed using STE SELECT-D, G-25 spin columns (Bio-Rad, Hercules, CA). The probes were stored at -20^0.

DNA-Protein Binding Electro phoretic mobility shift assay (EMSA): Nuclear extracts from HeLa cells that had been grown as monolayer or put in suspension for specific time periods or treated with the above inhibitors or their negative controls. Binding reactions (20ul) were prepared containing 300 ug/ml BSA, 55 mM KCl, 0.11 mM EDTA, 2 mM $MgCl_2$, 0.1 mM PMSF. 0.28 mM DTT, 100 ug/ml poly (dI-dC); poly (dI-dC), 19% glycerol, 4 mM Tris·Cl, 19 mM HEPES and 16 pg/ul P^{32}-FRE or 12 pg/ul P^{32}-AP1 double-stranded DNA probes with equal protein for each individual experiment was loaded on the gels. The reaction mixes were incubated at 30^0C for 30 min. DNA-protein complexes were separated from free probe by electrophoresis on 4% polyacrylamide gel (80:1 acrylamide: bisacrylamide) in 2.7 mM EDTA, 380 mM glycine, 2.5% glycerol, 50 mM Tris·Cl, pH 8.5. Gels were run at 100mV (for 2 h, constant voltage, in cold room), dried overnight, exposed to PhosphorScreen (Molecular Dynamics, Sunnyvale, CA), analyzed on a PhosphoImager and quantitated using ImageQuant software (Amersham Biosciences).

Western Blot Analysis: Whole cell extracts were collected as described above from Hela cells in suspension over different time periods and analyzed by Western blot for

phospho-ERK (pERK) and total ERK (tERK). Samples were run on a 15% polyacrylamide-SDS gel at constant 25 mAmp, 300V/gel for ~50 min at room temperature. The proteins were electroblotted to nitrocellulose membranes (Nitrobind; Osmonics, Westborough, MA) using Hoefer TE 50 apparatus at constant 400 mAmp, 17 V/gel for 2h. The membrane was stained with 0.2% Ponceau S in 3% trichloroacetic acid staining solution at room temperature for ~2 min to ascertain uniform transfer of proteins to the membrane. After destaining with water, the membrane was incubated for 1h at room temperature in blocking solution [5% BSA in TBS {1XTD (135.6 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 and 25 mM Tris), 0.7 uM $CaCl_2$ and 2 uM $MgCl_2$} + 0.2% Tween 20]. The blocking solution was then decanted and the membrane was washed 3 times for 5 min each with TBST (TBS + 0.2% Tween 20). The membrane was soaked in a blocking solution containing our first primary antibody (anti-tERK at a dilution of 1/1000) overnight at 4^0 C on a Rocker at speed 4. The membrane was again washed three times for 10 min each with TBST, and soaked in blocking solution containing secondary antibody (horseradish peroxidase-conjugated anti-mouse at a dilution of 1/10,000 from Santa Cruz Biotechnology) for 1h at room temp on shaker at speed 4. The membrane was then washed 3 times for 10 min each with TBST and finally stained with ECL substrate (Western blotting Analysis reagents, Amersham International, Buckinghamshire, UK) and analyzed by autoradiography. The membrane was then incubated at 37^0 for 1h in stripping buffer (62.5 mM Tris, 2% SDS and 100 mM beta-mercaptoethanol) and washed 3 times with TBS. This stripped membrane was reprobed for our second primary antibody (rabbit anti-pERK at a dilution of 1/1000) with the exact procedure described above followed again. After we had the pictures from autoradiography of both our antibodies the bands were quantified using Kodak Imaging System and results analyzed by Prizm Software.

RESULTS AND DISCUSSION

The FRE sequence is present in certain MMP promoters: A systematic search for the FRE sequence was performed in the proximal promoters of different MMP family members that are regulated by bFGF using the BLAST med and CLUSTEL programs. Interestingly the FRE sequence either in its entirety or with a difference of 2-3 base pairs was discovered within the identified sequences of MMP family members from both mouse and human species (Fig 1). Transcriptional elements, like the GATA and AP-1 transcription factor, similar to those observed in the proximal promoter of *mrp3*, were also found in the proximal promoters of the *mmp* genes. This result further confirmed the initial studies done in our lab on the presence of the FRE in the different MMP family members [313]. Because the FRE present in the *mrp3* promoters has been shown to regulate the expression of *mrp3* in response to bFGF, it was further hypothesized that the FRE sequences in the *mmp* promoters could be playing a similar role as a transcriptional element in response to bFGF in these genes.

Fig. 1

FRE-FREBP binding specificity: TRE, the response element for the AP-1 transcription factor is also present in the promoter of the *mrp3* gene along with the FRE [225]. We conducted further experiments to confirm the specificity of the FRE-FREBP binding and to exclude AP-1 as being part of the FRE-FREBP complex. Competition assays were performed by EMSA using nuclear extracts from HeLa cells (monolayer and suspension cultures) with labeled probes and unlabeled oligonucleotides containing FRE and AP-1 sequences. The AP-1 complex migrated more slowly through the gel than the FRE-FREBP complex. Excess

unlabeled FRE oligonucleotide competed successfully for the FRE-FREBP band and not with the AP-1 band. Excess unlabelled AP-1 oligonucleotide did not compete for FRE-FREBP binding but competed successfully for AP-1 binding (Fig 2 A). The data was further confirmed using quantitative analysis and a significant reduction (** = $p < 0.01$) was observed in the samples for FRE probe and AP-1 probe containing cold FRE oligonucleotides and AP-1 oligonucleotides for competition. The FRE-FREBP complex binding was competed successfully by the cold FRE oligonucleotide (Lane 3 and 4) and not by the cold AP-1 oligonucleotide (Lane 5 and 6). The AP-1-nuclear protein complex binding was similarly competed for successfully by cold AP-1 oligonucleotides (Lane 9 and 10) and not by the cold FRE oligonucleotides (Lane 11 and 12) (Fig. 2B). Together these results showed that FRE-FREBP binding is specific for the FRE sequence.

Fig. 2

FREBP activity is anchorage dependent: To confirm the observation that the FREBP activity is anchorage dependent and to chart out its time course, HeLa cells were suspended for different time periods over the course of 24 h, nuclear extracts were prepared and EMSA analysis was performed to analyze FRE-FREBP activity. Our results effectively demonstrate that FREBP is an anchorage dependent protein. EMSA analysis showed that FREBP activity decreases in a time-dependent manner until no detectable activity was observed at 24h post suspension (Fig 3A). Densitometric analysis using Imagequant was done in order to quantify the FREBP activity changes over the period of cell suspension analysis (Fig 3B). Furthermore we conducted short time course studies within a period of one hour to determine at what time point the loss of activity initiated. The data obtained showed that the activity

begins to decline within the first hour and continues until about 10 h (Fig 3C). Taken together these results suggested that the FREBP is regulated in an anchorage dependent manner. Based on published literature and the knowledge of current work in the lab we proposed the involvement of one or more of three major pathways; the MAP kinase cascade, FAK pathway and PI-3 Kinase pathway in the regulation of FREBP activity.

Fig. 3

Activity of the transcription factor (AP-1) is not anchorage dependent: AP1, an established transcription factor, was used to gauge the specificity of the effect of anchorage on the regulation of transcriptional activity [315]. The following experiments were done to determine whether AP-1 also followed a similar anchorage dependent response as the FREBP in HeLa cells after the cells were suspended. The activity of AP-1 was monitored in the same samples in which the FREBP activity was monitored (Fig. 3). EMSA analysis showed no reduction in the AP-1 activity in HeLa cell suspension studies even up to 12 h after initiation of suspension (Fig 4A) and these results, which were further quantified by analyzing PhosphoImager scans (Fig 4B), indicated no significant changes in activity of AP-1 with increase in time of suspension. Our results suggested that FREBP is uniquely an anchorage dependent transcription factor. A TRE (AP-1-binding site) is present in the promoter of *mrp3* gene and it was investigated for its role in regulating *mrp3* in response to bFGF by the following experiments. Serum and protein kinase C (PKC) can activate Jun and Fos transcription factors that are regulated through the TRE. It was shown that down regulation of serum and PKC did not affect the activation of *mrp3* in response to bFGF. Hence it was postulated that the TRE is not taking a part in the regulation of *mrp3* in

response to bFGF [225]. Our results further show that AP-1 activity unlike the FREBP activity is not anchorage–dependent in HeLa cells.

Fig. 4

MEK involvement in the regulation of FREBP activity: Signal transduction molecules that were known to participate in ECM regulated pathways were selected for our study. These were MEK, Src Kinase, PI-3 Kinase and Ras. Inhibitors were used for each of these molecules when the cells were grown in monolayer cultures. These studies were undertaken to assess the affect of each inhibitor on the FREBP activity and to determine if any of these molecule could be involved in regulating the FREBP. Nuclear extracts were prepared as described previously and DNA and DNA-protein complexes separated by running EMSA gels. The FREBP activity was analyzed by exposing the gels to PhosphoImager and bands quantified by using ImageQuant software. The well-characterized MEK inhibitor, PD 98059, attenuated FREBP activity in monolayer HeLa cells (Fig 5). This suggested that MEK, an important molecule of the MAP kinase pathway could be involved in regulating the FREBP activity.

Fig. 5

PI-3 Kinase, Src Kinase and Ras are not Involved in regulating the FREBP activity: In this set of experiments we investigated if the other selected molecules played any role in the development of FREBP activity. The inhibitors and doses that were used are as follows; LY294002 (PI3K inhibitor-50 μM), PP2 (Src kinase inhibitor-30μM) and Ftase (Ras inhibitor-20 μM). All doses were selected based on an extensive search of current and past

relevant literature. The inhibitors used had no significant effect on the FREBP activity in monolayer cells, which indicated that these molecules possibly do not regulate FREBP activity while the cells are in monolayer and still attached to the ECM. Further experiments with these inhibitors with cells in suspension were not undertaken.

These results narrowed down our scope of identifying the signal transduction pathway involved in regulating the FREBP. Because MEK is downstream of Ras in the MAP kinase pathway cascade certain questions arose from these experiments that remain unanswered at this point. In agreement of our earlier finding that inhibiting MEK decreases the FREBP activity. Inhibiting Ras should, via the inhibition of the MAP kinase cascade inhibit MEK which in turn should inhibit the FREBP activity. We did not see this decrease in FREBP activity is response to Ras inhibition. The possible explanation for this result could be that either MEK is being activated independent of Ras in our system as has been shown in certain other systems [17] or the time period of incubation with Ras inhibitor in our study was too short for the inhibitor to have its effects. These results need to be further confirmed by repeating these experiments. Even though PD 98059 is a very potent and specific inhibitor of MEK1, it is also capable of inhibiting ERK5 by binding to inactive MEK5 and also has a inhibitory effect on cyclo-oxygenase 1 and 2. Hence the results from studies using PD 98059 to inhibit MEK have to be confirmed by other possible experiments like using a dominant negative MEK and observing the effects on the FREBP activity. (Fig. 6).

Fig. 6

MEK activity decreases more slowly than FREBP activity after cells are placed in suspension: On the basis of the previous results (Fig. 5) it appeared that MEK could play an important role in the regulation of the FREBP. To further extend this hypothesis we looked at the activity of MEK once the cells are put in suspension cultures without any inhibitor over the same time course that we already had established for the decrease in FREBP activity. These experiments would give us information regarding the status of MEK activity and by comparing it to the FREBP activity over the same time course we can compare the rate of decline of FREBP activity to MEK activity. The direct downstream substrate of MEK is ERK1/2 and hence we looked at the ability of MEK to phosphorylate ERK as an indicative of the change in MEK activity after the induction of cell suspension. By performing Western blots we monitored the changes in total ERK (tERK) and phosphoERK (pERK) during the same time period that the FREBP activity decreases by performing Western blots. The results were quantified by densitometric analysis (Fig. 7B) and it was confirmed after normalizing the values for pERK to tERK that the proportion of ERK that is phosphorylated decreases more slowly than the activity of the FREBP. The r^2 value of 0.69 indicates a poor fit and the p value of <0.0001 show significant decrease in MEK activity. But since we are looking at the comparison of MEK activity with FREBP activity, this suggested that MEK activity decreases more slowly than FREBP activity after the cells are suspended (Fig 7A). These results suggest that it is unlikely that MEK is responsible for the decrease in FREBP activity in suspension cultures. This needed further clarification and for that we need to know what happened to FREBP activity in suspended cells when the activity of MEK is inhibited.

Fig. 7

Inhibiting MEK does not decrease FREBP activity in suspension cultures. Inhibiting MEK in monolayer has a dose dependent inhibitory effect on the FREBP activity but the decrease in activity of MEK does not correspond to the decreasing FREBP activity in suspension cultures. I tested the ability of a MEK inhibitor to decrease FREBP activity in suspended cells. HeLa cells were grown in monolayer cultures and suspended and treated with PD 98059 at the times identified. My results show that there is no effect of inhibiting MEK on FREBP activity in suspension cultures (Fig 8a and 8B). These finding taken together with our earlier findings indicate that although the MEK molecule may be involved in regulating the FREBP in monolayer HeLa cells, it does not regulate the FREBP activity directly in suspension cultures.

Fig. 8

Comparison of FREBP and AP-1 activity Decay. The half-life of a molecule is the average time required for the activity of a substance (proteins, radionucleotides, enzymes etc) to reduce to half of the amount that it started with originally. The calculation of half-life of proteins can give us valuable information regarding its activity status. The FREBP activity decays much faster compared to the MEK activity in suspended HeLa cells over the same time period. **(A)** FREBP and MEK activity over ~24h. **(B)** FREBP and MEK activity over ~8h. The half-life of MEK was calculated to be 18h and the half-life of the FREBP activity was calculated to be 2.8h.

Fig. 9

Effect of p38MAP kinase pathway inhibitor: SB 203580. Results from preliminary experiments show that the inhibiting the p38 MAP kinase pathway has a 40% reduction in the FREBP activity in monolayer HeLa cells. Experiments were not enough to do statistical analysis. Further studies have to be conducted to know the significance of these results (Fig. 10).

Fig. 10

Summary. The molecules targeted from the MAP kinase pathway, PI-3 kinase pathway and FAK pathway are illustrated along with the inhibitors used for each one of above mentioned molecules beside them.

Fig. 11

CONCLUSIONS AND FUTURE DIRECTIONS

The *mrp/plf's* and the *mmp's* are classified as delayed response genes that are activated in response to growth factors. Both families of genes encode secreted proteins that are involved in the biological processes of cell growth and angiogenesis [325]. Interestingly both *mrp/plf's* and *mmp's* are also expressed in the physiological as well as pathological events of tissue remodeling like wound healing and cancer. There are many similarities between the processes of wound healing and cancer like cell proliferation, cell migration and invasion, angiogenesis, degradation of basement membrane, activation of similar pathways, increased expression of growth factors, growth factor receptors, angiogenic factors along with increased expression of similar genes [326] [327] [146]. Recently a model to predict cancer has been proposed based on the expression of genes during a fibroblast cellular response to serum in cell culture [328]. Fibroblasts from different sites in the body shared a common gene expression pattern, which was called a genetic signature. This genetic signature was always found in certain cancers like prostate and liver-cell carcinomas and variably in others like breast, lung and gastric carcinomas. Patients having tumors containing the serum-activated wound-like signature had a worse prognosis compared to ones not having this signature [328]. The timing of expression of *mrp3* and *mmp's* are similar in during wound healing and cancer. Certain members of both *mrp/plf* (mrp3) and the *mmp* gene (stromelysin-1, collagenase type 1, gelatinase B) family are regulated by the bFGF. The *mmp-9* gene is expressed in the outer root sheath hair follicle cells in response to growth factors [329] and *mrp3* is expressed during wound healing in adults. Furthermore, MRP3 protein expression is found in the suprabasal keratinocytes at the wound healing sites and in the outer root sheath of the hair follicle during the hair growth (late anagen phase) [234]. The

FRE transcriptional element present in the proximal promoter of the *mrp3* gene was also found in many of the bFGF regulated promoter sequences in genes of the *mmp* family (stromelysin-1, collagenase type 1, gelatinase B) as well as those belonging to other protease families (cathepsinL, cathepsin B, tissue plasminogen activator). Interestingly, members from other protease families (elastase, metalloelastase, trypsin, and chymotrypsin) not regulated by bFGF did not contain the FRE or a related sequence [313]. This literature analysis closely mirrored our hypothesis of the FRE being involved in the bFGF induced physiological cellular processes. The involvement of similar pathways have been proposed like the mitogen activated protein kinase (MAPK) pathway in the regulation of MMP family members like MMP-1 [195] and *mrp/plfs* [330]. Owing to the co-incidental expression and the similarity of their encoding proteins involved in cellular processes, it is suggested that similar transcriptional element(s) might be involved in the regulation of the *mrp/plf's* and the *mmp's*.

The FRE binds to one or more nuclear factors derived from different cell lines and from tissues like the placenta (day 11) and fetus. The FRE element linked in three tandem repeats upstream of the minimal thymidine kinase CAT reporter gene was shown to be bFGF-responsive, hence it was proposed that the protein binding to this FRE is a bFGF activated protein. This nuclear factor that binds the FRE was further characterized and termed as the fibroblast growth factor response element binding protein (FREBP) with an approximate MW of 32-37 kDa. It was hypothesized that this is a transcription factor involved in regulating *mrp3* gene expression in response to bFGF [313]. Furthermore, FREBP activity was found in cells across several species including 3T3 cells from mouse;

CHO cells from hamster and Hela cells from humans. FRE-like sequences from different MMP family members like stromelysin-1 (human and rat), and collagenase (mouse) were capable of binding proteins in the nuclear extracts of the aforementioned cell lines bearing remarkable similarity to the established band mobility of FRE. In addition, successful competition binding was observed from the previously mentioned MMP family member sequences for the proteins binding to the FRE as well as for proteins binding to the FRE like sequences [313]. Taken together this data yielded the hypothesis that the same protein(s) could be binding to the FRE-like elements from the matrix metalloproteinase genes as binds to the FRE from the *mrp3* gene.

These findings together led in the direction of establishing the FREBP as a new transcription factor involved in the regulation of *mrp3* and certain *mmp's* (FRE-containing bFGF regulated genes) in tissue remodeling processes and cancer. The present study was done in order to provide further insight into the functional and regulatory aspects of FREBP. With these and other studies [308] we demonstrated that the FREBP binding is very specific for the FRE and that AP-1, which is present in the proximal promoter of the *mrp3*, was not a part of this complex. The results from EMSA's done with nuclear extracts from HeLa cells showed that AP-1-protein complex migrated more slowly than the FRE-FREBP through the acrylamide gel indicating that the two elements, the FRE and TRE, formed different protein complexes. Furthermore, excess unlabeled FRE oligonucleotide competed successfully for the FRE-FREBP complex but not for the AP-1 complex. In addition, excess unlabelled AP-1 oligonucleotide did not compete for the FRE-FREBP

complex but competed successfully for the AP-1 complex thus proving effectively that FRE-FREBP binding was independent of AP-1 activity.

It was then demonstrated that the FREBP was an anchorage dependent protein. Time course studies done with HeLa cells over a period of 0-24h after they were placed in suspension showed that the FREBP activity goes down gradually reaching its lowest levels by ~12h (Fig. 3B). This observation suggested that probably the FREBP activity was being regulated by an ECM dependent pathway. This property of anchorage dependence is increasingly becoming the focus of studies involving biological events like tissue remodeling, as the cells involved in these events become anchorage independent and acquire the ability to move from their original position to different areas. The exact mechanism by which the cells acquire this ability is still unclear but it is proposed to be one of the major factor regulating cancer cell pathophysiology [331].

Next our main focus was to identify the pathways and/or molecules that could be involved in regulating the FREBP activity in an anchorage dependent manner in these cells. This data would help provide a better understanding of the mechanism adopted by cancer cells in their ability to become anchorage independent. These results would also help in making the connection of FREBP activity to the *mmp's* since it has already been demonstrated that certain mmp's (*mmp-2,3,7 and 9)* are also regulated by interactions with the cell surface [322] [191]. In the subsequent experiments we showed, through timed suspension studies, that the time course of decline in FREBP activity starts within the first hour and decreases for the first 8-10 h. It was also demonstrated that the phenomenon of anchorage dependence was exclusive for FREBP activity and AP-1 activity did not change

similarly. EMSA analysis depicted no reduction in the AP-1 activity in HeLa cell suspension studies even up to 12 h after initiation of suspension (Fig 4A) and these results were further quantified using Densitometric analysis (Fig 4B) indicated no significant changes in activity with increase in time of suspension up to ~24h. Studies have shown that the AP-1 response element TRE is present in the promoter of *mrp3* gene [225] but it was shown that AP-1 is not part of the regulation of *mrp3* by bFGF as both serum and PKC can activate Jun and Fos transcription factors but down regulation of serum or PKC did not affect the bFGF regulation of *mrp3* [313]. Our finding are in corroboration with these earlier studies done describing the role of AP-1 in regulation of *mrp3* in response to bFGF. Our studies further strengthened the belief that the FREBP functions as a separate transcription factor by a mechanism distinct from AP-1 since the latter was not regulated in an anchorage dependent manner while the former was in the same experimental setup. The ramifications of decreasing FREBP activity in response to loss of anchorage could vary from the suppression of genes that it is directly activating and in increasing activity of genes it might be helping to suppress under normal conditions. The activation of genes that it could be suppressing further might lead to the activation of new genes.

These studies also helped in narrowing down the kind of pathway that could be involved in the FREBP regulation because it became evident that an extracellular matrix (ECM) regulated pathway was involved in regulating the FREBP in an anchorage dependent manner. Based on this observation and extensive literature review, the MAP kinase, phosphoionositol-3 (PI-3) kinase and the focal adhesion kinase (FAK) pathways were narrowed down as candidate for studies. These pathways are activated in response to growth

factors as well as in response to ECM via cell surface receptors like integrins. These pathways can each act on different transcription factors that in turn regulate myriad cellular processes. Recent investigations have shown that the MAP kinase pathway can regulate transcription factors like Elk-1, MEF2A, GATA-1, JunB and Rsk1 [332]. Based on further analysis we chose certain specific molecules from these pathways like MEK kinase, Src kinase, PI-3 kinase and Ras to be studied as possible modulators of FREBP activity in the cellular model of anchorage dependence. These molecules, like constitutively active MEKI, have already been well established for playing a role in regulating several genes, like *mmp-2*, involved in tissue remodeling and cancer in response to ECM adhesion [198].

Our studies in monolayer cultures indicated that among the various inhibitors we investigated, a MEK inhibitor PD 98059 decreases FREBP activity significantly in monolayer HeLa cells (Fig. 5). MEK, a member of the MAP kinase pathway, is considered very important because of its presence and high activity demonstrated in several cancerous lesions and it has been postulated as an important player in the cell's decision to become anchorage dependent [59] . The MEK inhibitor PD 98059, has been studied extensively and is at present part of a clinical trial to be developed as a therapeutic measure against development of cancerous pathology [333]. PD 98059 inhibits the MAP kinase pathway *in vitro* and *in vivo* by the specific interaction with its target kinase and not by disruption of the ATP metabolic mechanisms in the cell [334]. The activation of plasminogen activator inhibitor-1 gene is induced by ECM attachment via two transcriptional elements present in its promoter: serum response element (SRE) and hypoxia response element (HRE). It was shown that the binding ability of these transcriptional elements to their nucleoproteins was cell adhesion dependent and also MEK dependent [335]. Although we had already

established that the FREBP activity is anchorage dependent, we further demonstrated its functional connection with MEK.

We further extended our hypothesis to include that MEK could be involved in regulating the FREBP activity not only in monolayer cells but also by regulating FREBP in an anchorage dependent manner. In order to answer this question we determined the effect on MEK activity of placing HeLa cells in suspension over a similar time course to that used to measure the rate of decline of FREBP activity. Furthermore, studies were also done to investigate the effect on FREBP activity in suspended HeLa cells when MEK is inhibited. The activity of MEK in suspension cultures was examined over a time course similar to that where we had observed a decreasing FREBP activity. Antibodies for the direct substrates of MEK, tERK and pERK were used. Results were quantified by normalizing the pERK values to tERK values. Our data indicated that the pERK activity did not decrease significantly over the period of time that we had observed a significant drop in FREBP activity (Fig. 7). Based on these results it was established that the process of FREBP activation was independent of MEK activity in the case of suspended HeLa cells. In the next study we tested if inhibiting MEK in suspension cultures decreased FREBP activity. MEK inhibitor PD98059 was used to inhibit MEK in HeLa cells that had been placed in suspension for 30 min and 12 h and the effect on FREBP activity was analyzed. The results showed that inhibiting MEK does not inhibit FREBP activity in suspension cultures (Fig. 8) further establishing the hypothesis that in suspended HeLa cells the FREBP activity is not being directly regulated by MEK.

Analysis of the half-life of the decrease in activity of the FREBP and MEK showed that the FREBP activity in suspended HeLa cells decayed at a much faster rate than the MEK activity (Fig. 9). I believe there can be two possible causes for these results to be

observed. First, that even though MEK may regulate FREBP in monolayer HeLa cells, it is not directly involved in the regulation of FREBP in suspension cultures. This explanation can be clarified by investigation of the indirect role of MEK in suspension cultures and involvement of other co-regulators. Secondly, the results of studies done by others on the factors affecting the nucleocytoplasmic distribution of MAP kinase pathway suggest an explanation for our results. It has been demonstrated that integrin mediated cell adhesion differentially regulates the nucleocytoplasmic distribution and translocation of ERK and the subsequent phosphorylation of its nuclear substrate Elk-1 at serine 383. The ability of activated ERK to localize to the nucleus and to phosphorylate its nuclear substrates was markedly decreased when the cells were in suspension versus attached and ERK activity was dependent on an intact actin cytoskeleton [55]. Interestingly, it was further established that this effect on ERK was restricted to the ERK-MAPK cascade and does not extend to the JNK or p38-MAPK kinase pathway. Anchorage-independent activation of the JNK and p38-MAPK pathway in NIH 3T3 cells showed that the ability of JNK to localize to the nucleus and phosphorylate substrates (c-Jun and Elk-1) mirrored the activation characteristics of p38 and was the same in suspended and adherent cells [62]. Based on these studies it is possible that once in suspension the ability of ERK to phosphorylate the FREBP is markedly attenuated in comparison to the cells that are in monolayer cultures. Further clarification can be afforded by determining the effect of cell suspension on ERK activity in nuclear fractions from HeLa cell extracts. Using the same nuclear extracts used for the FREBP study will give us data to compare the FREBP and ERK activity in the nucleus of HeLa cells in suspension. Preliminary studies have been also been done to determine effects of inhibiting p38 MAP kinase on the FREBP activity. Attenuating the p38MAP kinase pathway using a specific

inhibitor SB 203580 in monolayer HeLa cells did not have any affect on the FREBP activity (Fig.10).

In summary these results showed that inhibiting MEK activity decreases the FREBP activity in monolayer cells yet the MEK inhibitor PD 98059 has no effect on FREBP activity in suspension systems. Hence MEK could be playing an important role in the regulation of FREBP in monolayer cells but it is not directly involved in the FREBP regulation in suspension cultures. We need to further elucidate the role of MEK in FREBP regulation, whether it is a direct relation and if not what are the other molecules participating in this phenomenon.

Future work investigating the mechanism that regulates the FREBP in an anchorage dependent manner and other studies to further investigate the effect on FREBP activity of MEK inhibition in monolayer HeLa cells need to be done. Simultaneously, further experiments need to be performed in order to establish the connection of FREBP to the *mmp* genes and determine its role if any in the regulation of the FRE containing *mmp's*. These studies would be very useful as not only will they establish whether the FREBP regulates expression of the FRE-containing genes in response to bFGF but they will also give us more understanding to the type of molecules participating in the cell's ability to become anchorage dependent.

In this regard the following approaches and experiments are suggested. Studies mutants like dominant negatives of MEK or constitutively active MEK can be done to further confirm whether MEK is involved in regulating the FREBP activity. Those studies should be compared with the inhibitor studies and the data analyzed together. In order to establish if

ERK could be involved in the phosphorylation of FREBP, the activity status of ERK in the nuclear extracts needs to be established. For these experiments antibody against a known substrate of ERK, ELK-1 can be used in HeLa cell nuclear extracts and compared with the inhibitor studies. Using an ERK inhibitor in monolayer HeLa cells can further confirm the role of ERK on FREBP activity in our system. Establishing the time course of the expression of *mmp's* containing the FRE by doing RT-PCR in HeLa cells over the same time course as decreasing FREBP will give us a very useful tool in assessing whether there is any decline in the expression of the *mmp's* and how does it correspond to the decline in FREBP activity. The preliminary studies done with the p38 MAP kinase inhibitor show a 40% decline in the FREBP activity in monolayer HeLa cells. These studies need to be extended more and results obtained from enough experiments in order to establish any significance. It is of prime importance before starting any other investigation to get the purified FREBP. Then its sequence can be compared to the databases for any similarities with the already established transcription factors and based on those results future course decided. Then we need to have the cloned cDNA encoding FREBP and this will open a door to many possibilities. This will enable us to characterize the structure of FREBP and its function. Anti-FREBP antibody could be prepared and by doing Northern blots the presence of its mRNA in tissues and cells can be mapped out. It can be determined if the protein regulates the genes containing the FRE in their promoters like the *mrp3* and *mmp's*. Characterization of the DNA binding domain of the FREBP to finding its co-regulators and also if it functions in conjunction with other transcription factor by forming heterodimers with them can be achieved. Mutagenesis studies with FREBP showing the effects if any on the *mrp3* and *mmp* gene expression can be planned in promoter-reporter experiments. Preparation of dominant negatives of MEK and

FREBP and performing microarray experiments would identify the kind of genes being expressed and whether there is an association with the *mmp's*.

FIGURES

Figure 1. ***Promoter Analysis.*** The FRE sequence from the proximal promoter of mrp3 was compared with different *mmp* promoters, known to be regulated by bFGF, using the search engine BLASTN and CLUSTAL from Pub Med.

Fig 1

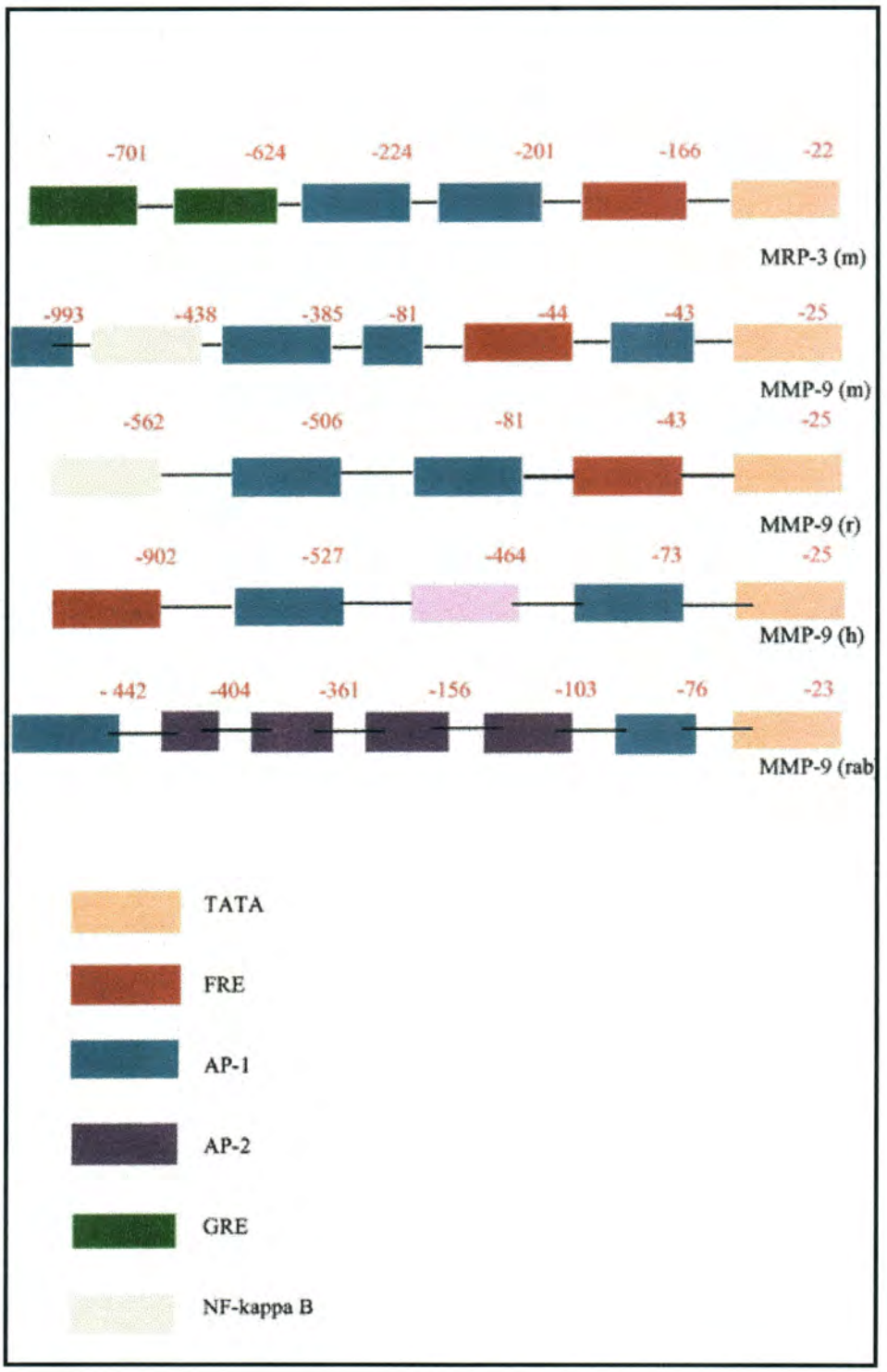
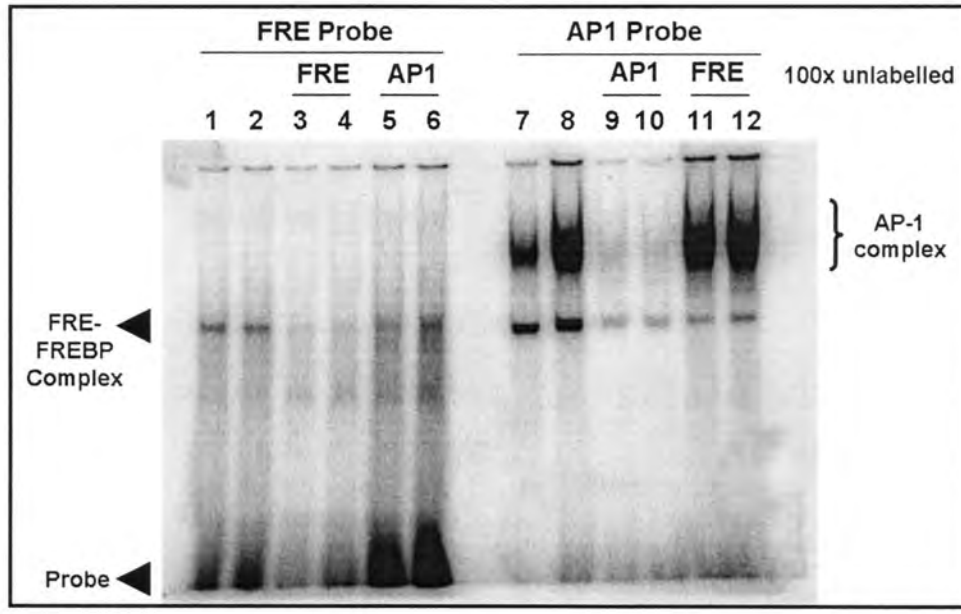

-701
-624
-224
-201
-166
-22
MRP-3 (m)
-993
-438
-385
-81
-44
-43
-25
MMP-9 (m)
-562
-506
-81
-43
-25
MMP-9 (r)
-902
-527
-464
-73
-25
MMP-9 (h)
- 442
-404
-361
-156
-103
-76
-23
MMP-9 (rab
TATA
FRE
AP-1
AP-2
GRE
NF-kappa B

Figure 2. ***Competition studies for FRE and AP-1.*** **(A)** FRE-FREBP binding specificity was analyzed by competition using the EMSA assay. The FRE and AP-1 oligonucleotide sequences were used as probes (radiolabeled) and as competitors (unlabeled and 100 times the probe concentrations). The positions are labeled of complexes formed by the FRE (with FREBP) and AP-1 (with AP1 proteins) with the same nuclear extracts from HeLa cells. METHODS: nuclear extracts from HeLa cells were prepared as described in material and methods and incubated with radiolabeled oligonucleotides of either FRE or AP-1. Some of the incubation mixtures contained 100-fold excess of unlabeled oligonucleotide competitor as indicated on the figure. The samples were resolved by gel electrophoresis and the radioactivity present in each band was detected by a Phosphoimager and analyzed by Imagequant software. **(B)** A quantitative summary of data representing band shift analysis of the complexes depicted in Figure 2A.

STATISTICAL ANALYSIS: Data represents N=2-4 Mean ± SEM and ** = $p < 0.05$ represents significant differences between control and treated groups based on one-way ANOVA using Bonferroni's Multiple Comparison Test.

Fig 2

A


FRE Probe
AP1 Probe
FRE
AP1
AP1
FRE
100x unlabelled
1 2 3 4 5 6 7 8 9 10 11 12
AP-1 complex
FRE-FREBP Complex
Probe

B

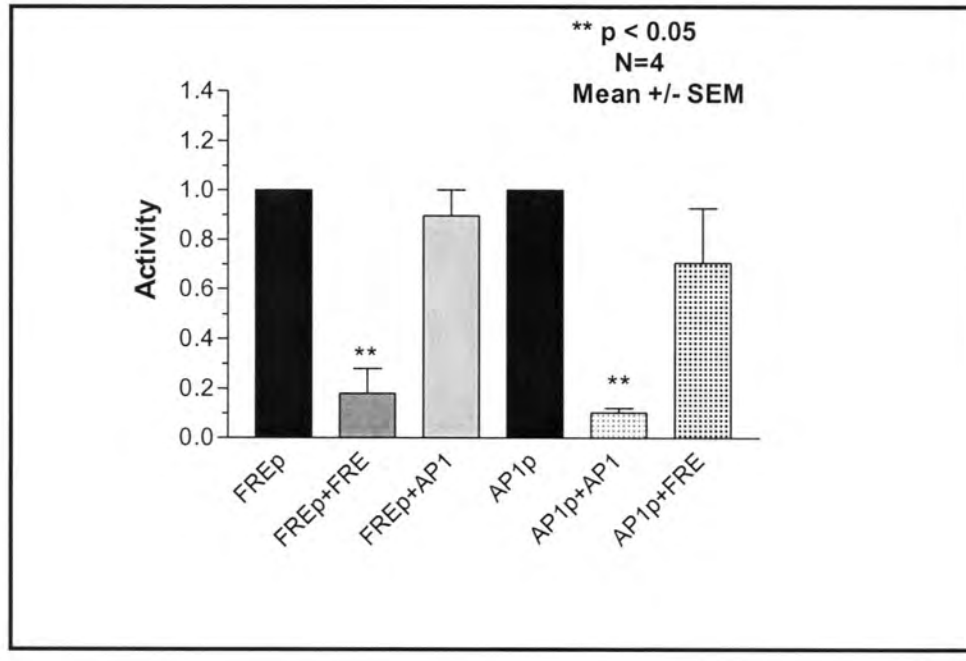
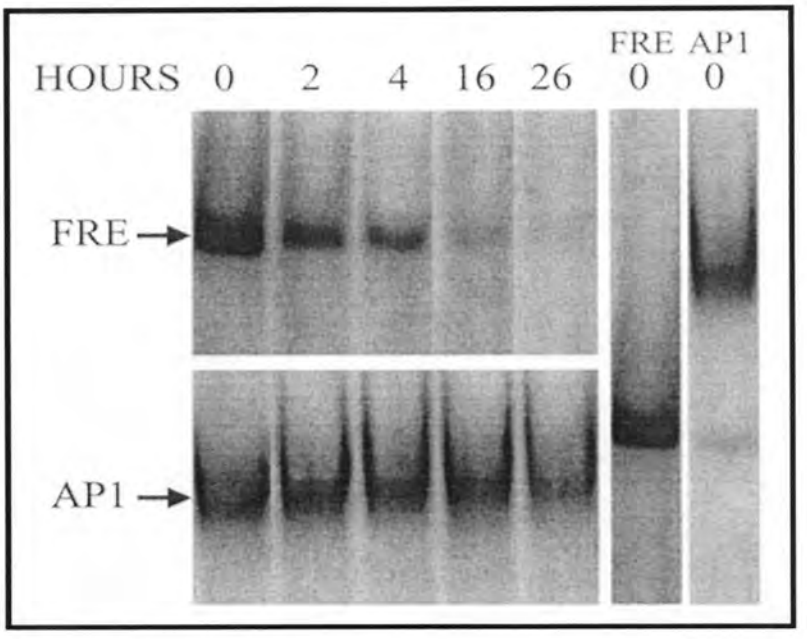

** p < 0.05
N=4
Mean +/- SEM
Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
FREp
FREp+FRE
FREp+AP1
AP1p
AP1p+AP1
AP1p+FRE

Figure 3. ***FREBP activity is anchorage dependent.*** **(A)** It was established through a series of experiments in which the FREBP activity was measured after the cells were suspended that the FREBP is an anchorage dependent protein. **(B)** A time course describing the decline in its activity was established from 0-24h. METHODS: HeLa cells were grown in monolayer cultures and put in suspension (using spinner flasks) over the time points identified on the gel. Nuclear extracts were prepared as described in material and methods and incubated with radiolabeled FRE oligonucleotide. The DNA and DNA-protein complexes were separated by EMSA (Electro phoretic mobility shift assay). The amount of shifted FRE in each sample was analyzed by exposing gels to Phosphoimager and quantified by Imagequant Software. Equal amounts of nuclear protein were loaded in each well.

Fig 3

A


HOURS 0 2 4 16 26
FRE AP1
0 0
FRE
AP1

B

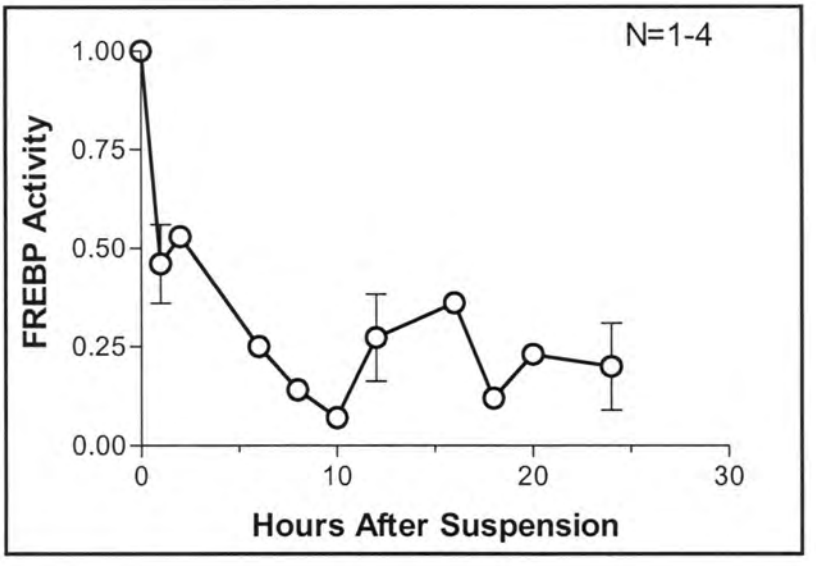
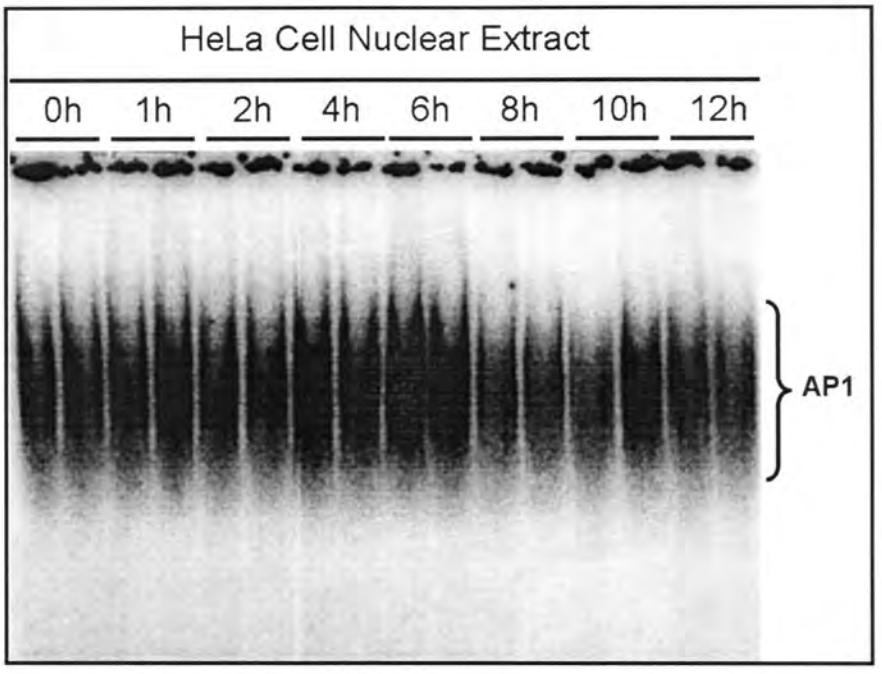

N=1-4
FREBP Activity
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Hours After Suspension

Figure 4. ***AP-1 is not anchorage dependent.*** A time course for the AP-1 activity after placing HeLa cells in suspension was established that shows that AP-1 unlike FREBP is not an anchorage dependent protein in HeLa cell extracts. **(A)** Depicts a representative EMSA showing no significant changes in AP-1 activity in HeLa cell extracts over a period of 12h post suspension and **(B)** depicts a quantitative summary analysis of the AP-1 complex density measurements showing no significant changes in AP-1 activity over the entire period of measurement from 0-24h. METHODS: Nuclear extracts analyzed previously for FREBP were incubated with radiolabeled AP-1 oligonucleotide. The DNA and DNA-protein complexes were separated by EMSA. The mobility shifts were analyzed by exposing the gels to Phosphoimager and quantifying by Imagequant Software. ANOVA analysis did not reveal any significant differences in AP1 activity up till 24h.

STATISTICAL ANALYSIS: Data represents N=1-4 Mean ± SEM. One way ANOVA analysis using Dunnett's Multiple Comparison Test showed no significant differences between analyzed groups as depicted below.

Fig 4

A


HeLa Cell Nuclear Extract
0h
1h
2h
4h
6h
8h
10h
12h
AP1

B

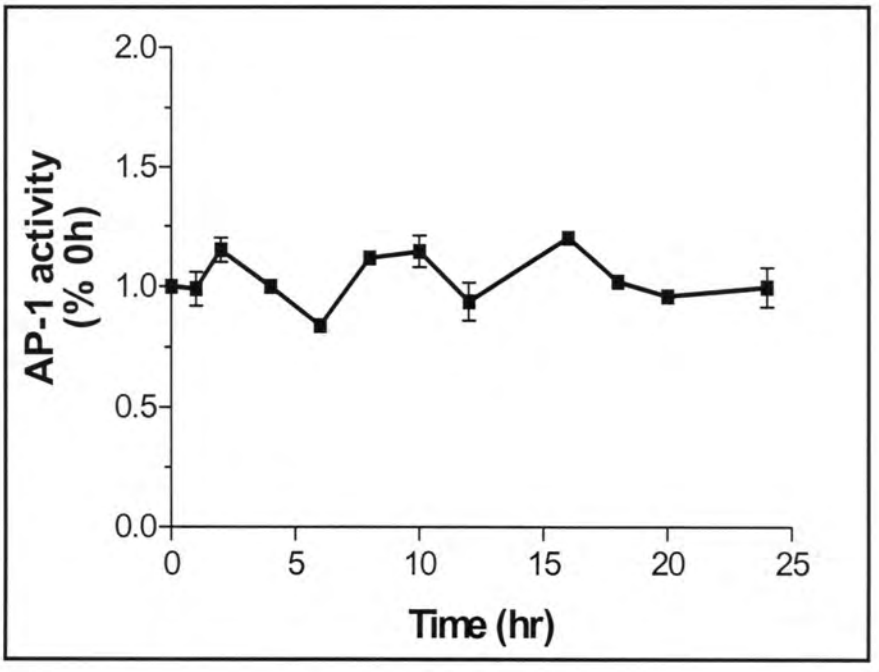

AP-1 activity
(% 0h)
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Time (hr)

Figure 5. ***Inhibiting MEK inhibits the FREBP in monolayer HeLa cell cultures.*** METHODS: HeLa cells were grown in monolayer (in DMEG, 10% CS and 10 u/ml P/S) and treated with for 2h and 4h with PD98059 in DMSO for the previously identified concentrations or with DMSO alone. Nuclear extracts were prepared and incubated with radiolabeled FRE oligonucleotide. The DNA-protein complexes were analyzed by EMSA. The DNA-protein complexes were quantitatively analyzed by exposing the gels to Phosphoimager and quantifying by Imagequant Software. The PD 98059 decreased the FREBP activity as compared to the activity levels seen in the control DMSO treated cells.
STATISTICAL ANALYSIS: Data represents N=4 Mean ± SEM. One way ANOVA analysis using Bonferroni's Multiple Comparison Test yielded significant differences between and DMSO and treatment groups *** = $p < 0.001$.

Fig 5

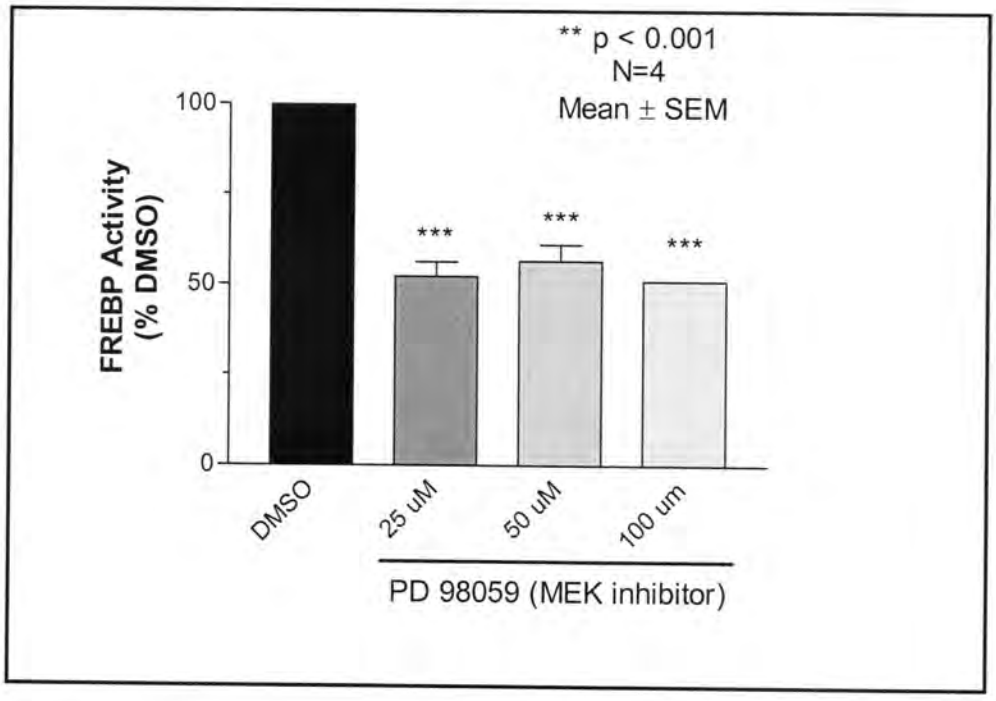

** p < 0.001
N=4
Mean ± SEM
FREBP Activity
(% DMSO)
100
50
0
***
***
***
DMSO
25 uM
50 uM
100 um
PD 98059 (MEK inhibitor)

Figure 6. ***Inhibiting PI-3 kinase, Src kinase and Ras does not change the FREBP activity.*** Inhibitors for PI-3 kinase (LY 294002), Src Kinase (PP2) and Ras (FTase) did not have any effect on the FREBP binding as seen in HeLa cell monolayer studies. METHODS: HeLa cells were grown in monolayer (in DMEG, 10% CS and 10 u/ml P/S) and treated with LY 294002 (50 uM), PP2 (30 uM) and FTase inhibitor (20 uM) for 2h. Nuclear extracts were prepared as described in material and methods and incubated with radiolabeled FRE oligonucleotide. The DNA and DNA-protein complexes were separated by electrophoresis and the mobility shifts analyzed by exposing the gel to a PhosphoImager and quantifying by Imagequant Software. Although the inhibitor treatments did show some reduction in values, it was not statistically significant. The negative controls for LY294002 (LY30) and PP2 (PP3) showed no difference from the inhibitors.
STATISTICAL ANALYSIS: Data represents N=4 Mean ± SEM. One way ANOVA analysis using Bonferroni's Multiple Comparison Test yielded no significant differences between and DMSO and treatment groups.

Fig 6

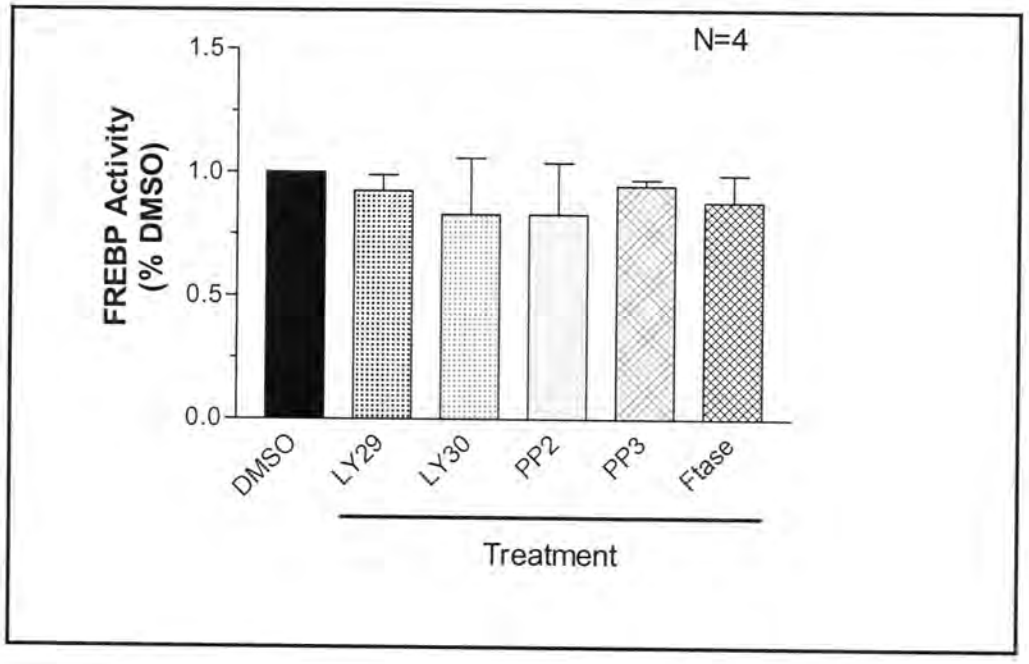
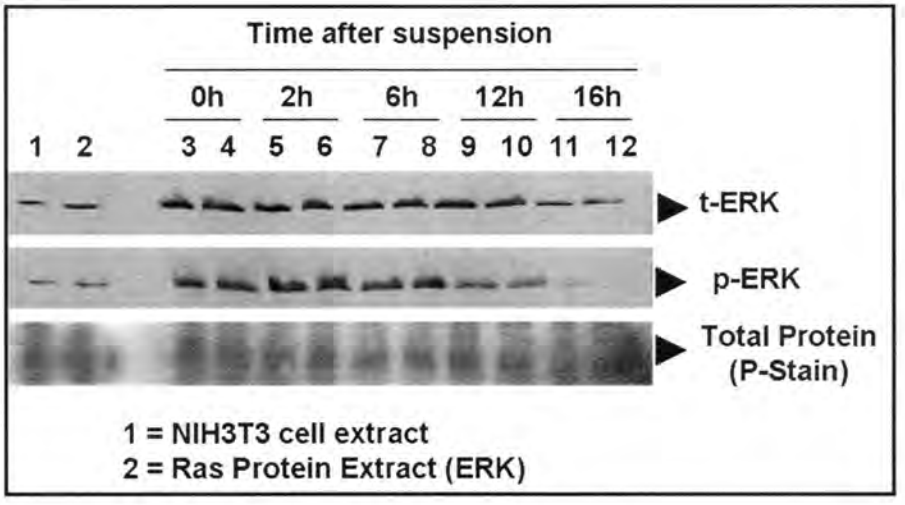

N=4
FREBP Activity
(% DMSO)
1.5
1.0
0.5
0.0
DMSO
LY29
LY30
PP2
PP3
Ftase
Treatment

Figure 7. ***MEK activity decreases more slowly than the FREBP activity when cells are placed in suspension cultures.*** **(A)** Gel pictures depict the time course of MEK activity in suspended HeLa cells for the time points identified. Antibodies for ERK, the direct substrate of MEK, were used (tERK: total ERK and pERK: Phospho ERK) and each sample normalized for tERK. **(B)** Summary of the results for MEK activity. METHODS: HeLa cells were grown in monolayer and put in suspension (using spinner flasks) for the time points identified. Whole cell extracts were prepared as described in Materials and Methods and the samples were analyzed by Western Blots. Antibody for tERK and pERK were used on the same membrane. The membrane was first stained with Ponceau S, then blotted for tERK, stripped and then blotted for pERK respectively. ECL detection kit was used for detecting proteins and the bands were quantified by using Kodak Imaging system.
STATISTICAL ANALYSIS: Data represents N=4 Mean ± SEM. One way ANOVA yielded no significant differences between analyzed groups at $p > 0.05$.

Fig 7

A


Time after suspension
0h
2h
6h
12h
16h
1 2 3 4 5 6 7 8 9 10 11 12
t-ERK
p-ERK
Total Protein
(P-Stain)
1 = NIH3T3 cell extract
2 = Ras Protein Extract (ERK)

B

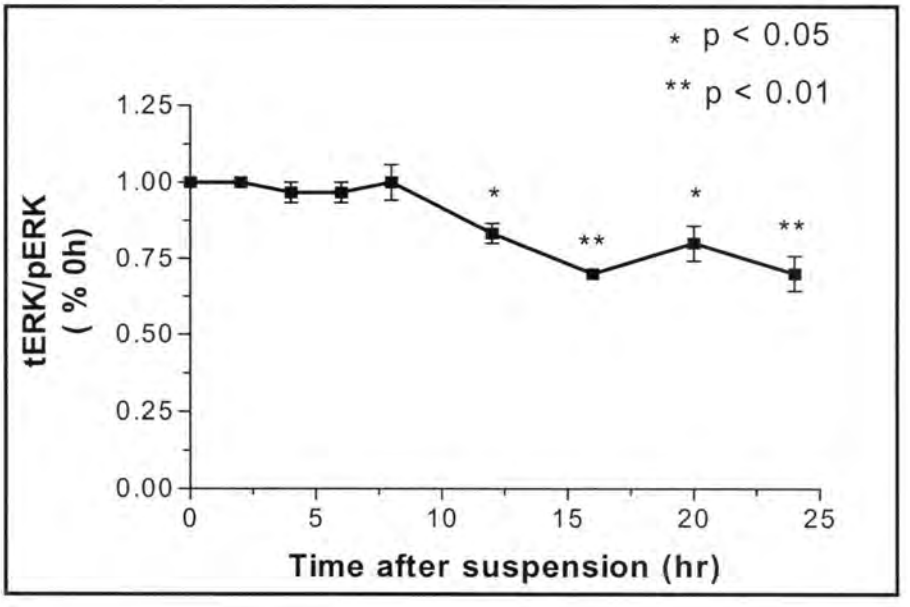

* p < 0.05
** p < 0.01
tERK/pERK
(% 0h)
1.25
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
Time after suspension (hr)

Figure 8. *FREBP activity is not affected by inhibiting MEK in suspension.* **(A)** Summary of the results for the FREBP activity after treatment with PD 98059 for 30 min or 12h. METHODS: HeLa cells were grown in monolayer and put in suspension (using spinner flasks and treated with 25 uM PD 98059 for the time points identified). Nuclear extracts were prepared as described in Materials and Methods. Samples were incubated with radiolabeled FRE oligonucleotide and DNA and DNA-protein complexes were separated by EMSA gel. The DNA-protein complexes were analyzed by exposing to Phosphoimager and quantified by Imagequant Software. Paired t-test analysis showed no significant differences between DMSO and PD+DMSO samples at 30 min and 12 h.
STATISTICAL ANALYSIS: Data represents N=4 Mean ± SEM. One way ANOVA analysis and paired t-test yielded no significant differences between analyzed groups at $p > 0.05$.

Fig 8

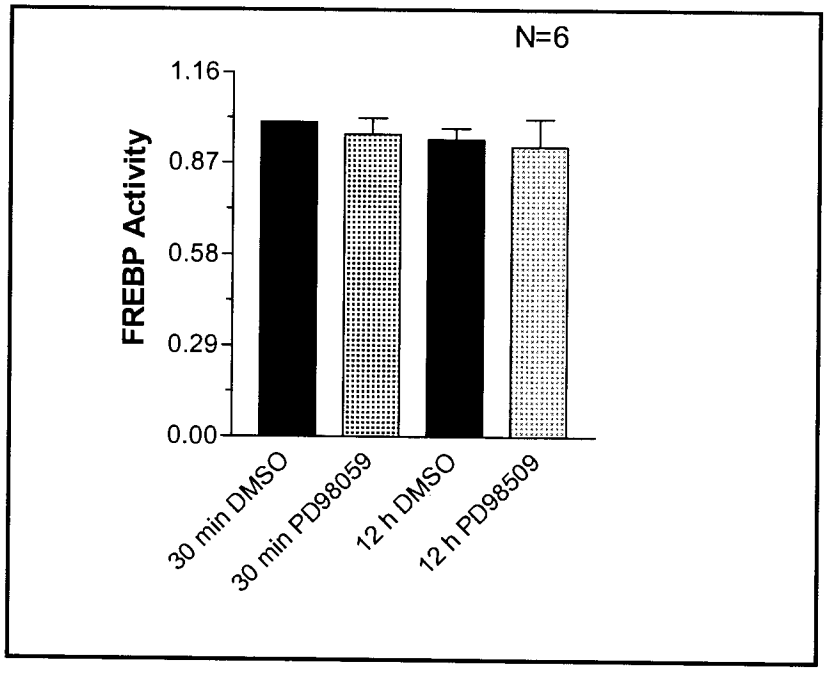
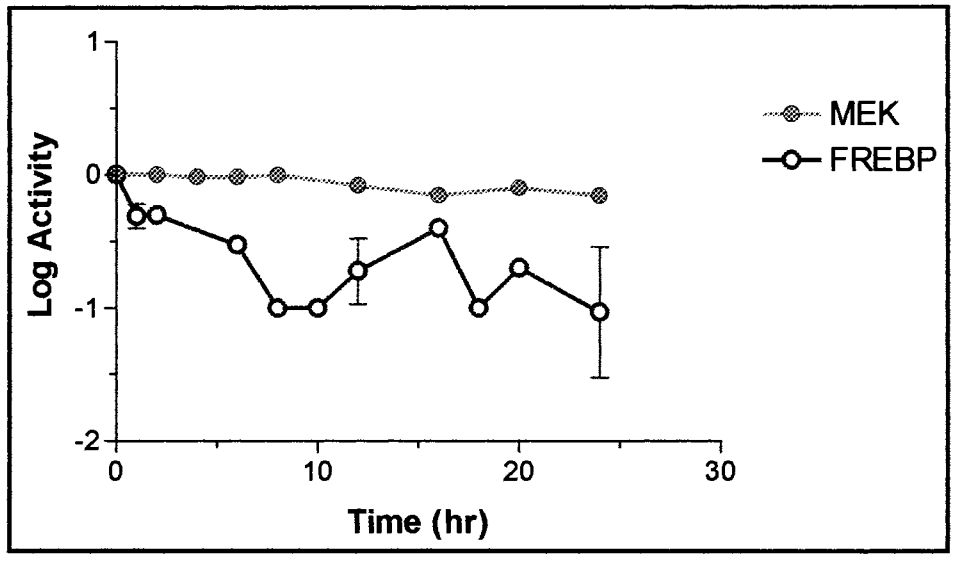

N=6
1.16
0.87
0.58
0.29
0.00
FREBP Activity
30 min DMSO
30 min PD98059
12 h DMSO
12 h PD98509

Figure 9. *Comparative analysis of decay in FREBP and MEK activity.* The FREBP decay is much faster compared to the MEK activity decay in suspended HeLa cells over the same time course. **(A)** Comparison of FREBP and MEK activity over ~24h. **(B)** Comparison of FREBP and MEK activity over ~8h. The Half-life of MEK is 18h and FREBP is 2.8h.

STATISTICAL ANALYSIS: Data represents N=5 Mean ± SEM. Half life was calculated using non-linear regression curve fitting using Graph Pad PRISM® Software.

Fig 9

A



Half Life
MEK: 18 hr
FREBP: 2.8 hr

B

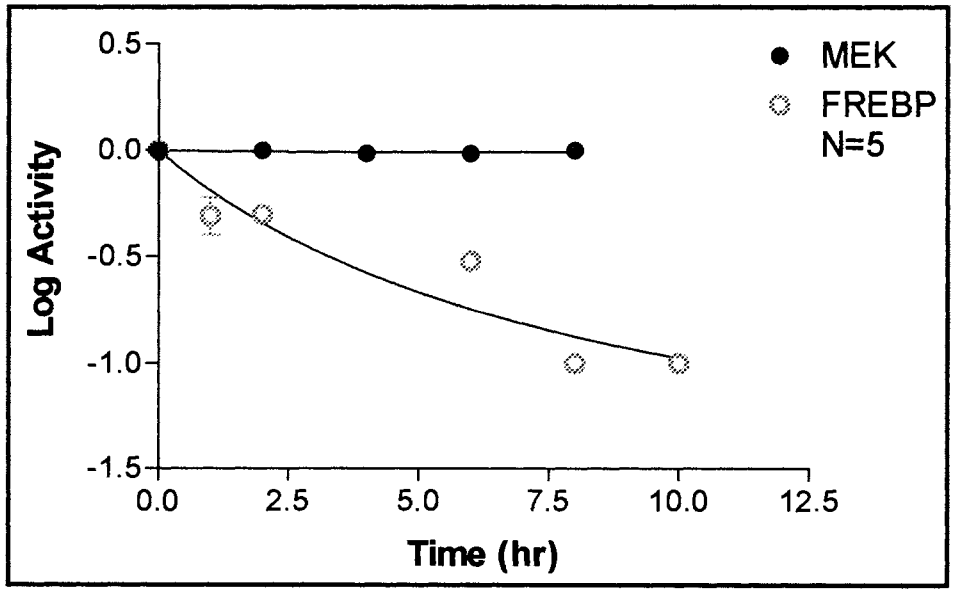


Figure 10. ***Effect of inhibiting p38 MAP kinase pathway on the FREBP activity.*** METHODS: HeLa cells were grown in monolayer (in DMEG, 10% CS and 10 u/ml P/S) and treated with for 2h with SB203580 in DMSO for the previously identified concentrations or with DMSO alone. Nuclear extracts were prepared and incubated with radiolabeled FRE oligonucleotide. The DNA-protein complexes were analyzed by EMSA. The SB 203580 decreased the FREBP activity by 40% as compared to the activity levels seen in the control DMSO treated cells. The significance of these results could not be determined as there were not enough experiments for statistical analysis.

Fig 10

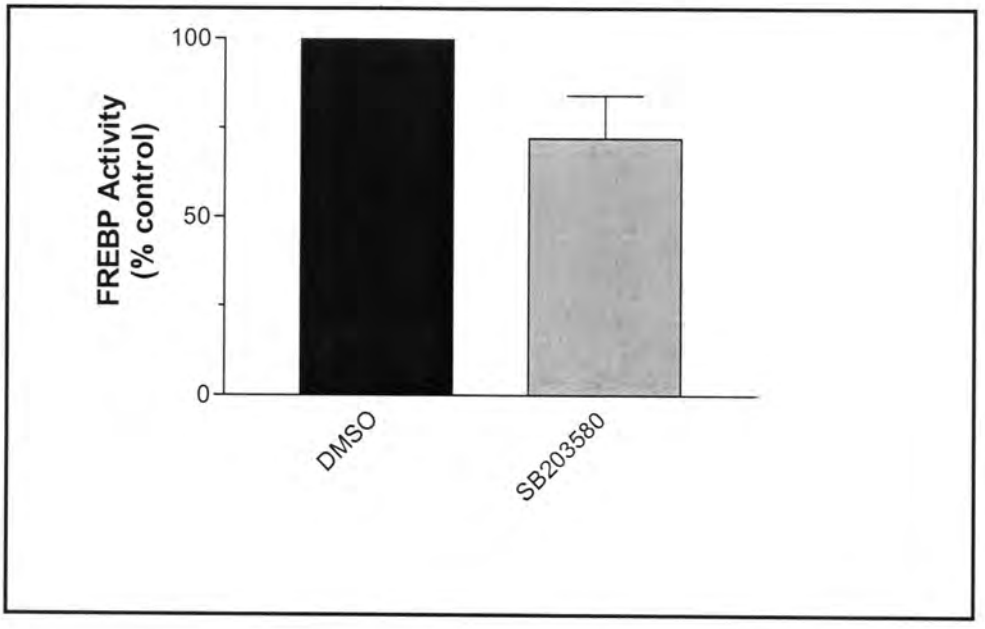
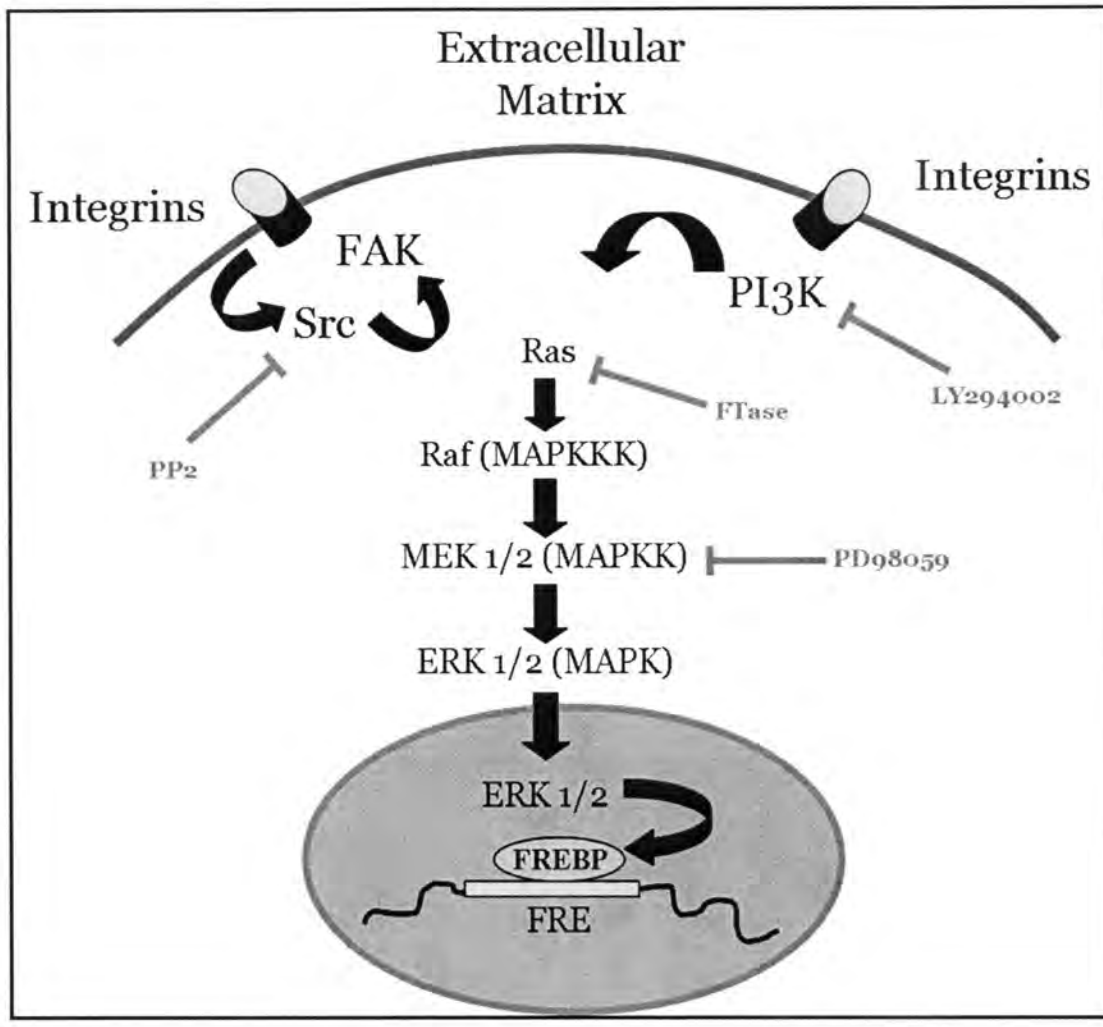


Figure 11. Summary chart. The molecules targeted from the MAP kinase pathway, PI-3 kinase pathway and FAK pathway are illustrated along with the inhibitors used for each one of above-mentioned molecules beside them.

Fig 11


Extracellular
Matrix
Integrins
Integrins
FAK
Src
PI3K
Ras
FTase
LY294002
PP2
Raf (MAPKKK)
MEK 1/2 (MAPKK)
PD98059
ERK 1/2 (MAPK)
ERK 1/2
FREBP
FRE

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