University of Strathclyde, Glasgow

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"Novel Cellular and Molecular Mechanisms of Action of Insulin-Like Growth Factor Binding Protein-5 (IGFBP-5) in Human Breast Cancer Cells"

By

Sureshbabu Angara

A thesis presented in fulfillment of the requirements for the degree of Doctor of Philosophy

2010

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Dedicated to...

My Parents

For their enormous support, for their abundant patience and for their endless love

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Abstract:

Insulin-Like Growth Factor Binding Protein-5 (IGFBP-5), added as exogenous protein or via adenoviral transduction, induced an increase in cell to substratum adhesion even in the presence of IGF-I. Adhesion induced by IGFBP-5 typically involves 3- or 4- point cell attachment. Further to this a mutant, comprising the c-terminal domain of IGFBP-5, partially mimicked the cell to substratum adhesion in MCF-7 cells indicating the adhesion property of IGFBP-5 resides in c-terminal domain. IGFBP-5 increased cell adhesion on collagen or fibronectin but it failed to do so when cells were cultured in the presence of laminin. Using function-blocking antibodies to various integrins, I showed that both β1 and α2- integrins were essential for this action of IGFBP-5. I have demonstrated the enhanced survival effects of IGFBP-5 in the presence of nutrient poor conditions in MCF-7 cells. Consistent with this, I demonstrated that IGFBP-5 increased expression of integrin-linked kinase (ILK), sustained phosphorylation of Akt, p53 and decreased phosphorylation of p38MAPK and β-catenin. Focal Adhesion Kinase (FAK) was not activated and consistent with this, IGFBP-5 significantly inhibited the migration of MCF-7 cells in response to IGF-I, EGF and serum. Inhibitors of PI-3 kinase, or Erk1/2 kinases failed to inhibit the actions of IGFBP-5 whereas an inhibitor of p38MAPK actually enhanced the actions of IGFBP-5. Using immunofluorescence, I demonstrated that altered expression of E-cadherin, and actin stress fibres in IGFBP-5-treated MCF-7 cells. These results suggest that IGFBP-5 binds to α2β1 integrins with high affinity interaction and induces cell to extracellular matrix adhesion and facilitates epithelial cell survival and repair at sites of injury, whilst restricting their migration and thereby limits epithelial-mesenchymal transgressions.

Chapter I

Introduction

INTRODUCTION

1. Breast Cancer Metastasis

Breast cancer is the most common cause of cancer-related deaths and the most frequently diagnosed malignancy among women worldwide (Jemal et al., 2007). More than 1.1 million women worldwide are newly diagnosed with breast cancer annually, representing about 10% of all new cancer cases and 23% of all female cancers. With more than 410,000 deaths each year, breast cancer accounts for about 14% of all female cancer deaths and 1.6% of all female deaths worldwide (http://www.who.int/). Worldwide, 40-70% of patients ultimately develop metastatic disease (Smith 2006). Early detection of breast cancer and optimal combination of surgery, chemotherapy, hormone therapy, and radiation therapy result in a decrease of local recurrence or development of metastatic cancer, contributing to the reduction of deaths by more than 50% (Hortobagyi, 2000). Although statistics do not show the entire scenario about the impact of breast cancer, there is a factor driving the need to improve detection and treatment. Advanced breast cancer is still a serious healthcare problem as the metastatic disease is incurable by today's standard of treatment. Thus, identifying new therapeutic targets and solving the problems of endocrine (hormonal therapy) and chemoresistance (chemotherapy) is of paramount importance. The recent (limited) success of interference with the HER2 receptor (Erbb2) and tyrosine kinase inhibitors has indicated that growth factor receptors and peptide growth factors may be potential targets of clinical importance (Hirata et al., 2005). Recently, Valastyan and his colleagues identified a

micro RNA miR-31 and detected an inverse correlation between its expression with breast cancer metastasis (Valastyan et al., 2009).

A more comprehensive knowledge of each step of very heterogeneous breast cancer disease is pivotal for the development of therapeutic strategies for breast cancer prevention and cure. To this end, we need to understand the biology of mammary gland in-depth including its normal development, physiology and disease.

Mammary Gland Development: Overview

The mammary gland is a dynamic bilateral organ that undergoes a series of unique developmental features. It goes through embryonal, puberty, pregnancy, lactation and post-lactation phases, and many of the genetic determinants that regulate cell specification, proliferation, differentiation, survival and death have been studied in great detail. Hence, it provides an excellent source for experimental biologists to study both normal development and tumorigenesis.

Mammary glands are tubuloalveolar exocrine glands composed of three tissues: (i) Epithelial tissue, (ii) Adipose tissue, and (iii) Connective tissue, stroma. The rat mammary gland epithelium comprises of continuous, branching ducts emanating from the nipple and leading to smaller ducts, called ductules that terminate in terminal end buds (TEBs), blunt end buds, or alveoli (Masso-Welch et al., 2000). In general, the development of mammary gland is broadly separated into embryonic, adolescent and adult stages, each of which is differentially orchestrated with the presence of number of growth factors and hormones.

The Cell Populations of the Mammary Gland:

In the developing human mammary gland, cell populations are broadly divided into two compartments: (i) the epithelial, also known as parenchymal compartment and (ii) the stromal, also known as mesenchymal compartment. The inner luminal cell population is separated from the basement membrane by an outer basal or myoepithelial layer. Myoepithelial cells secrete continuous basement membrane that separates the epithelium from the connective tissue-rich stromal compartment. Basal cells are in the ducts in a basal position from the myoepithelial cells in the acini, which are true secreting cells. As per the definition, a group of acini arising from one terminal duct and embedded in intralobular stroma is known as terminal duct lobular unit (TDLU). TDLU is generally considered as the functional unit of breast. The absence of myoepithelial cells and its derived extracellular matrix is observed during neoplastic development of mammary gland and indicates invasive carcinoma (Fernig et al., 1991).

Table 1.1: ***General representation of histological and cellular changes in the stages of human mammary gland***

Stage	Ductal System	Epithelial Changes	Stromal changes	References
Prenatal	-	Condensation in thoracic/pectoral region of embryo where breast bud forms	Epithelial - mesenchymal reciprocal interactions	(Howard and Gusterson, 2000)
Postnatal	-	Bilateral epidermal ridge; Appearance of distinct placodes, mammary bulbs and cone budding	Epithelial - mesenchymal interactions	(Sternlicht, 2006)
New Born Baby	Rudimentary; Dilated ducts ending	Secretory type epithelial cells		(Vorherr, 1974)

	in short ductules.	with eosinophilic cytoplasm		
Adolescent/Peri pubertal woman	Dichotomous branching; Type 1 lobules - High Type 2 lobules - Moderate	Lateral bud formation with bulbous and solid epithelial tips	Increase in fibrous and fatty tissue; Stromal expansion	(Russo and Russo, 2004)
Adult/Nulliparous woman	No additional changes were observed	-	Concentric arrangement of fibroblasts around TDLU; Flattened stromal cells.	(Russo and Russo, 2004)
Pregnant/Parous woman	Acini are evident; Type 3 lobules - High	Alveoli separate into secretory cells and release milk	Collection of fat gobules. Rise in secretory cells	(Russo and Russo, 2004)
Lactation	Enlargement and stimulation of ductal system	Dilated acini containing milk	Prolactin and oxytocin active	(Howard and Gusterson, 2000)
Premenopausal involution	-	-	ECM remodelling, inflammation	(Asztalos et al., 2010)
Postmenopausal involution	-	Glandular epithelium is replaced by adipocytes	Interlobular stroma is replaced by collagen	(Howard and Gusterson, 2000)

Insulin/Insulin-Like Growth Factor (IGF) system

During the past ten years, multiple epidemiologic studies have correlated high circulating IGF-I levels with greater risk for different types of cancer (Pollak, 2008). The possible involvement of IGFs in cancer was observed initially in cell culture experiments (Macaulay, 1992) and transgene animal technology (DiGiovanni et al., 2000). Nevertheless, increased understanding of IGF system members and their

divergent pathways will similarly lead to effective therapeutic strategies for breast cancer patients.

The IGF axis comprises two cell membrane receptors namely IGF-IR & IGF-IIR with their respective polypeptide ligands, IGF-I & IGF-II and six well characterized high-affinity IGF-binding proteins (IGFBPs), isolated and characterized from a variety of vertebrate species, including humans. In addition, a large group of IGFBP proteases hydrolyze IGFBPs, resulting in the release of bound IGFs that then resume their ability to interact with IGF-IR.

Table 1.2: ***Molecular features of members of the IGF family.*** *(Herbert Yu et al. 2000)*

IGF Member	M.W. (kd)	No. of Aminoacids	Gene Location	Gene Size, Kb	No. of exons
IGF-I	**7.7**	**70**	**12q22–12q24**	**100**	**6**
IGF-II	**7.5**	**67**	**11p15**	**30**	**9**
IGF-IR	**225**	**α subunit: 706** **β subunit: 626**	**15q25–15q26**	**100**	**21**
IGF-IIR	**270**	**2450**	**6q25–6q27**	**140**	**Unknown**
IGFBP-1	**25.3**	**234**	**7p12–7p14**	**5.2**	**4**
IGFBP-2	**31.4**	**289**	**2q31–2q34**	**32**	**4**
IGFBP-3	**28.7**	**264**	**7p12–7p14**	**8.9**	**5**
IGFBP-4	**26.0**	**237**	**17q12–17q21**	**12**	**4**
IGFBP-5	**28.6**	**252**	**2q31–2q24**	**33**	**4**
IGFBP-6	**22.8**	**216**	**12q13**	**4.7**	**4**

Insulin-Like Growth Factors (IGFs)

The IGFs were discovered on the basis of their ability to increase cartilage sulfation and to replace the "sulfation factor activity" of growth hormone (Jones and Clemmons, 1995). IGFs are single chain polypeptides that share structural homology with insulin.

These IGFs are mitogens that play a critical role in regulating cellular proliferation, survival, differentiation, and prevention of apoptosis. This paramount function of IGFs has led to speculation concerning their possible involvement in cancer development and growth (Herbert. Yu., 2000) and, there has been mounting evidence suggesting that the IGF system is a potential target for breast cancer therapy (Lann, 2008).

IGF Receptors

IGFs bind mainly to two cell surface receptors IGF type I receptor (IGF-IR) and IGF type II receptor (IGF-IIR) and with the insulin receptor (IR). However, IGFs bind to IGF-IR at significantly higher affinity than IR or IGF-II R (Milazzo et al., 1992).

IGF-IR

The IGF-IR is a tyrosine kinase-containing transmembrane protein that plays a pivotal role in regulation of cell growth. IGF-IR is a disulphide-linked heterotetrameric glycoprotein composed of two ligand-binding α-subunits of 706 amino acid and two transmembrane β-subunits of 627 residues. The human protein is produced by mRNAs derived from the single 21-exon IGF-I-R gene, located on chromosome 15q25-q26 (Abbott, 1992; Ullrich, 1986).

The IGF-I receptor is ubiquitously expressed with highest levels seen during embryonic development and organogenesis and plays an important role in tissue homeostasis via regulation of proliferation, differentiation, and cell survival. (Himpe, 2009). Mice homozygous for a null mutation of this receptor are small in size and die at birth. Their

inability to develop properly is largely due to respiratory failure associated with underdeveloped respiratory muscles as well as poorly developed lungs and brains, and decreased bone ossification (Baker et al., 1993; Liu, 1993a).

IGF-IIR

The IGF-II-R, also known as cation-independent Mannose-6-phosphate (M-6-P) receptor is a single-chain membrane-spanning glycoprotein. The IGF-II-R is highly conserved among different species, with approximately 80% homology being found among bovine, rat, mouse, and human receptors (Kornfeld, 1992). The mature human receptor contains 2,451 amino acids that can be divided into three regions, a large 2,264-residue extracellular domain, a 23-amino acid transmembrane region, and a 164-residue carboxyl-terminal cytoplasmic domain (Morgan, 1987; Oshima, 1988). The extracellular domain of each receptor is comprised of 15 contiguous segments of 134-191 residues repeats that share 16-38% identity (Lobel et al., 1988; Morgan, 1987; Szebenyi and Rotwein, 1994; Zhou et al., 1995). The extracellular part of the IGF-IIR binds ligand, and the cytoplasmic region encodes segments responsible for interaction with different subcellular compartments (Lobel et al., 1988; Rohrer et al., 1995), including those involved in endocytosis (Lobel et al., 1988), and potentially for coupling to inhibitory GTP-binding proteins (Nishimoto I, 1987; Okamoto T, 1990).

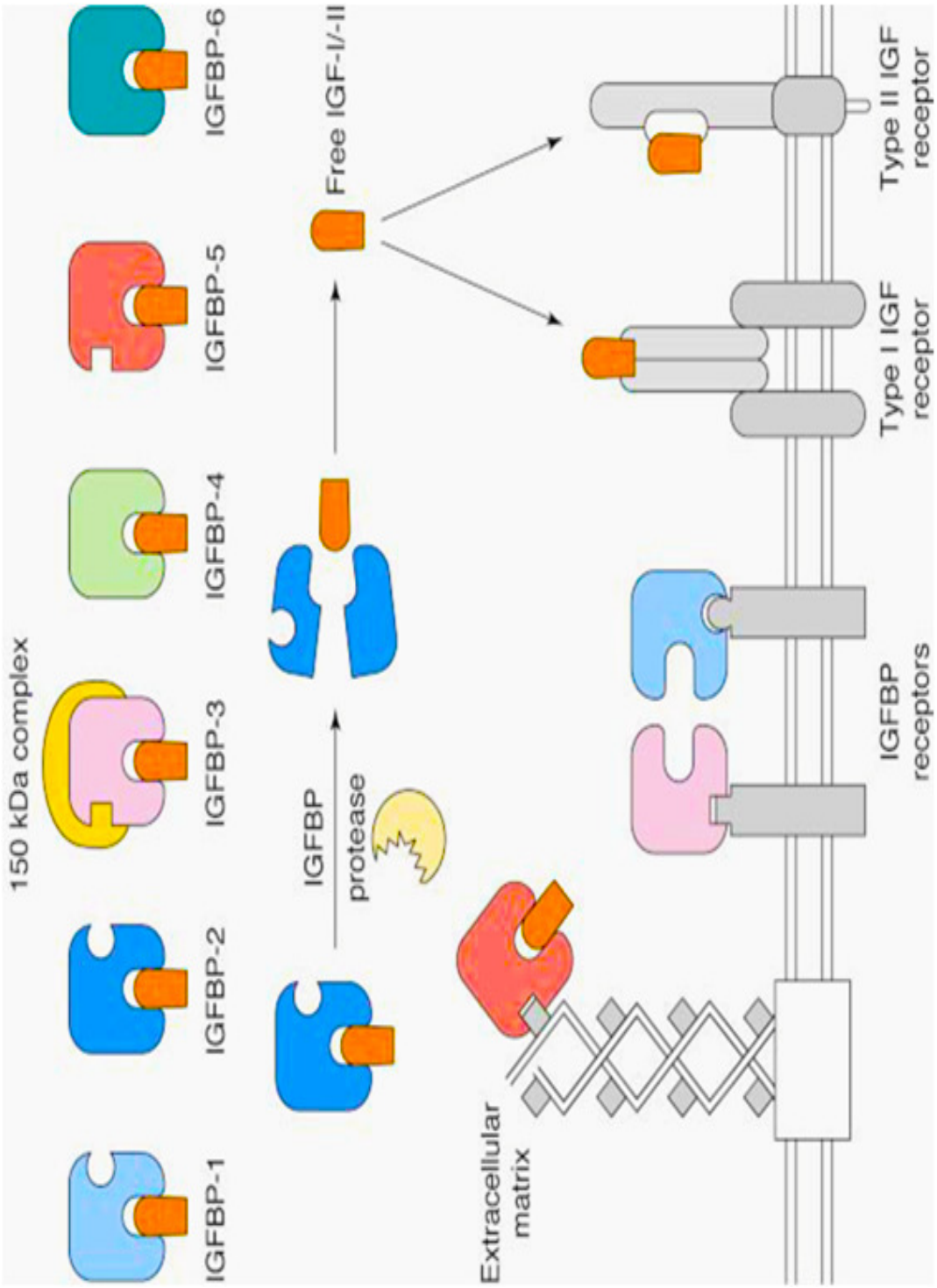


Fig 1.1: ***Schematic representation of components of the IGF system*** *(Allan et al., 2001).*

Functional Aspects of IGFs

The *in vivo* effects of IGFs involve mainly stimulation of glucose uptake and glycogenesis in fat and muscle cells (Froesch et al., 1996) and a growth promoting effect through mediation of growth hormone effects (Salmon and DuVall, 1970). Stimulation of cell proliferation in a large variety of organs and tissues by IGFs has also been observed including nervous system development (Heidenreich, 1993), osteoblasts (Hock et al., 1988), chondrocytes (Ohlsson, 1992), bone endothelial cells (Fiorelli, 1994) hormone synthesis in ovary (Christman, 1991; Davoren, 1985; Erickson, 1991; Hernandez, 1988; Talavera, 1991) spermatogonial cells in testes (Soder, 1992) and various cancer cells (LeRoith, 1995).

The IGFs have been well documented to play an important role in cellular growth, differentiation and apoptosis (Kim, 2002; Valentinis, 2001). IGFs stimulate a mitogenic response in many cell types whereas in haematopoietic cells and in some carcinoma cell lines they can function as survival factors preventing apoptosis. *In vitro*, IGFs promote cell cycle progression from the Go\G1 to the S phase, resulting in DNA synthesis and cell proliferation (LeRoith and Roberts, 1993). Proliferation and apoptosis signals may share some common pathways as they both induce similar changes in the morphology of the cells (chromatin condensation, loss of cell-cell contact inhibition, nuclear disintegration). This is further supported by the observation of over-expression of proteins normally associated with cell proliferation in various cell types (Askew, 1991; Hartwell, 1994; Steller, 1995; White, 1991). However, IGF-I has also been shown to support viability in non-proliferating cells in culture (Beck et al., 1994; Carlsson-

Skwirut et al., 1989; LeRoith and Roberts, 1993; Svrzic and Schubert, 1990) suggesting that stimulation of cell proliferation and anti-apoptotic actions may be two distinct signals (Rubin and Baserga, 1995).

IGFs stimulate cell differentiation in osteoblasts (Hall and Chambers, 1990), chondrocytes (Geduspan, 1993), adipocytes and neural cells (Pahlman et al., 1991), and induce an acute insulin-like effect on protein and carbohydrate metabolism, especially in myoblasts (Dimitriadis et al., 1992) They also regulate the hormone secretion or induction of chemotactic migration in specific cell types (El-Badry et al., 1990; Stracke et al., 1989).

IGF-mediated cell survival

There are multiple cellular pathways through which the IGF system can regulate cell survival (Vincent and Feldman, 2002). Activation of IGF-IR on epithelial cells occurs through growth hormone (GH) acting on the growth hormone receptor (GHR) which induces IGF-I from the stromal cells. This signaling is particularly important to mediate epithelial cell survival and proliferation during development. Similarly, other hormones like estrogen can also induce IGF-I expression, which may then act on adjacent mammary epithelial cells. The basement membrane provides an interface between stroma and epithelial cells, and it can contribute to the signals required for mammary development through integrin receptors. Epidermal growth factor (EGF) can synergize with IGF-I, and IGF-I can transactivate the EGF receptor (EGFR) and estrogen receptor (ER).

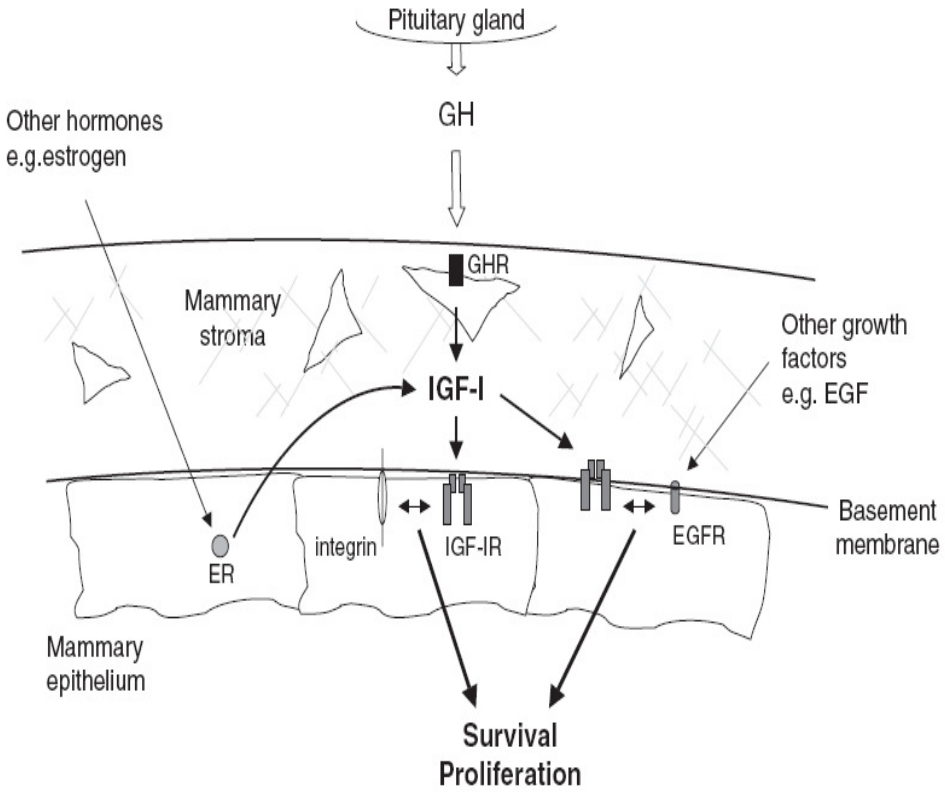


Fig 1.2: ***Schematic diagram representing IGF-I signaling in the mammary gland*** *(Marshman and Streuli, 2002).*

Insulin-Like Growth Factor Binding Proteins (IGFBPs)

The pleiotropic activities of the IGF system are regulated by interaction with a family of six conserved and well characterized IGFBPs, IGFBP-1 to IGFBP-6. These IGFBPs are ubiquitously expressed by a variety of different tissues, and each tissue has specific patterns of IGFBP secretion. IGFBPs are traditionally thought to act as carrier proteins that regulate the IGFs activity by prolonging their half life and circulation turnover. These IGFBPs range in size from 216 (IGFBP-6) - 289 (IGFBP-2) amino acids, typically translated from four exons, with molecular masses between 24 and 44 kDa (Beattie et al., 2006; Firth and Baxter, 2002). All six proteins contain 16 - 20 cysteine residues revealing the concept these are cysteine-rich proteins (IGFBP-4 contains two additional cysteines in the central domain). They share a highly conserved protein domain structure consisting of three domains (N, L and C). The N and C domains contain IGF-binding sites, and the L (central) domain comprises sites affected by posttranslational modifications including phosphorylation and proteolytic degradation. Analysis of the amino acid sequence reveals that the IGFBPs are clearly distinct but share regions containing strong homology. That these proteins play an important role in cellular metabolism is apparent as they associate and modulate the biological actions of IGFs by interference with receptor binding and by regulating tissue availability and turnover of these growth factors (Akkiprik et al., 2008; Bach et al., 2005).

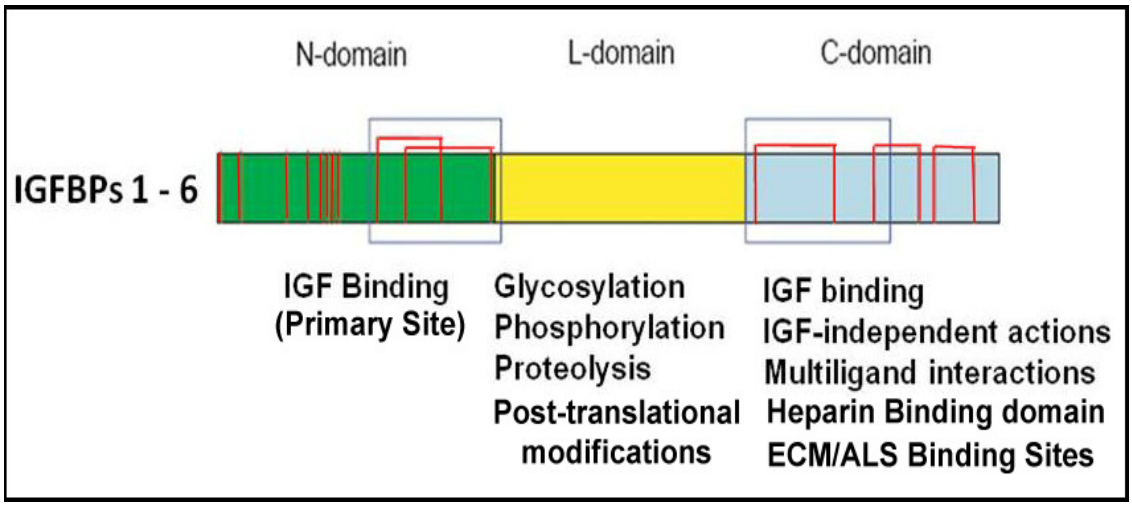


Fig 1.3: ***General Domain Organization of IGFBPs*** *(Bach et al., 2005).*

Regulation of IGFBP gene transcription is complex and tissue-specific. A number of hormones including estrogens, glucocorticoids, parathyroid hormone, FSH, GH, thyroid hormone, insulin, vitamin D, and cortisol and multiple growth factors, including FGF, EGF, TGF-β, PDGF, and IGFs themselves, as well as retinoic acid have been found to regulate the expression of IGFBPs (Akkiprik et al., 2008).

IGF Binding Motifs

IGFBPs are highly structured molecules and hence three dimensional structures provide a solid baseline to understand their IGF-dependent biological functions. Although the structure of IGFBPs have not been determined by X- ray crystallography, it is believed that the N- and C-terminal domains of approximately 30 kDa IGFBP proteins make contact with approximately 7.5kDa IGF molecules. Thus, the general structure of the IGFBP protein may be envisioned as two highly folded terminal domains in close contact, connected by a flexible central domain. To date, many researchers have performed experiments aimed at identifying the IGF binding motifs in N- and C-terminal domains of IGFBPs (Bach et al., 2005).

Structure of N-terminal domains of IGFBPs

The only defined function of the N- terminal domain is its high affinity to IGF-binding. The three-dimensional structure of an N- terminal domain region of IGFBP-5 (mini BP-5) has been elucidated. Mini BP-5 that is important for IGF binding, consists of a compact three-stranded β-sheet and a short α- helix, stabilized by two disulfide bonds (Kalus et al., 1998; Zeslawski et al., 2001). As shown in Figure 1.4, a crystal structure of the IGF-I–mini-IGFBP-5 complex revealed that the side chains of Glu3, Phe16, and

Leu54 of IGF - I were inserted into two clefts on the surface of mini BP-5 consisting of solvent-exposed hydrophobic side chains (Zeslawski et al., 2001). Based on this study, Imai and colleagues mutated several amino acids within this hydrophobic patch between residues Val49 and Leu74 in both IGFBP-3 and -5. They found that the substitutions Lys68Asn, Pro69Gln, Leu70Gln, Leu73Gln, and Leu74Gln in IGFBP-5 (changing one charged residue, Lys68, to a neutral one and the four hydrophobic residues to nonhydrophobic residues) resulted in an ~1000-fold reduction in the affinity of IGFBP-5 for IGF-I. In conclusion, this study showed residues 68, 69, 70, 73, and 74 in IGFBP-5, as well as the homologous residues in IGFBP-3, appear to be critical for high affinity binding to IGF-I. (Imai et al., 2000).

Structure of C-terminal domains of IGFBPs

The three dimensional structure of the C- terminal domain region of IGFBP-6 (C-BP-6) has also been elucidated. C-BP-6 adopts a thyroglobulin type-1 fold, with an initial α-helix followed by a three-stranded antiparallel β-sheet (Headey et al., 2004). In the Headey study, the IGF-II-binding site on C-BP-6 comprises a largely hydrophobic surface patch involving the a-helix, the first b-strand and the first and second loops. In contrast to N- domain binding site, binding interaction of C- terminal domain and IGF-II involves several relatively weak contacts. This binding site is largely consistent with mutation studies indicating that the proximal half of the C- terminal domain is important for IGF binding (Forbes et al., 1998). However, these authors also identified a greater reduction of affinity to both IGFs, when 63 residues were deleted from the C- terminal domain of bovine IGFBP-2. Therefore, these studies also identified a critical region between amino acids 222-236 of bovine IGFBP-2 that is involved in binding IGFs.

Figure 1.5 shows the binding interaction of C- terminal domain region of IGFBP-6 and IGF-I and mini BP-5 (Bach et al., 2005).

Further to the IGF binding sites, considerable interest was generated by observations from Baxter's group and his colleagues. They demonstrated using fluorescently labelled IGFBP-3 and IGFBP-5 translocates to the nucleus via β-importin dependent pathway in T47D breast cancer cells (Schedlich et al., 2000).

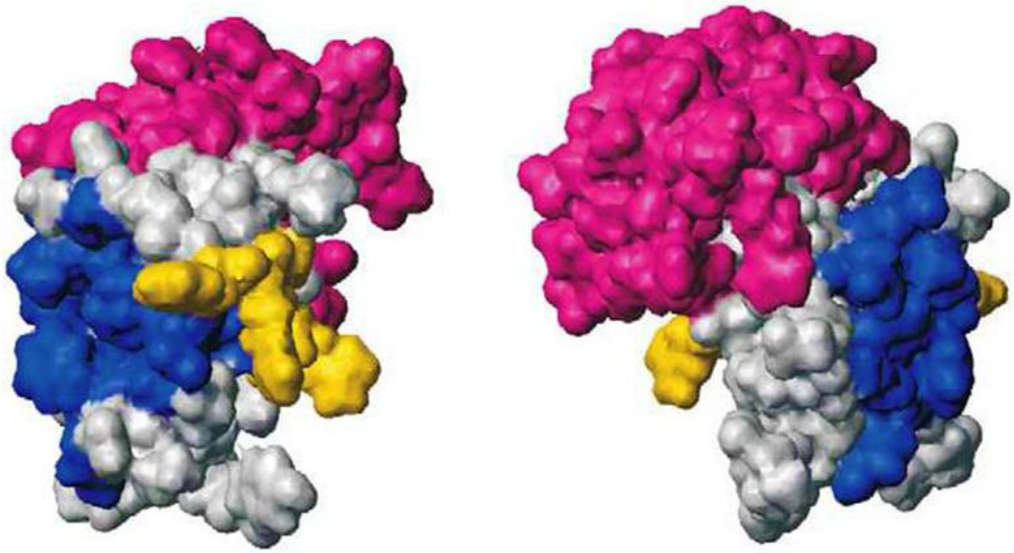


Fig 1.4. ***The general 3D structure showing binding of N- domain of IGFBPs to IGF-I. Mini-BP5 (in pink) complex with IGF-I (in white, yellow and blue. The right view is rotated ~ 120^{o}about the vertical axis relative to the left*** *(Bach et al., 2005).*

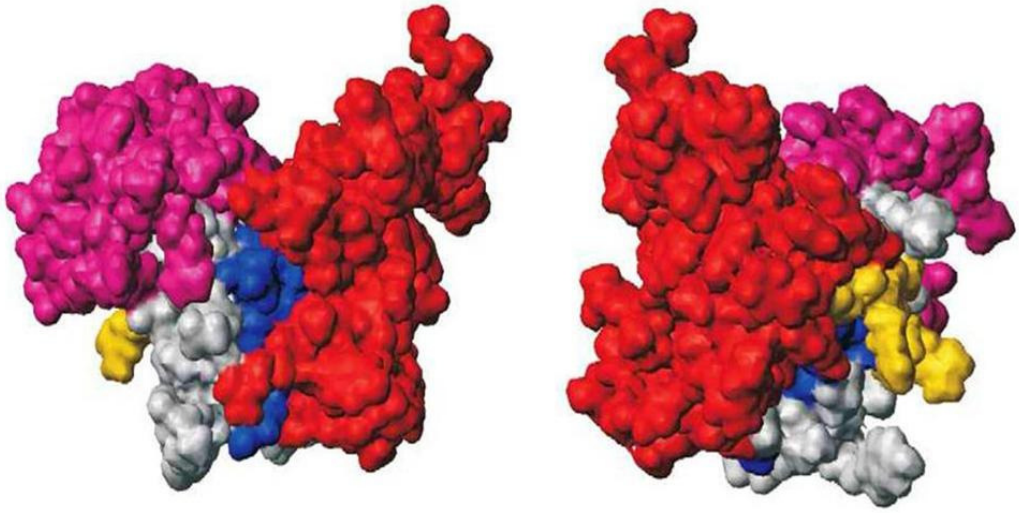


Fig 1.5. ***The general 3D structure of C-BP-6–IGF-I–mini-BP-5 complex. This model structure was generated by docking NMR structures of C-BP-6 (in red) with the IGF-I–mini-BP-5 crystal structure using high ambiguity driven protein–protein docking program*** *(Bach et al., 2005; Dominguez et al., 2003).*

Biological Functions of IGFBPs

IGFBPs regulate cellular metabolism by both IGF-dependent and IGF-independent effects.

IGF-dependent functions of IGFBPs

When binding to IGFs, IGFBPs exhibit three major functions: 1) transporting IGFs 2) protecting IGFs from degradation and that protection can promote the action of IGFs by increasing their bioavailability in local tissue 3) regulating the interaction between IGFs and IGF-IR. The schematic representation of binding of IGFBP-5 to IGF-I and sequential blockade activation of IGF signaling is depicted in Figure 1.6 (Akkiprik et al., 2008). If IGFBP-5 is degraded by IGFBP proteases, the concentration of free IGF increases in the circulation and leads to enhanced IGF actions.

IGF-independent functions of IGFBPs

During the last decade, there has been increasing evidence of IGF-independent effects of IGFBPs. IGF-independent actions of IGFBPs are briefly discussed below:

Ligand-independent actions of IGFBPs include effects on cellular migration, growth and apoptosis (Mohan and Baylink, 2002). In addition to stimulatory effects, recent evidence demonstrated that IGFBP-1 treatment caused an increase in apoptosis in breast cancer cells by a mechanism involving IGFBP-1 binding to integrin and dephosphorylation of focal adhesion kinase (Perks et al., 1999).

IGFBP-3 studies by Villaudy's group first suggested that IGFBP-3 inhibited mouse fibroblast growth factor-stimulated DNA synthesis in chick embryo fibroblasts lacking functional IGFs (Villaudy et al., 1991). The lack of involvement of the IGF-I receptor in growth inhibition by IGFBP-3 has been shown more definitively by the use of a mouse fibroblast cell line with a disrupted IGF-IR gene (Valentinis et al., 1995). In addition to its effect on growth inhibition, there is substantial evidence showing that IGFBP-3 induces apoptosis via an IGF-independent mechanism (Hong et al., 2002; Rajah et al., 1997; Zadeh and Binoux, 1997).

Recently, IGFBP-4 studies by Zhu's group first reported that IGFBP-4 enhanced cardiomyocyte differentiation *in vitro* (Zhu et al., 2008). The effect of IGFBP-4 as a cardiogenic growth factor was independent of its IGF-binding activity but was intervened by the inhibitory effect on canonical Wnt signaling.

Similarly, there has been increasing evidence that the biological actions of IGFBP-5 treatment involving increased differentiation in osteoblasts are mediated via a mechanism independent of IGFs (Richman et al., 1999). Figures 1.6 and 1.7 gives a schematic representations of IGF- dependent and independent effects of IGF respectively. Thus, several lines of evidence suggest that IGFBPs act as multifunctional regulators with actions ranging from the traditional role of prolonging the half-life of the IGFs to functioning as growth factors independent of the IGFs.

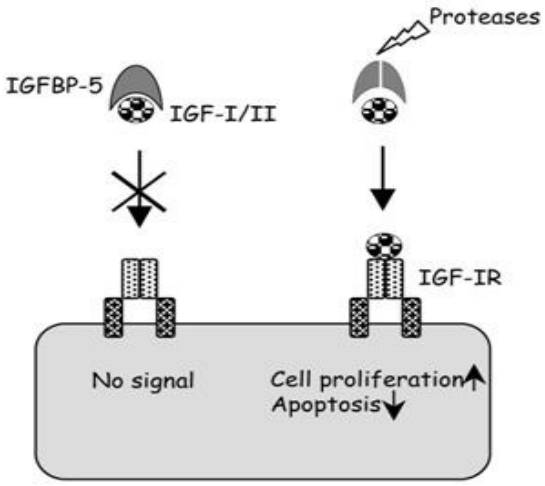


Fig 1.6: ***Schematic presentations of insulin-like growth factor (IGF)-dependent effects of IGFBP-5 in IGF-signalling.***

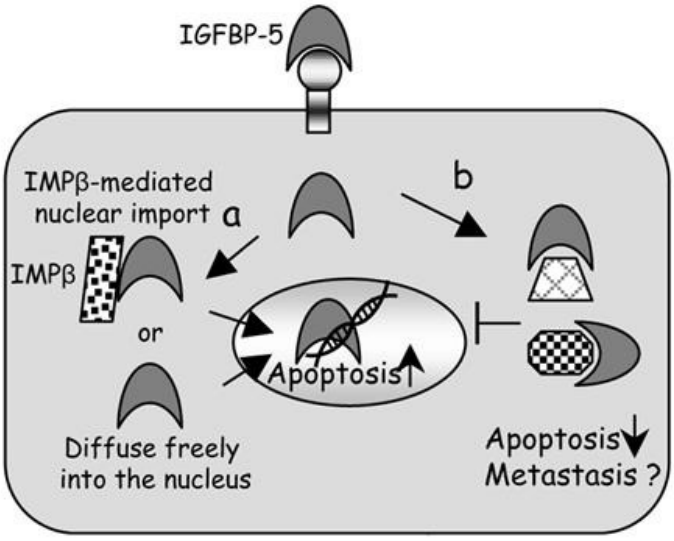


Fig 1.7: ***Schematic presentation of IGF-independent effects of IGFBP-5 in breast cancer. (a): nuclear import of IGFBP-5 – there are two mechanisms through which IGFBP-5 enters the nucleus: importin-β (IMPβ)-mediated or diffusion. (b): cytoplasmic accumulation of IGFBP-5 by interaction with other proteins, and stimulation of antiapoptotic effects and metastasis.****(Akkiprik et al., 2008)*

IGFBP-5 Gene and Protein

Allander and colleagues first cloned and characterized the genomic structure of IGFBP-5 (Allander et al., 1994; Zhu et al., 1993). The length of IGFBP-5 gene is 33 kb and located on chromosome 2 in humans. This gene comprises four exons which encodes a soluble protein of approximately 29 kDa expressed in various types of tissues. The IGFBP-5 gene is located on the same chromosome as the IGFBP-2 gene but is oriented in a tail-to-tail fashion (transcription directs in opposite direction). The IGFBP-1 and IGFBP-3 genes also show the same structural orientation on chromosome 7 (Ehrenborg et al., 1992).

Transcriptional regulation of IGFBP-5 expression

Schemer and colleagues examined a significant increase in IGFBP-5 concentration in the conditioned medium and only a slight increase in cellular extract when T47D breast cancer cells were incubated with IGF-I (Shemer et al., 1993). In contrast, IGF-I treatment had no significant effect on the IGFBP-5 mRNA level. In addition, blocking the IGF-IR using a specific monoclonal antibody did not change the results, suggesting that IGF-I regulates IGFBP-5 through both receptor-dependent and receptor-independent mechanisms (Shemer et al., 1993).

Kou and colleagues analyzed the promoter sequence of IGFBP-5 by luciferase assays in the mouse C2 myoblasts, and found that 156 nucleotides upstream of the start codon is critical for the promoter activity of IGFBP-5 (Kou et al., 1994; Kou et al., 1995).

Transcription factor AP-2 regulates expression of human IGFBP-5 gene through binding the proximal GCCNNNGGG-like sequences in fibroblasts (Duan and Clemmons, 1995). They also found that this region of the IGFBP-5 promoter is responsible for the cAMP responsiveness of this gene.

Gabbitas and colleagues demonstrated that cortisol decreases IGFBP-5 gene expression, and showed that the region responsive to cortisol is from base pairs –70 to –22 and that E-box binding proteins or c-myb-related nuclear factors may be involved in its regulation (Gabbitas et al., 1996). Analysis of the IGFBP-5 promoter region revealed that myb-binding sites and also serine/threonine kinase-binding- elements regulates the promoter region of IGFBP-5 transcription (Tanno et al., 2002).

On the other hand, studies showed that IGFBP-5 expression is regulated by retinoic acid and vitamin D treatments. Retinoic acid treatment upregulates IGFBP-5 expression in different cell lines, including neuroblastoma (Cesi et al., 2005), cervical cancer (Higo et al., 1997), and osteoblastic cells (Dong and Canalis, 1995). In contrast, retinoic acid reduced IGFBP-5 transcription in T47D (Shemer et al., 1993) and PC-3 cells (Hwa et al., 1997). This difference could be possibly due to cell type specificity and context dependency of IGFBP-5.

Rozen and colleagues found that vitamin D and its compounds increased IGFBP-5 expression in conditioned medium and also had the same effect on its mRNA level in MCF-7 cells (Rozen et al., 1997). Vitamin D regulates calcium homeostasis and also has

potential antiapoptotic activities (Colston et al., 1989). Therefore, it is possible that IGFBP-5 contributes to the anti-proliferative action of vitamin D in MCF-7 breast cancer cell line.

IGF and IGFBP-5 in Mammary Gland Development and Involution

The mammary gland, unlike most other tissues of the body, exhibits maximum development during specific reproductive-associated cycles in adult life under the control of steroid and peptide hormones. It experiences multiple cycles of proliferation, differentiation, and regression resulting from repeated cycles of pregnancy, lactation and involution. Hormones, growth factors, and the ECM are well-known to influence the growth and function of mammary gland. Therefore, mammary gland differentiation is a good model for understanding the roles of IGFBPs in cancer development.

One of the most critical and interesting systems involved in the regulation of mammary epithelial cell function is the IGF system (Hadsell et al., 2002; Neuenschwander et al., 1996). The mitogenic and survival function of IGFs is observed not only in normal mammary cells, but also in breast cancer cells. Generally, all six IGFBPs are present in the mouse mammary gland, and IGFBP-3 and IGFBP-5 are expressed most prominently in pregnant gland tissue (Wood et al., 2000).

Development of the mammary gland occurs in defined stages namely embryonic, prepubertal, pubertal, pregnancy, lactation, and involution. The expression level of IGFBP-5 increases dramatically during the early stages of involution with an apoptotic

relationship (Flint et al., 2000; Marshman et al., 2003; Tonner et al., 2002; Tonner et al., 1997). Involution involves two distinct phases. The first phase is reversible and controlled by apoptosis. The second phase is irreversible and is characterized by remodeling of the lobuloalveolar architecture, which is regulated by proteases and macrophages (Sutherland et al., 2007). In addition to many critical pathways - such as NF-κB, PI3-K, Stat3, TGF-β (Baxter et al., 2007), matrix metalloproteinase, and the TIMP family, IGFBP-5 gene has shown to play a crucial role in both the stages of involution (Sutherland et al., 2007). In addition, Chapman and colleagues, using conditional knockout mice, showed a large increase in IGFBP-5 by the activation of Stat3 in the involution phase induce a mammary epithelial apoptosis (Chapman et al., 1999). Recently, Ning and colleagues using IGFBP-5 knockout mice found that IGFBP-5 is not essential to normal growth but does have an important role in mammary gland involution and also can regulate mammary gland differentiation by different mechanisms (Ning et al., 2007).

IGFBP-5 in Breast Cancer

The role of IGFBP-5 in human breast cancer is very complex and is not completely understood. The IGF-I signaling pathway is activated in breast cancer and activates two main downstream signal transduction pathways which are responsible for an aggressive breast cancer phenotype:

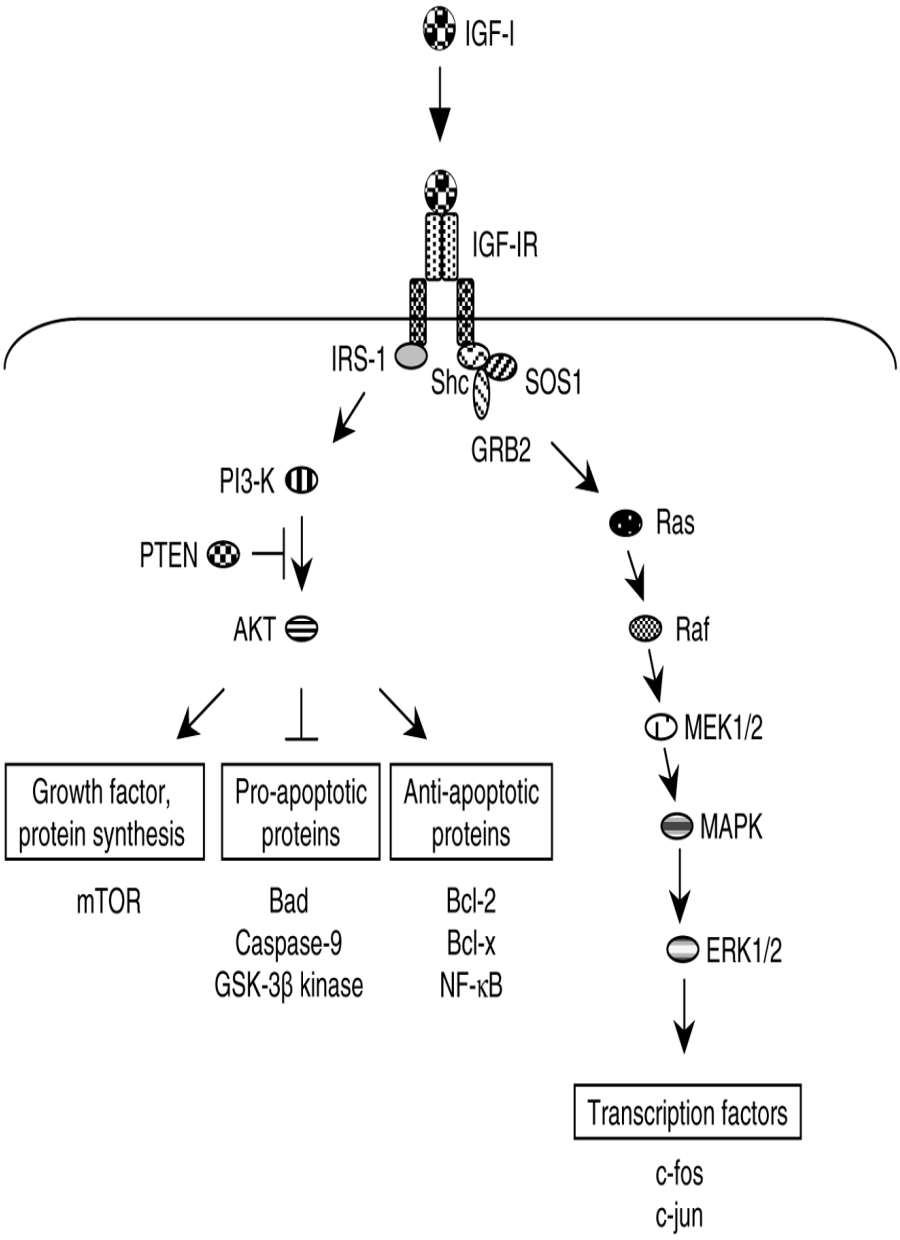


Fig 1.8: ***Overview of the IGF-I signaling pathway involved in breast cancer. IGF-I activates both the Ras/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3-K)/serine–threonine kinase (AKT) pathways, and causes cell growth and proliferation.*** (Akkiprik et al., 2008).

the IRS-1/PI3K/serine - threonine kinase (AKT) pathway and the Ras/mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) pathway (Esteva et al., 2004; Sivaraman et al., 1997; Wu et al., 2008). IGFBP-5 can regulate these pathways by IGF- dependent effects. Pekonen and colleagues have detected elevated levels of IGFBP-5 mRNA in 47 breast cancer tissue specimens by RT-PCR, and found higher levels of IGFBP-5 content in the tumour specimens than in adjacent normal tissues (Pekonen et al., 1992). In several independent studies, IGFBP-5 was among the differentially expressed genes that were positively associated with metastasis (Hao X et al., 2004; Nishidate et al., 2004; Van 't Veer et al., 2002).

Recently, Li and colleagues detected a positive correlation between the mRNA levels of IGFBP-5 to the invasion of axillary lymph nodes and the presence of the ER. The group also found an association with poor outcome between breast cancer patients who had positive lymph nodes and negative ER and increased mRNA levels of IGFBP-5 (Li et al., 2007). Mita and colleagues also investigated the predictive effects of IGFBP-5 on prognosis and endocrine therapy outcome. They also observed a negative correlation between the level of IGFBP-5 mRNA and HER2 overexpression (Mita et al., 2007). Undoubtedly, these data suggest that IGFBP-5 may play an important role in the metastasis of breast cancer.

IGFBP-5, Integrins and Breast Cancer

The integrins are a large family of transmembrane heterodimeric cell adhesion receptors which mediate cell-surface interactions either to the extracellular cellular matrix or to specific cell-surface receptors during cell-cell interactions (Hynes, 2002; Miranti and

Brugge, 2002). They are composed of α and β dimers in a noncovalent complex, comprising 24 combinations of 18 α and 8 β subunits. These receptors function as important regulators that control a whole set of cellular processes including cell spreading, retraction, migration and proliferation. Integrins contain the specialized binding regions for physical attachment of cells to the ECM, and through intracellular domains they form connections to various components of the actin cytoskeleton and a wide variety of inter-connecting signalling adaptors (Zaidel-Bar et al., 2007).

There has been considerable interest in the cross talk between IGFBPs and integrins. Mounting evidence documented in the last decade suggests that the direct binding of IGFBPs to integrins has both inhibitory and stimulatory biological effects (Schutt et al., 2004; Wang et al., 2006). It is interesting to note that IGFBP-3 and 5 can indirectly bind to extracellular proteins and are able to influence integrin activity (Beattie et al., 2010).

In tissues such as mammary gland, the binding of integrins to underlying basement membrane is essential for the structural and functional integrity of the epithelial component of the gland. There is documented scientific evidence suggesting integrin expression is regulated transcriptionally and post-transcriptionally during mammary gland development. Huang and Ip have shown increased expression of β1 and β4 integrins during pregnancy in rat mammary epithelial cells. Surprisingly, the expression of β4 integrin was found to be downregulated whereas, β1 integrin expression levels were found to be high during lactation (Huang and Ip, 2001).

2. Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary fibrosis (IPF) is a non-neoplastic chronic lung syndrome that is characterized by aberrant accumulation of fibroblasts/myofibroblasts and progressive abnormal remodelling of lung parenchyma with subsequent scarring and disruption of its structure and function. It is also known as cryptogenic fibrosing alveolitis in the United Kingdom (U.K.) and often referred as idiopathic because of its unknown aetiology and varying degrees of inflammation and fibrosis. It belongs to a broad category of 200 disorders called Diffuse Pulmonary Lung Diseases (DPLD) or simply Interstitial Lung Diseases (ILD). This is further classified into a subgroup known as Idiopathic Interstitial Pneumonia (IIP) where IPF is one of the seven diseases which is most prevalent and is associated with this pernicious disorder. It is sub-classified with a pathological condition known as usual interstitial pneumonia (UIP) which is synonymous to IPF. Major symptoms include chronic dyspnea (shortness of breath) induced by a 6 minute walk test, persistent dry cough, reduced lung volume and impaired gas exchange which interferes with daily life activities. Constitutional symptoms include weight loss, fatigue, finger clubbing and malaise (2000). IPF has a poor prognosis and is five times more prevalent than cystic fibrosis and amyotrophic lateral sclerosis with no FDA approved treatments. Despite recent considerable progress, current therapies are largely ineffective and none of the therapies to date have shown promise to either reverse or reduce the severity of this disease (Strieter, 2005). Current treatment regimens are largely unpromising with a median survival rate of less than three years from the date of diagnosis in affected patients (Tzilas et al., 2009).

IPF Incidence & Pathophysiology

IPF usually an elderly disease occurs after five decades of life and the probability of incidence markedly increases thereafter. The onset of disease is slow but with age and time, the symptoms get worse. There are five million people that are affected by IPF (Sharma and Chan, 1999). The number of affected patients has doubled in the past decade (Verma and Slutsky, 2007) and inevitably incidence will increase in developed countries due to an increasing aged population. The disease is slightly more prevalent in men (Gross and Hunninghake, 2001) and strongly associated with cigarette smoking (Baumgartner et al., 1997). Recent statistics of large U.S. and U.K. population based studies indicated a significant increase in mortality (Gribbin et al., 2006; Olson et al., 2007). Annually, 40,000 and 4,000 new cases were diagnosed each year in U.S. and U.K.

IPF can occur due to a variety of stimuli ranging from life style habits like cigarette smoking (Baumgartner et al., 1997), occupational exposure to polluted environments, notably asbestos or silica (Governa et al., 1999), induced by pharmacological agent (Teixeira et al., 2008), radiation exposure (Newman et al., 2005), genetic predisposition (Gross and Hunninghake, 2001), associated with other autoimmune abnormalities such as collagen vascular diseases (Hubbard et al., 2008; Lynch, 2009) and pathology of unknown origin.

Identification and a better understanding of the signalling pathways and pathogenic events that are responsible for the onset of this devastating disease holds great promise

and could lead to the development of effective therapeutic agents. This section describes a novel role of IGFBP-5 in the development of fibrosis and also reviews the critical role of TGFβ and other cytokines involved in the progression of this pathologic disease.

Wound healing and fibrosis

The term wound has been defined as a disruption of normal anatomical structure and function as a result of an injury (Lazarus et al., 1994). Wound healing is the universal response to the inflammation that succeeds injury. Acute wounds usually heal in a very sequential and efficient manner characterized by four distinct, but overlapping events: these include hemostasis, inflammation, cell proliferation and tissue remodelling. The healing process that is initiated as a result of a wound is a multifarious association of various inflammatory cells, cytokines and growth factors which helps in the reestablishment of tissue function (Diegelmann and Evans, 2004).

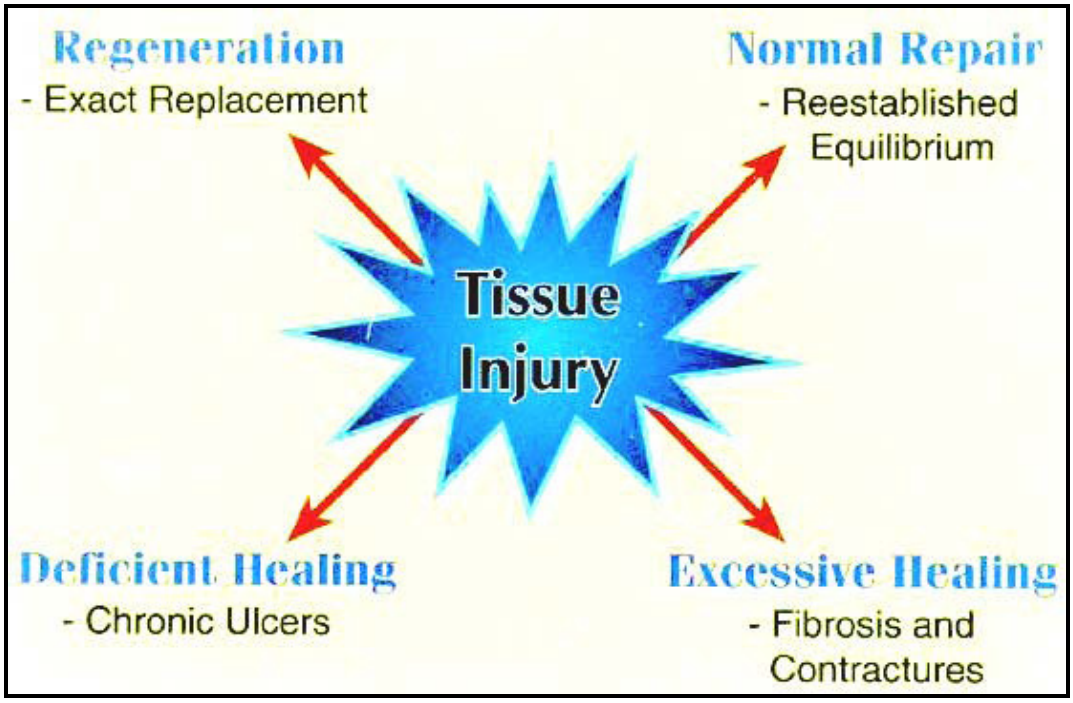


Fig. 1.9: ***The four possible responses following tissue injury (Diegelmann and Evans, 2004)***.

Fibrosis as Dysregulated repair

Fibrosis is defined as the replacement of normal structural elements of the tissue by distorted, non-functional and excessive accumulation of scar tissue (Diegelmann and Evans, 2004). In a nutshell, fibrosis is wound healing gone wrong with dysregulated repair mechanisms. Keloids and hypertrophic scars in the skin are examples of fibrosis (Bock and Mrowietz, 2004; Rahban and Garner, 2003). Scleroderma (SSc), Crohn's disease, oesophageal strictures, urethral strictures, liver cirrhosis, atherosclerosis, transmission blockage following nerve injury are some of the classic examples of fibrosis. The hallmarks of the these fibrotic disorders include fibroblast activation, with increased production of collagen and fibronectin, and transdifferentiation of fibroblasts into contractile myofibroblasts (Allan et al., 2008; Darby and Hewitson, 2007).

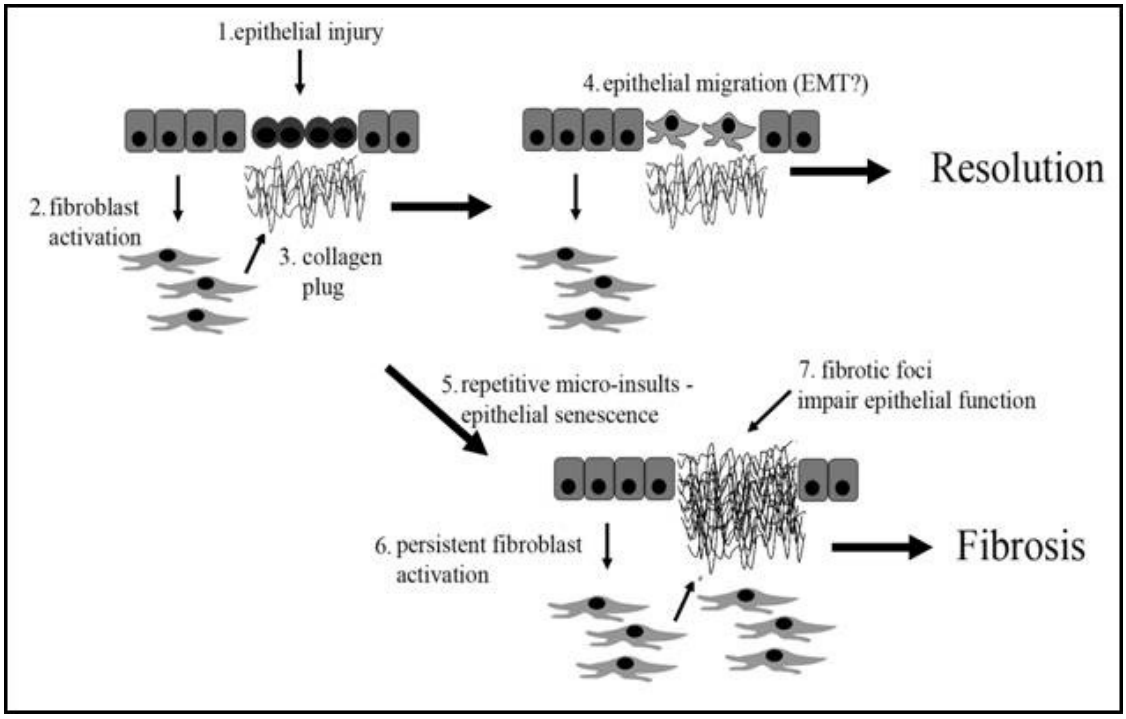


Fig 1.10: ***Schematic model of wound healing versus pathological fibrosis***.

Fibroblasts/Myofibroblasts in Fibrosis

Effective therapies to prevent tissue fibrosis requires a complete understanding of the mechanisms involved in the development of the disease, and one mechanism of particular interest to the field is fibroblast development. Fibroblasts and myofibroblasts are central cell types to the process of wound healing and in the pathogenesis of fibrosis.

Fibroblasts express receptors for a number of cytokines including PDGF (Alpers et al., 1994), TGF β1 (Noronha et al., 1995) and TNF-α (Noronha et al., 1995). These cytokines and others may mediate their recruitment and activation during injury. Myofibroblasts are mesenchymal cells with characterisitics of both fibroblasts and smooth muscle cells (Sappino et al., 1990). In addition to their involvement in skin wound healing (Gabbiani et al., 1971; Hebda et al., 1993; Majno et al., 1971), myofibroblasts are most commonly identified and well-characterized in idiopathic pulmonary fibrosis (Gharaee-Kermani et al., 2009). The role of myofibroblasts has been extensively studied in skin wound healing where they are known to aid in wound contraction (Grinnell, 1994; Majno et al., 1971).

Table 1.3: ***Principal Pathologic Disorders in which Myofibroblasts have been observed*** *(Darby and Hewitson, 2007).*

Pathology	References
Skin wound healing	(Darby et al., 1990; Hebda et al., 1993; Majno et al., 1971)
Pulmonary fibrosis	(Blum, 1994; Gharaee-Kermani et al., 2009; Zhang et al., 1994)
Hepatic fibrosis	(Bhathal, 1972; Schmitt-GraV et al., 1993; Tanaka et al., 1991)

Tumors	(Kalluri and Zeisberg, 2006)
Cardiac overload	(Leslie et al., 1991)
Renal fibrosis	(Alpers et al., 1994; Essawy et al., 1997)

EMT as an origin for Myofibroblasts

Determination of the cellular source of the myofibroblasts is crucial in understanding the pathogenesis of tissue fibrosis. Myofibroblasts appear to have at least three possible origins. Figure 1.11 represents the schematic diagram of the origins of myofibroblasts in pulmonary fibrosis as an example of organ fibrosis. Resident fibroblasts can respond to a variety of profibrotic mediators and differentiate into myofibroblasts (Scotton and Chambers, 2007). TGFβ1 induces transdifferentiation of fibroblasts through a Smad3-dependent mechanism to myofibroblasts (Hu et al., 2003). Over-expression of IGFBP-5 *in vivo* induces increased expression of α SMA and vimentin in dermal fibroblasts suggesting the fibroblast transdifferentiation (Yasuoka et al., 2006a).

Epithelial-myofibroblast transdifferentiation from epithelial cells is a specialized version of epithelial–mesenchymal transition (EMT), a physiological process in which epithelial cells can acquire the invasive and motile properties of mesenchymal cells (Radisky, 2005).TGFβ1 induces EMT in alveolar epithelial cells *in vitro* (Willis BC, 2006). IGFBP-5 induces phenotypic EMT in MCF-7 cells *in vitro* (Allan et al., 2008). In addition, circulating bone marrow derived fibrocytes, behave like mesenchymal stem cells and migrate into sites of lung injury and become myofibroblasts. These fibrocytes express type 1 collagen and α SMA and contribute to lung fibrosis (Hashimoto et al., 2004).

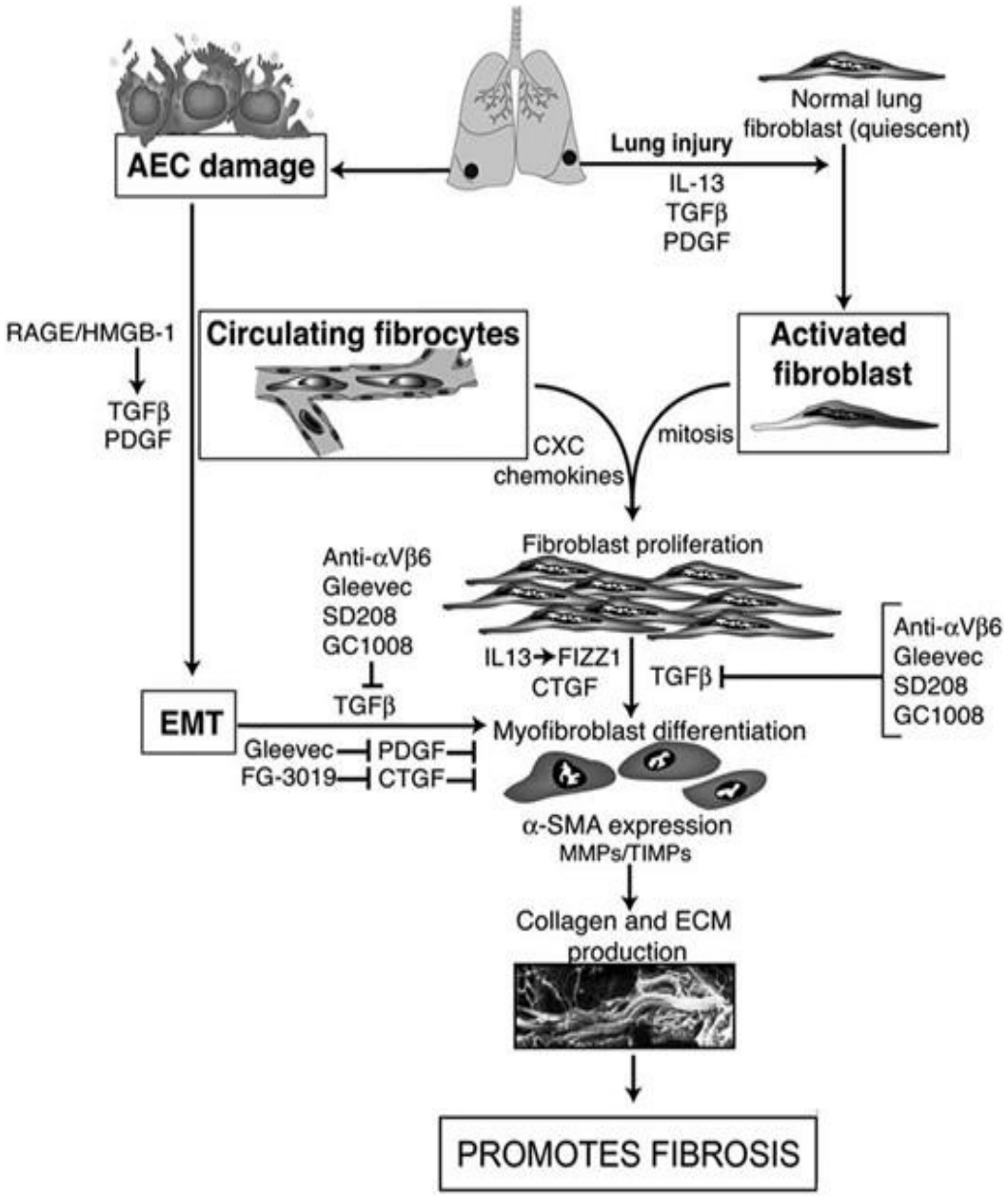


Fig 1.11: ***Schematic presentation of sources of myofibroblasts in pulmonary fibrosis. A number of inhibitors were employed to inhibit fibroblast matrix production and myofibroblast differentiation. GC1008, a monoclonal antibody that neutralizes all three isoforms of TGFβ; avβ6, blocking antibody that blocks TGFβ; SD-208, a TGFβ receptorkinase (ALK5) inhibitor; Gleevec, a protein kinase inhibitor that blocks both the PDGF receptor and TGFβ2; and FG-3019, anti-CTGF antibody.***

(Gharaee-Kermani et al., 2009).

TGFβ in Fibrosis

Transforming growth factor β (TGFβ) is a family of multifunctional, profibrotic regulatory cytokines that regulates fibroblast proliferation, myofibroblast differentiation and increased expression of collagen deposition during fibrosis. TGFβ1, out of three highly homologous isoforms (TGF β1 - 3), is thought to play the most significant role in wound healing and fibrosis (Flanders, 2004; Lee CG, 2006; Roberts and Sporn, 1996).

In pulmonary fibrosis, active TGFβ acts as a chemoattractant for fibroblasts and macrophages, and induces fibroblast proliferation via the expression of PDGF. TGFβ also stimulates these cells to synthesize a number of pro-inflammatory cytokines such as TNF-γ, PDGF, IL-1 β and IL-13(Flanders, 2004; Lee CG, 2006; Roberts and Sporn, 1996). Differentiation of fibroblasts to myofibroblasts induced by TGFβ has been shown to be a key mediator of collagen deposition in lung fibrosis (Thannickal et al., 2003). TGF β is one of the most potent inducers of ECM production, including collagens and other components. TGF β reduces the breakdown of matrix deposition both by inhibiting the generation of matrix metalloproteases (MMPs), plasminogen activators, as well as enhancing the expression of tissue inhibitors of metalloproteases (TIMPs) and plasminogen- activator inhibitors (PAIs) (Kunugi et al., 2001; Mcguire et al., 2003; Selman et al., 2000). Gene expression studies in human tissue showed upregulation of TGFβ responsive genes in IPF patients (Kaminski et al., 2000; Kjetil et al., 2006). Finally, transgenic studies showed overexpression of TGFβ alone is sufficient to induce a progressive fibrosis independent of inflammation (Thannickal et al., 2003)

IGFBP-5 in fibrosis

Recently, IGFBP-5 increased expression has also been described in fibrosis (Feghali and Wright, 1999; Pilewski et al., 2005). Increased expression of IGFBP-5 at mRNA and protein levels was also evident *in vitro* in primary fibroblasts cultured from disease-affected skin of patients with SSc, compared with the skin from their healthy twins. Yasuoka and co-workers demonstrated that the role of IGFBP-5 to induce collagen and fibronectin production from fibroblasts and to induce fibroblast or myofibroblast transdifferentiation *in vitro* and *in vivo (Yasuoka et al., 2006a; Yasuoka et al., 2006b)*. Finally, *in vivo* overexpression of IGFBP5, using replication-deficient adenovirus, induced skin fibrosis in mice which included increased thickness of the dermis and increased collagen bundle thickness (Yasuoka et al., 2006a) and pulmonary fibrosis (Yasuoka et al., 2006b). Increased expression of α-SMA and vimentin in dermal fibroblasts was also evident with overexpression of IGFBP-5. Collectively, these findings suggest that upregulated expression of IGFBP5 could be an initiating event in ECM production and the development of fibrosis.

Integrins, IGFBP-5 and Fibrosis

Activation of integrins can initiate intracellular signalling or influence signalling through other receptors. Bhowmick and co-workers demonstrated that β1 integrins are critical for TGFβ1 mediated transcription and epithelial cell plasticity *in vitro* (Bhowmick et al., 2001). Recently, Kim and colleagues demonstrated that the integrin α3β1 is involved in myofibroblast formation by specific deletion of α3 integrin in lung epithelial cells and that these cells contribute to pulmonary fibrosis. Furthermore, it suggests a mechanism

whereby integrins could contribute to differential induction of EMT on specific matrices depending on their ability to function as integrin ligands (Kim et al., 2009). Therefore, there has been pressing a need to understand the role of integrins in the regulation of EMT and fibrosis.

Focal Adhesion Proteins - Interactions with IGFBP-5

Focal adhesions are contacts of large protein complexes by which cells attach to the substratum and serve as physical linkage between extracellular matrix and cytoskeleton. The associated proteins of focal adhesion assembly or disassembly include functionally diverse structural proteins that regulate signalling pathways of cell adhesion, survival and migration. Adhesive interactions are principally regulated by a system of actin-rich anchor proteins and some of which include Cadherins, Vinculin, Paxillin and Zyxin (Streuli and Akhtar, 2009; Zaidel-Bar et al., 2004). Cadherins are group of calicium ion dependent protein complexes that are important for adherens junction. Vinculin is a classic membrane cytoskeletal protein involved in focal adhesion plaques. Paxillin is referred as adaptor protein with signalling properties that targets focal adhesions. I have attempted to consider how IGFBP-5 uses focal adhesions to interact aforementioned signalling complexes involved in integrin function, induction of cell adhesion, survival and migration processess.

Matricellular proteins – Interactions with IGFBP-5

As progressive accumulation of extracellular matrix production (ECM) is observed in all the fibrotic disorders, it is important to understand the biology of ECM and the effect of IGFBP-5 on ECM synthesis. The ECM is an intricate arrangement of glycoproteins,

collagens, proteoglycans and growth factors that act not only as a physical scaffold for the adhesion and organization of the cellular structures, but also as a mediator of intracellular signaling through cell surface receptors that recognize these ECM molecules (Joanne, 2001). Matricellular proteins function as adaptors and modulators of cell: matrix interactions. These structurally diverse proteins include thrombospondins 1 and 2 (TSP 1,2) osteopontin (OPN), osteonectin, tenascins-C and -X, secreted protein, acidic and rich in cysteine (SPARC) support a state of intermediate adhesion, characterized by disruption of focal adhesions and a reorganization of actin stress fibers (Bornstein and Sage, 2002). Much interest has been focused on the biological roles of OPN and TSP as they have been implicated in the pathogenesis of various diseases such as cancer, fibrosis, inflammation, vascular remodeling and kidney diseases (Tuck and Chambers, 2001).

Since matricellular proteins such as osteopontin (OPN), and thrombospondin (TSP) act as potential regulators influencing pathways involved in the control of cell adhesion, migration, and because IGFBP-5 has been shown to bind to these factors (Clemmons et al., 1995; Nam et al., 2000) it is possible that IGFBP-5 is also a member of this family of proteins. The fact that IGFBP-5 also activates fibroblasts (Pilewski et al., 2005) and is secreted by epithelial cells during cell death (Beattie et al., 2006) adds further weight to this hypothesis. Thus, it was hoped that this project would also examine the molecular interactions between osteopontin, thrombospondin, tenascin-X and -C with IGFBP-5.

3. Cellular Senescence and Autophagy

Cellular senescence is typically characterized by an irreversible cell cycle/proliferation arrest and potentially caused by a myriad of cellular stress factors, including exhaustive overload of cell division and cancer-causing genetic alterations (Adams, 2009). For the first time, Leonard Hayflick and his co-workers defined the term "cellular senescence" to describe the phenotypic effect of primary human fibroblasts irreversible proliferation arrest in culture (Hayflick, 1965). Three major forms of cellular senescence are of particular interest:

1) Replicative Senescence (RS) - defined by the loss of ability to proliferate/divide due to telomere attrition.
2) Oncogene-Induced Senescence (OIS) - independent of telomere length and defined by the activation of oncogene(s) that subsequently leads to the irreversible cell cycle arrest.
3) Accelerated Cellular Senescence (ACS) - refers to the senescence induced by genotoxic stress such as irradiating cells with substantial dosage and time.

Cell senescence is usually associated with altered secretion involving inflammatory cytokines, chemokines and matrix proteases and activated by various cellular stresses - oxidative stress and activated oncogenes. Certain oncogene expression in a primary cell can initiate proliferation arrest by inducing senescence or programmed cell death (Mooi and Peeper, 2006) . Inactivation of both p53 and pRB signalling pathways typically terminates senescence-associated proliferation arrest in spite of an initial senescence trigger in human cells (Campisi and d'Adda di Fagagna, 2007).

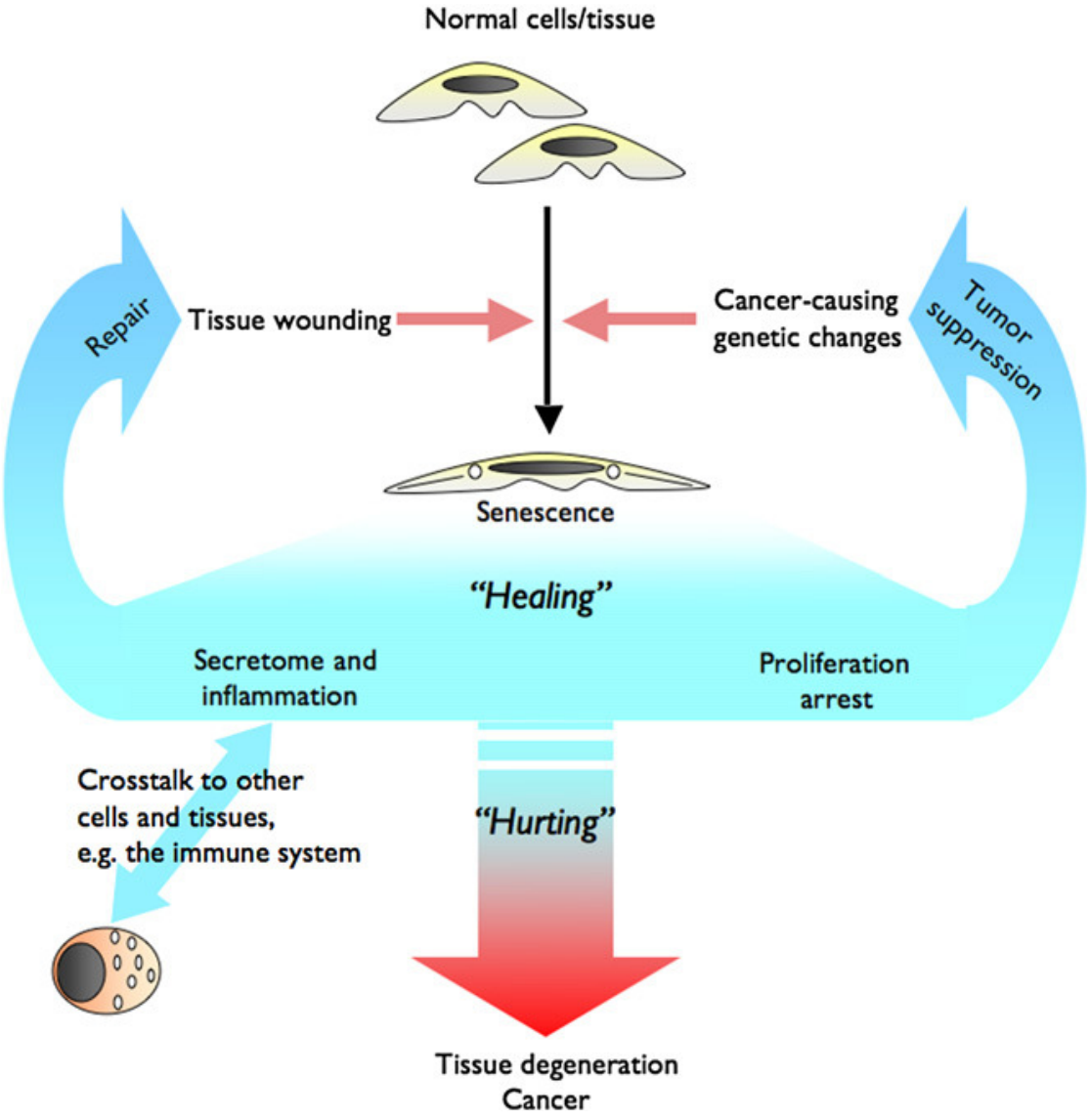


Fig 1.12: ***Schematic presentation showing oncogenic alterations and tissue wounding activate cell senescence. In addition to positive element of tissue healing, senescence may lead to tissue degeneration i.e., aging (Adams, 2009).***

Recently, Wajpeyee and co-workers have demonstrated the role of IGFBP-7 in the induction of cellular senescence and apoptosis through autocrine/paracrine pathways. During this study, they showed IGFBP-7 inhibits BRAF-MEK-ERK signalling and irreversibly induces apoptosis following transient exposure (Wajapeyee et al., 2008). Similarly, there are studies demonstrating upregulation of IGFBP-5 and IGFBP-3 expression in senescent human diploid fibroblasts (Goldstein et al., 1991; Yoon et al., 2004). The above studies implicate a role of IGFBPs in the induction of cellular senescence.

There are quite a few publications reporting integrin regulation of cellular senescence. Roth and her co-workers have demonstrated recombinant alpha 2 integrin induces cell senescence both in vitro and in vivo and inhibits melanoma (M21) cell growth in vivo (Roth et al., 2005). On the other hand, Liu and co-workers have shown an increase in β4 integrin levels during HUVECs senescence (Liu et al., 2007).

Autophagy and Senescence-associated autophagy

Autophagy is an intracellular degradation catabolic system that delivers cytoplasmic constituents or organelles to the lysosome. Autophagy literally means “self - eating” and is a multistep cell-recycling process that plays an important role in cell growth, development and homeostasis and is also implicated in several pathologies including ageing (Klionsky, 2004; Mizushima, 2007; Mizushima et al., 2008). There are three types of autophagy which are distinct from endocytosis-mediated lysosomal degradation of extracellular and plasma membrane proteins.

1) Macroautophagy/Autophagy - is characterized by the formation of double or multi-membrane autophagosomes that sequester cytosol and long-lived organelles such as mitochondria
2) Microautophagy - is characterized by the direct engulfment of cytoplasm by invagination, protrusion and septation of the lysosomal limiting membrane.
3) Chaperone-mediated autophagy (CMA) - a selective form of autophagy that modulates soluble cytosolic protein turnover. In this process, proteins possessing consensus peptide sequences recognize and bind to hsc70-containing chaperone. This CMA substrate/chaperone complex determines the delivery to the lysosomal surface (Bejarano and Cuervo).

Autophagy comprises multiple sequential steps: sequestration, degradation and aminoacid/peptide generation and the process is therefore multifunctional. Typically, nutrient starvation triggers autophagy and in this sense lack of any type of essential nutrient can potentially induce autophagy. Mammalian target of rapamycin (mTOR) is a master regulator of nutrient signalling and suppresses autophagy (Mathew et al., 2007). Treatment with inhibitors of TOR such as rapamycin and CCI-779 induces autophagy in mouse models of Huntington's disease (Ravikumar et al., 2004). Similarly, some signals can induce autophagy in a mTOR independent manner. More recently, small molecules which are independent of mTOR called SMERS (small-molecule enhancers of the cytostatic effects of rapamycin) enhance autophagy in Huntington's disease models (Sarkar et al., 2007).

Autophagy is an important facet of the senescence program. Very recently, Young and co-workers have demonstrated autophagy as a new effector mechanism of senescence. Furthermore, they have shown upregulation of a subset of autophagy-related genes (ATG) which include ULK1 and ULK3 and activation of LC3 through detection of autophagic vacuoles and lipidation (Young et al., 2009).

Activation of autophagy in association with senescence appears to be dependent on negative feedback-mediated repression of PI3K-AKT-mTOR. Oncogenes like Akt are activated by phosphorylation and inhibit FOXO3. Recent studies have reported FOXO3 is a transcriptional activator for some autophagy regulated genes (ATG) and autophagy regulators (Mammucari et al., 2007). Taken together, repression of PI3K-AKT-mTOR pathway likely activates autophagy through mTOR and AKT dependent mechanisms (Adams, 2009).

Recent studies reported autophagy as a tumour suppression mechanism (Bialik and Kimchi, 2008; Mathew et al., 2007). Thus, autophagy's contribution to cell senescence might, at least in part, account for autophagy's tumour suppression mechanism. It would be interesting to examine how autophagy regulates complex human pathologies like cancer metastasis and fibrosis.

Aims of the present study

Cancer metastasis and fibrosis represent a devastating group of disorders associated with ageing, with poor prognosis and survival rates. Attempts to develop effective therapeutic strategies for this wide range of diseases may have much in common since the mechanisms involved, cell migration and invasion for example, are common to them all

As described earlier, increased expression of IGFBP-5 is evident in two human fibrotic diseases namely SSc and IPF as well as in breast cancer, ovarian cancer and prostate cancer metastases. I have attempted to investigate the role of IGFBP-5 in fibrosis and cancer metastasis.

An initial aim was to express recombinant IGFBP-5 employing a bacterial system and also to demonstrate IGFBP-5 expression at mRNA and protein level using an adenovirus transduction system. Subsequent aims include the demonstration of the effect of IGFBP-5 on epithelial-mesenchymal transition and integrin expression. The central aim is to understand the underlying mechanisms of IGFBP-5 action in normal and tumorigenic epithelial and mesenchymal cells in the context of both fibrosis and cancer metastasis.

Research Objectives

Given that IGFBP-5 appears to be able to activate fibroblasts and stimulate fibronectin and collagen production it seems reasonable to assume that it plays a role in wound-healing responses involving epithelial tissue injury. This puts IGFBP-5 amongst the

matricellular proteins, several of which it interacts with. Thus, the major goals of this thesis will be to:

- Determine if IGFBP-5 influences epithelial-mesenchymal transition in fibrotic responses involving epithelial injury.
- Determine the effect of IGFBP-5 on integrin expression in epithelial and dermal fibroblasts.
- Determine the effect of IGFBP-5 on extracellular matrix production in epithelial cells and fibroblasts.
- Examine the ability of IGFBP-5 to influence the cellular migratory actions in epithelial and dermal fibroblasts.
- Understand the mechanisms of action of IGFBP-5 in the context of epithelial to mesenchymal cellular interactions.
- Elucidate IGFBP-5 signalling mechanisms of epithelial cell adhesion and survival and migration.
- Determine the aforementioned signalling mechanisms with the use of C-terminus and N-terminus mutants of IGFBP-5.

Chapter II

Materials & Methods

MATERIALS & METHODS

General Chemicals and Reagents

Agarose, Crystal Violet, Ethidium bromide, GeneJammer transduction reagent (Agilent Technologies Catologue # 204130), Nitrocellulose membrane (Amersham BioSciences), Paraformaldehyde, Penicillin/Streptomycin (Lonza), Urea. Unless otherwise specified all the chemicals, solvents and reagents used were of highest purity commercially obtained from Sigma.

Culture Media

Mammalian tissue culture cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) or DMEM/HAMS F12 with phenol red supplemented with 2mM glutamine (Cambrex Cat # 17-603E), 100 U/ml penicillin/100 µg/ml streptomycin and 10% fetal calf serum, hereafter referred to as 10% FCS, or 2% FCS or 5% Horse serum supplemented with 1% ready-mixed Pen/Strep/Glu and glutamine. Primary cells such as human dermal fibroblasts (HDFs) were maintained in either 2% FCS and supplemented with ready-mixed low serum growth supplemented kit (Cascade Biologics, Cat. # C-013-5C) which includes fetal bovine serum, recombinant human basic fibroblast growth factor or heparin, hydrocortisone, recombinant human epidermal growth factor.

Mammalian Cell lines

Cell lines & primary cells employed in various biological assays included the human breast adenocarcinoma non-invasive epithelial-like cell line MCF-7, human breast carcinoma highly invasive estrogen receptor negative epithelial HS 578T cells, human

breast adenocarcinoma highly invasive epithelial MDA-MB-231 cell lines, normal human mammary epithelial cell line MCF-10A, primary human dermal fibroblasts. Cells were cultured in a humidified atmosphere of 5% CO_2/95% air cell culture incubator under physiological pH (7.2 - 7.4).

Maintenance of mammalian cell lines

- **MCF-7 cells** (ECACC # 93616) were cultured in 10% FCS DMEM (Lonza, Cat. # BE 12-917F) and passaged every three to four days, or when confluence was reached, using either trypsin/EDTA, 0.5 mg/ml trypsin, 5.3 mM EDTA (Biosera, Cat. # L0940) treatment. Before Trypsin/EDTA treatment, existing medium was discarded and the adherent cells were washed with DPBS (Lonza, Cat. # BE 17-512F) before exposing them to Trypsin/EDTA mix for approximately 5 minutes at 37^{o}C. Disaggregated cells were then washed with 10% FCS DMEM to remove residual trypsin before plating at a concentration of 1/2, 1/4 and 1/8 of the original cellular concentration in standard 75 cm^2 flasks.

- **MCF-10A cells** (ATCC® Cat. # CRL - 10317) were cultured in 5% Horse Serum (Biosera, Cat. # S0900/500) DMEM/HAMS F12 (1:1 mixture) (Biosera, Cat. # L0094) with added 10 μg/ml Insulin (Sigma 16634-100MG), 20ng/ml Epidermal growth factor (Sigma E-4127-1MG) and 0.5 μg/ml Dexamethasone (Sigma, Cat. # D-4902). Resuspension medium was carried out in DMEM/F12 (1:1 mixture) with 5% Horse Serum.

- **HS 578T cells** (ATCC® Cat. # HTB - 126) were cultured in 10% FCS DMEM, 2mM L-glutamine, 10 µg/ml Insulin, 100 U/ml penicillin/100 µg/ml streptomycin in 75-cm2 flasks at 37°C in a 5% CO2 atmosphere. These cells were passaged every three to four days, or when confluence was reached, using trypsin/EDTA, 0.5 mg/ml trypsin, 5.3 mM EDTA treatment.

- **MDA-MB-231 cells** (ATCC® Cat. # HTB - 26) were cultured in 10% FCS DMEM, 2mM L-glutamine, 10 µg/ml Insulin, 100 U/ml penicillin/100 µg/ml streptomycin in 75-cm^2 flasks at 37°C in a 5% CO2 atmosphere. These cells were passaged every three to four days, or when confluence was reached, using either trypsin/EDTA, 0.5 mg/ml trypsin, 5.3 mM EDTA treatment.

- **HDF cells** (Cascade Biologics, Cat. # C-013-5C) were cultured in 2% FCS supplemented with Low Serum Growth Supplement (Cascade Biologics, Cat. # C-013-5C) (LSGS) containing fetal bovine serum, hydrocortisone, human epidermal growth factor, basic fibroblast growth factor with heparin and Amphotericin solution. These cells were split every five to six days, in a similar fashion using Trypsin/EDTA (Cascade Biologics, Cat. # R-001-100) and Trypsin Neutralizer (Cascade Biologics, Cat. # CC-5002) under standard conditions.

Cell Count

10 µl of resuspended cell suspension was placed into a hemocytometer and counted. The mean of 4 outer squares was taken and the cell number was calculated as follows:

Cell number per ml = Average cell count in 4 squares x 10^4

Once cell count was determined, the cell suspension was diluted to appropriate density and seeded as per the requirements of the experiment.

Procedure for fixing and crystal violet staining (for 96well plate):

Upon appropriate incubation time, the media was aspirated from the plate. It was then fixed by treating with 4% paraformaldehyde for about 20-30 min at room temperature. Then the paraformaldehyde from the plate was tipped off and washed in order to remove any debris. The fixed cells were stained with 100µl of 0.01mg/ml of crystal violet (BDH Chemicals, C.I 42555) for 10-15 min. This step was followed by washing three times with 200µl of double distilled water until clear and photography was carried out. The dye from the cells was eluted by using 200µl of 0.1% Triton X-100 (Sigma, T-8787). The absorbance was then measured at 560nm using a spectrophotometer (Labsystems iEMS Reader MF).

WST-I metabolic assays: Cell proliferation reagent WST-1 was purchased from Roche Diagnostics GmbH, Germany. WST-1 was diluted 10X and 10 µl was pipetted into cell culture wells and incubated for 45 min at 37 ^{o}C. The absorbance was measured at 450 nm using spectrophotometer.

Virus Culture:

HEK 293 cells were infected with adenovirus stock and the purification procedure was carried out using AdEasy™ Purification Kit 500 (Stratagene; Cat # 240243) and the

adenoviral titers were determined by using AdEasy™ Viral Titer Kit (Stratagene; Cat # 972500). The concentrated viral aliquots were stored at -80°C for further use.

The adenovirus vector system employed for transfecting the above cell lines is replication-incompetent, i.e. each virus particle can only infect a single cell and no virus progeny will be generated.

Overview of AdEasy Purification:

For adenovirus purification and titer's, Stratagene's AdEasy™ viral titer kit (Catalog # 972500) was used. At first, 80% - 90% cytopathic HEK 293 cells in 75 cm^2 flask were trypsinised and centrifuged retaining both pellet and supernatant. The pellet was resuspended in 10 ml of supernatant and cells were lysed by three freeze/thaw cycles. To remove the unwanted nucleic acids, Benzonase nuclease was added to the supernantant and incubated at 37 °C for 30 minutes. Then after, the membrane of the Sartobind® filter unit was equilibrated with PBS before loading virus. Immediately, without any bubble formation, the prepared supernatant was passed slowly drop-by-drop through the Sartobind® filter unit. After that the filter was washed twice with the wash buffer.

Then, elution of purified viral particles was carried out by passing elution buffer slowly through the Sartobind unit with a syringe using the special elution tip. For maximum recovery of viral particles, elution was carried out at a flow rate less than 1 ml/minute. In order to increase the concentration of viral particles, centrifuge concentrators were used which were supplied in the manufacturer's kit. Concentrated virus obtained was recovered and resuspended gently for viral titer determination. Stratagene's AdEasy

Viral Titer Kit was employed to determine the viral titer and carried out according to the manufacturer's instructions.

Recombinant IGFBP-5 expression employing Bacterial system

Expression of wild type (wt) recombinant IGFBP-5 proteins was carried out using conditions identical to those described by Allan and co-workers (Allan et al., 2002). cDNAs for mouse wt IGFBP-5 without the signal peptide-encoding sequence, were cloned into the pGEX 6P-1 vector (Amersham Pharmacia Biotech, Arlington Heights, IL) between BamHI and EcoRI in the multiple cloning site, so that the proteins would have an N-terminal glutathione S-transferase (GST) tag. 50ng of this construct was then used to transform the Origami ^{TM} B (DE3) P Lys S competent strain (Novagen) of Escherichia coli, and the cells were incubated overnight at 37 ^{o}C in 10 ml Lysogeny Broth (LB) medium containing 12 µg/ml ampicillin and 30 µg/ml chloramphenicol. After a 40-fold dilution into fresh LB/ampicillin/chloramphenicol, the cells were re-grown to mid-log phase (E600nm=~0.6), then the expression of IGFBP-5, as a glutathion-s-tranferase (GST) fusion protein, was induced by addition of 1 mM isopropyl β-D-thiogalactoside and allowed to proceed at 25 ^{o}C overnight.

Purification of recombinant IGFBP-5

GST-affinity Chromatography:

Origami B cells were harvested by centrifugation at 1,500 × *g* for 15 min, washed once in 50 ml PBS, and resuspended in 10 ml PBS containing protease inhibitors (Roche,

Indianapolis, IN). The suspension was frozen and thawed once to lyse the cells, then the bacterial DNA was sheared by three 30-sec cycles of sonication (KT-40, Kontes Co., Vineland, NJ; 4-mm prove, full power) with cooling on ice. Insoluble material was removed by centrifugation at 11,000 × *g* for 30 min, then the supernatant was filtered through a 0.45-µm pore membrane and incubated overnight at 4°C with 1 ml (packed volume) washed glutathione-Sepharose (Amersham Pharmacia Biotech). The suspension was decanted into a disposable plastic column (Bio-Rad Laboratories, Inc., Hercules, CA), and unbound material allowed to flow through, then the glutathione-Sepharose was washed twice with 10 ml PBS and once with 10 ml cleavage buffer [50mM Tris (pH 7.0), 150 mm NaCl, and 1mM EDTA]. The column was sealed, and the glutathione-Sepharose was resuspended in 2 ml cleavage buffer containing 160 units of PreScission protease (Amersham Pharmacia Biothech). After 4h at room temperature with hourly resuspension, the column was reopened, and the cleaved IGFBP-5 was recovered in the eluate. GST and PreScission protease remained bound to the glutathione-Sepharose. IGFBP-5 remaining in the column was recovered by washing with 10 ml cleavage buffer. A 400-ml bacterial culture typically yielded about 1 mg of IGFBP-5 protein.

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC):

IGFBP-5 proteins were further purified by RP-HPLC using a polymeric column (PLRP-S: 300Å; 8 µm bead; 4.6 mm diam X 150 mm Polymer Laboratories Ltd, Church Stretton, UK) equilibrated with a mobile phase consisting of 3 part solution A [0.1% trifluoroacetic acid (TFA) in water] and 1 part solution B (0.1% TFA in acetonitrile).

Aliquots (1-2 ml) of GST-affinity purified protein (2-3 mg) in cleavage buffer was injected onto the column which was run at 1ml/min throughout. After 5 min of isocratic elution, a linear gradient from 25 % solution B to 40 % solution B was applied over the next 50 min. Absorbance of column effluent was monitored at 220 nM; IGFBP-5 proteins were collected, typically between 25 and 30 min after sample injection, and fractions were assessed for protein purity by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie blue staining was employed to visualise protein bands after electrophoretic separation and to estimate their relative concentration. Gels were stained in 0.3% (w/v) Coomassie Brilliant Blue R250, 5% (v/v) methanol, 7.5% (v/v) glacial acetic acid by shaking gently for approximately 30 min at room temperature. To enable the detection of protein bands from the stained background, gels were destained in 30 % (v/v) methanol, 10% (v/v) glacial acetic acid for 1 hour with shaking, followed by fresh destaining, without shaking overnight. The gel was then dried using the EasyBreeze system (Hoefer Scientific Instruments, San Francisco, USA) according to the manufacturer's instructions. Proteins in all the fractions were also quantitated by Bradford assay. Fractions were lyophilized and stored at –70 °C. Proteins were re-dissolved in Tris.HCl/Tween 20, pH 7.4 buffer for use.

Protein analysis using Bradford assay:

Proteins were quantified using the Bio-Rad protein assay system (Pierce, Chester, UK) which is adapted from Bradford *et al*. 1976. Five dilutions of a BSA standard in 0.5% acetic acid-2M Tris (pH 7.0) which was an appropriate blank for the protein solutions to be tested were prepared as a standard solution. The linear range of the assay was 5.0 µg/ml to approximately 100 µg /ml. Standard and protein sample solutions were

normally assayed in triplicate. 160 µl of each standard and sample solution were pipetted into separate microtitre plate wells, and then 40 µl of diluted dye reagent concentrate was added to each well and mixed thoroughly using a multi-channel pipette. Samples were incubated for 10min at room temperature, and loaded into a TitreTeck® multi-scan and absorbance measured at 600nm.

Western Blotting

Preparation of samples for immunoblot analysis:

The soluble medium from IGFBP-5-transducedMCF-7/HDFa cells was collected on a daily basis. For cell homogenates the IGFBP-5-transduced cells were lysed with RIPA buffer (50mM Tris-HCL, pH 7.4; 150mM NaCl; 0.5 mM EGTA; 0.5 mM EDTA pH 7.4; 1% Igepal CA-630; 0.25% Na-deoxycholate; 1mM PMSF; 1mM Na-orthovandate; 1mM NaF; 1µg/ml Protease Inhibitor Cocktail). The protein concentrations of cell lysate samples were determined by using BCA™ Protein Assay Kit for immunoblot analysis.

For protein analysis, 10µg of target protein was taken in 10µl of eluant and mixed with 5µl of 4X Laemmli buffer and 1mM dithiothreitol. In the case of conditioned medium samples, 15µl was mixed with 5µl of sample buffer. The samples were denatured at 70^{o}C for 10 minutes and allowed to cool before loading on to a 10% NuPAGE^{R} Novex^{R} Bis-Tris Poly Acrylamide Gel Electrophoresis gel (Cat # NP0302BOX).

PAGE for subsequent immunoblotting was carried out as described below with the exception that the NuPAGE^{R} LDS sample buffer uses lithium dodecyl sulphate which stays in solution better than the sodium form. The following buffer was used:

1X NuPAGE MOPS (3-(N-morpholino) propane sulfonic acid) SDS Running Buffer is used in conjunction with Bis-acrylamide (Bis[2-hydroxyethyl] imino-tris [hydroxymethyl] methane)-HCl, polyacrylamide) gel.

Each well was loaded with sample and the gel was subjected to electrophoresis at a constant voltage of 200V for approximately 50 - 60 min run time.

Immunoblotting:

Cellular protein extracts of IGFBP-5 were separated by NuPAGE^{R} Bis-Tris Electrophoretic system and transferred onto Hybond^{TM}-C Extramembrane for immunochemical analysis. The following buffers and reagents were used:

1X NuPAGE^{R} Transfer Buffer was used specifically for blotting onto a Hybond^{TM} - C Extramembrane using 100ml methanol and made up to 1 litre with distilled water.

Tris-Buffered-Saline-Tween (pH 7.5) (TBST) was prepared by the addition of 0.025% v/v Tween-20 (Sigma) to TBS.

Blocking Solution was made by the addition of 3% Bovine Serum Albumin in TBST.

Primary Antibody was prepared with either α-IGFBP-5 sheep antibody at 1:2000 in 1X TBST/3% BSA in the case of the IGFBP-5 blot and fibronectin rabbit antibody at 1:4000 in the case of the fibronectin blot.

Subsequent secondary antibody was prepared with either α sheep horseradish peroxidase (HRP) conjugate antibody at 1:5000 in 1X TBST/5% skimmed milk in the case of the IGFBP-5 blot or α rabbit horseradish peroxidase (HRP) conjugate antibody at 1:5000 in 1X TBST/3% BSA for fibronectin.

The image was developed by exposing the membrane for 10 - 15 min with ECL solution and imaging via image reader. The Western blot analysis was done by using *Image J* densitometric analysis software from National Institutes of Health, USA.

Mouse IGFBP-5 ELISA (Enzyme Linked ImmunoSorbent Assay):

The conditioned medium levels of recombinant mouse IGFBP-5 were measured employing duoset sandwich ELISA development system (R&D Systems, Cat. # DY578) in accordance with the manufacturer's instructions.

For the measurement of IGFBP-5 concentration, MCF-7 cells, MCF-10 A cells and HDFs were transduced in serum-free conditions with appropriate empty virus and IGFBP-5 virus 50, 200 and 1000 m.o.i. (multiplicity of infection). After 3 h post-transduction foetal bovine serum was added to transduced cells depending upon the cell-type culture growth recommendations. 24 h post-transduction, conditioned medium was replaced from cell monolayers with the recommended cell culture medium and briefly

centrifuged to remove any cellular debris. The obtained conditioned medium was saved every 24 h for up to 10 d and the samples were stored at - 80 ^{o}C.

Briefly, sufficient wells for a complete experiment were taken and placed in a frame with dummy strips to complete the plate. The wells were then coated at 100 µl of capture antibody diluted 1:180 in PBS to each well. The plate was then sealed with a microplate sealer (Greiner bio-one, Cat. # 676001) and incubated overnight at room temperature. Wells containing capture antibody buffer was aspirated and washed three times with wash buffer (see below). Subsequently, wells were blocked with 300 µl of blocking buffer and incubated for a minimum of 1 hour at room temperature. Blocking buffer was then decanted and wells were given a further three washes with wash buffer and dried thoroughly.

Thereafter, 100 µl of standards and adenovirus-transduced human epithelial and fibroblasts conditioned medium samples diluted 1:10 in 10% FCS were added in duplicates. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature. The washing step was repeated.

Then, detection antibody diluted 1:180 in reagent diluent (see below) was added at 100 µl to each well. And the plate was allowed to incubate for a further 2 hours at room temperature. The washing step was repeated.

After that, 100 µl of 1:200 diluted streptavidin conjugated to horseradish peroxidase using reagent diluent was added to each well and incubated for 20 minutes at room temperature. The tipping off/wash step was repeated.

Wells then received 100 µl of substrate solution (see below) and were incubated for 20 - 30 minutes at room temperature. Finally, the reaction was stopped by adding 50 µl of 2N sulphuric acid. The optical density readings were recorded immediately at 450nm through the use of a microplate reader.

Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2 – 7.4

Block Buffer: 5% Tween® 20 in PBS w/v 0.05% Sodium azide.

Reagent Diluent: 5% Tween® 20 in PBS, pH 7.2 – 7.4

Substrate Solution (R&D Systems, Cat# DY999): 1:1 mixture of color reagent A (Hydrogen Peroxide) and color reagent B (Tetramethylbenzidine).

Preparation of samples for RT-RT-PCR

Total RNA was isolated from adenovirus transduced MCF-7/HDFa cells using high pure RNAeasy® plus mini kit (Qiagen, USA, Cat# 74134). Approximately, 3 x 10^6 harvested cells were disrupted by adding 350µl cell lysis buffer (RLT* buffer). The homogenized lysate was transferred onto a gDNA Eliminator spin column and centrifuged at 8,000 x g for 30 sec. To the lysate one volume of 70% ethanol was added and mixed by gentle pipetting. Thereafter, around 700 µl of lysate was transferred into an RNeasy spin column and centrifuged at ≥ 8,000 x g for 15 sec. To the column containing the sample, 700 µl of wash buffer (RW* 1 buffer) was added and centrifuged for 15 sec at ≥ 8,000 x g. This was followed by addition of 500 µl elution buffer (RPE* buffer) and centrifuged at ≥ 8,000 x g for 2 min. To the spin columns, 2ml collection tubes (supplied by Qiagen kit) were placed and centrifuged at 10,000 x g for 1 min to dry the RNeasy silica gel membrane. Finally, RNA was eluted by adding 30 µl RNase free water directly onto the spin column membrane and centrifuged at 10,000 RPM for 1 min to elute the RNA. The resultant RNA was quantified spectrophotometrically using a Biophotometer (Eppendorf) and used for gene expression studies.

*RLT, RW 1, RPE, RNeasy spin columns and gDNA eliminator spin columns were supplied by the manufacturer (Qiagen, USA).

For the reverse transcription reaction, complementary DNA is synthesized using first strand cDNA synthesis kit (AMV, Roche). Total RNA was reverse-transcribed into cDNA as per the protocol provided by the supplier (Roche, Mannheim, Germany).

Provisionally, 2 µg of RNA was used as a starting template for the RT-PCR reaction having a final volume of 20 µl. The reaction components of this single step reverse transcription-polymerase chain reaction were as follows:

Table 2.1: ***Reaction components of first step reverse transcription-polymerase chain reaction.***

Component	Volume	Final Concentration
RNase-free water	Variable	--
10X Reaction Buffer	2 µl	1X
25mM $MgCl_2$	4 µl	5 mM
dNTP mix	2 µl	1 mM
Random Primers $p(dN)_6$	2 µl	0.08 A_{260} units
RNase Inhibitor	1 µl	50 units
AMV Reverse Transcriptase	0.8 µl	≥ 20 units
RNA Template	Variable	2 µg
Final Reaction Volume	**20 µl**	

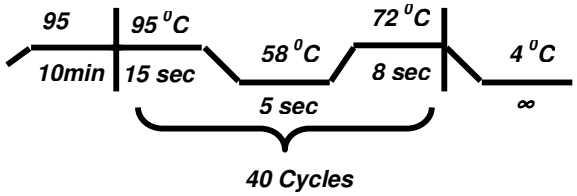
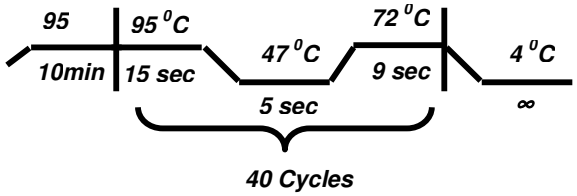
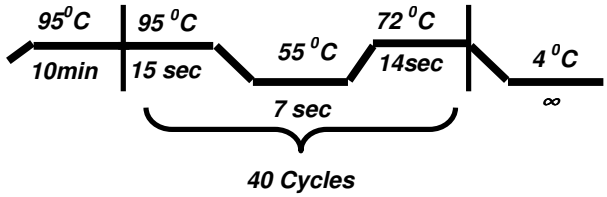
Because of the small volume of reagents required, a larger amount of the master mix was prepared depending upon the number of samples and centrifuged under pulse rate. Subsequently, the master mix was aliquoted into reaction tubes before adding the RNA template. cDNA sequences from different genes were obtained. For detection of cyclophilin, IGFBP-5, fibronectin and laminin 5 mRNA, the following primers were designed for the present study:

Table 2.2: ***shows the primer sequences used for cDNA amplification.***

Gene	Primer Sequence
Cyclophilin	**Sense:** 5' - CATCCTAAAGCATACGGGTCCTG - 3'
	Antisense: 5' - TCCATGGCCTCCACAATATT - 3'
IGFBP-5	**Sense:** 5' - CAAACACACCCGCATC - 3'
	Antisense: 5' - CAGGTACACAGCACGG - 3'
Fibronectin	**Sense:** 5' - TTCAAGTGTGATCCCCATGAA G - 3'
	Antisense: 5' - CAGGTCTACGGCAGTTGTCA - 3'

The resulting cDNA was amplified by real-time PCR using light cycler DNA technology (Roche). Fast start Taq DNA polymerase was activated by preincubation at 95^{o}C for 10 min. PCR was run for 40 cycles and the threshold cycle points (C_t) were calculated using the Light Cycler software.

Table 2.3: ***shows the gene amplification conditions of quantitative real-time polymerase chain reaction.***

Gene	Quantitative RT-PCR conditions
Cyclophilin	
IGFBP-5	
Fibronectin	

The resulting threshold cycle points (quantitative endpoints) were obtained from IGFBP-5 virus treated samples and untreated control samples. The fold change is calculated using the formula:

$$2^{-\Delta CT} = [(C_T \text{ gene of interest} - C_T \text{ internal control gene})]$$

SuperArray PCR System

Preparation of Samples for SuperArray PCR:

Human extracellular matrix and adhesion molecules gene expression profiling studies were carried out employing RT^2 Profiler™ PCR Array System (SA Biosciences, Cat. # PAHS-013C). This PCR super array system profiles the expression of 84 genes important for cell-cell and cell-matrix interactions. By real-time PCR, this array was used for a reliable expression of these genes.

Briefly, MCF-7 cells were seeded at a density of 5 x 10^5 cells/ml and adenovirally transduced with empty and BP-5 virus at 1000 m.o.i. The routine growth medium was replaced every 24 hours for a period of 3 - 4 days for a substantial RNA yield. Total RNA was extracted using Qiagen RNAeasy® Mini kit (Qiagen, USA, Cat. # 74134). The RNA yield was determined spectrophotometrically by measuring the optical density at 260 nm (Biophotometer, Eppendorf, Germany).

Preparation of First Strand cDNA Synthesis:

First strand cDNA synthesis was performed using 1 µg of Empty virus/IGFBP-5 total RNA and RT^2 First Strand Kit (SA Biosciences™, Cat. # C-03). As per the instructions, total RNA was mixed with 2 µl of 5X gDNA elimination buffer and made to final volume of 10 µl with RNase-free H_2O.

Following brief centrifugation, the contents were incubated at 42 ^{o}C for 5 minutes to eliminate any genomic DNA and kept immediately on ice to stop the reaction.

Preparation of RT Cocktail:

Table 2.4. : ***Shows reaction components of RT cocktail for the preparation of first strand cDNA synthesis reaction:***

Components of RT Cocktail	Volume of 2 Reactions (µl)
BC3 (5X RT Buffer 3)	8
P2 (Primer & External Control Mix)	2
RE3 (RT Enzyme Mix 3)	4
Rnase-free H_2O	6
Final Volume	20

Once the RT cocktail was prepared, 10 µl of it was mixed thoroughly with 10 µl of genomic DNA elimination mixture. Following brief centrifugation, the mixture was incubated at 42 ^{o}C for 15 minutes and immediately, the reaction was stopped by heating at 95 ^{o}C for 5 minutes using thermocycler (Techne). After the reaction was stopped, 91 µl of double distilled H_2O was added to each 20 µl of cDNA synthesis samples and kept on ice.

Experimental Cocktail Preparation:

RT^2 qPCR Components	Volume
2X RT^2 qPCR Master Mix	1275 µl
Diluted First Strand cDNA Synthesis Reaction	102 µl
ddH_2O	1173 µl
Total Volume	2550 µl

25 µl of experimental cocktail was added to each well of 96-well PCR array plate, sealed with a sealer, and kept on ice.

Once the cycling program has been set up, the 96-well PCR array plate kept in the ABI 7500 thermocycler and started with the manufacturer's recommended two cycling program.

Cycles	Duration	Temperature
1	10 minutes	95 ^{o}C
40	15 seconds	95 ^{o}C
	1 minute[2]	60 ^{o}C

PCR was performed for 40 cycles and the threshold cycle points (C_t) were calculated by setting the baseline and using ABI 7500 software. Obtained threshold cycle values were exported to excel spreadsheet using manufacturer's data analysis template excel file.

Biological Assays:

β-galactosidase transduction assay

Human dermal fibroblasts were seeded into a 96 well plate at a density 1 x 10^5 cells/ml in serum free conditions. Adenovirus containing β-galactosidase was diluted to obtain 50 & 200 viral particles per cell (m.o.i). To the virus, 1% Gene Jammer transduction reagent (Stratagene, USA) was added and incubated for 10 min at room temperature. Subsequently, virus with transduction reagent was added and incubated for 3 hrs. Thereafter, 2% FCS DMEM was added to make up to final concentration.

The appropriate level of transduction was determined employing the X-gal staining technique for transduced cells.

Preparation of X-gal staining solution

X gal (Bromo-4-Chloro-3-Indolyl α-D-Galactopyranoside) was prepared by dissolving 1 ml of 20 mg/ml X- gal stock solution (prepared by dissolving 100 mg Xgal in 5 ml DMSO) in 200 µl of 500 mM potassium ferro and ferricyanide and 20 ul of $MgCl_2$ and 18.58 ml 1X PBS. 50 µl of X-gal staining solution was put into 100 ul of HDFs & MCF-7 cell suspension for staining (2- 20 hrs).

Cell Adhesion Assays

To study the effect of IGFBP-5 on cell: substratum adhesion and cell: cell adhesion, MCF-7 and MCF-10A cells were transduced with adenovirus that has been genetically

engineered to produce IGFBP-5 endogenously and treated in serum-free and with increasing concentrations of serum and with exogenous treatment of 100 ng/ml IGF-I.

Day 1:

5 x 10^5 MCF-7 cells or MCF-10A cells/ml were transduced with empty virus and IGFBP-5 virus at 200 m.o.i. and seeded onto a 6 well plate in serum-free conditions. To improve the adenoviral transduction efficacy, 1:10 diluted 1% Gene Jammer was added to empty virus and IGFBP-5 virus and incubated for 10 min at room temperature. The wells containing MCF-7 cells or MCF-10A cells with empty and IGFBP-5 virus were incubated for 3 hours in CO_2 incubator at 37 ^oC. After incubation, 10% FCS DMEM was added to MCF-7 cells and 5% HS DMEM/F12 was added to make up to the final concentration.

Day 2:

Wells were rinsed and trypsinised and centrifuged in serum-free medium. Cells were resuspended in serum and serum-free conditions and adjusted to 200,000 cells/ml in case of MCF-7 cells and 250,000 cells/ml in case of MCF-10A cells. The wells containing MCF-7 cells were treated with increasing concentrations of serum in the order serum free, 0.01%, 0.1%, 1% and IGF-I at 0.1µg/ml. Similarly, the wells containing MCF-10A cells were also treated in the order serum-free, 0.05%, 0.5%, 5% and IGF-I at 0.1µg/ml. Simultaneously, 100µl of cell suspension was added to a separate 96 well plate containing 10ul of 10% FCS (final conc. 1% serum) as a start value. Both the plates

were cultured overnight for endogenous production of IGFBP-5 and t=0 plate was stopped after 24 hrs as a "start" value for cell number.

Day 5:

Thereafter, the cell-adhesion assay plates were stopped after 3 days depending upon the responses. The wells were fixed, stained, photographed and solubilized. Before and after solubilisation values were measured at absorbance 540 nm.

Cell Migration Assay

Cell culture inserts (Catalogue # 80209, Ibidi GmbH Integrated BioDiagnostics, Germany) were seeded with defined cell seeding of 50,000 cells/chamber in case of MCF-7 cells and 5,000 cells/chamber in case of HDFs. After overnight incubation, the inserts were removed using forceps carefully. The cells were topped up with DMEM depending upon the requirements of the experiment.

Cell Survival Assay

Adenovirally-transduced MCF-7 cells were seeded at different seeding densities optimum i.e., 250,000 cells/ml; low i.e., 50,000 cells/ml and high i.e., 500,000 cells/ml in 96 well plate at low and high serum concentrations. Similarly the cells were seeded at low and high seeding density and treated with and without exogenous IGFBP-5. The cells were stopped and fixed using 4% PFA at regular time intervals on the basis of cell de-adhesion. Then, the fixed cells of all the cell survival experiments were stained with

crystal violet and photographed and solubilised using 0.1% Triton X 100. Solubilised data was measured spectrophotometrically at 540 nm.

Cell Viability Assay

Cell viability assay or Live/Dead staining assay was employed to study the effects of IGFBP-5 on cell viability in nutrient-deprived conditions. Briefly, adenovirally-transduced MCF-7 cells were analysed for cell viability using Live/Dead or Viability/Cytotoxicity kit (Cat # L3224; Invitrogen Molecular Probes®, USA). After various periods of culture, both empty and IGFBP-5-transduced MCF-7 cells were rinsed into serum-free medium and then incubated with Live/Dead stain for 15 minutes at room temperature. The cells were measured for live and dead fluorescence absorbances at excitation wavelength 495/517 nm and 528/617nm respectively using fluorimeter (Spectramax, Germany). The cells were photographed using fluorescence microscope.

Integrin Binding Cell-mediated Adhesion Assays

α/β integrin binding assays (Avanticell science, Ayr, UK.) were employed to study the expression of different integrins in IGFBP-5-transduced cell systems. This α/β integrin-mediated cell adhesion assay uses panel of mouse monoclonal antibodies generated against human alpha (α1, α2, α3, α4, α5, αV and αVβ3) and beta (β1, β2, β3, β4, β6 and αVβ5 and α5β1) integrins along with a negative control that are immobilized onto a pre-coated microtitre plate. This plate captures only those cells expressing these integrins on

their cell surface and uses wells coated with non-specific antibodies as a negative control.

In serum-free culture medium, 2 x 10^5 MCF-7 cells or HDFs/ml were added onto the 8 wells of alpha-integrin and beta-integrin binding plates. In addition to this, a t=0 plate was prepared in a 96 well plate simultaneously with the same seeding density containing 1% FCS in case of MCF-7 cells and 2% FCS in case of HDFs as final concentration. Both α/β integrin binding plates were incubated for 3 hours in humidified atmosphere of 5% CO_2 incubator at 37 ^oC. After 24 hours, the t=0 plate was fixed, stained and the absorbance determined at 540nm.

After incubation, MCF-7 cells were aspirated and the wells were washed with wash buffer II 3 times. Wash buffer I, II, Cell stain solution and extraction solution were supplied in the kit (Avanti cell science, Ayr, Scotland). To the wells, cell stain solution was added to stain the cells and incubated for 10 min at 37^oC. Excess stain was then removed by washing with wash buffer II for about 3 - 5 times. To the stained cells, 100 µl of extracting solution was added and incubated for 5 - 10 min in an orbital shaker in order to solubilize the cell-bound stain. The absorbance values were measured spectrophotometrically at 540 nm.

Effect of function-blocking integrin antibodies on MCF-7 cell adhesion.

$3x10^4$, MCF-7 cells were added to wells containing 5µg/ml of integrin antibodies in the presence or absence of 10ug/ml of IGFBP-5 in 0.1% BSA: DMEM. Function-blocking antibodies used were β1 (Catalog # MAB1987Z), αVβ6 (Catalog # MAB2077Z), αVβ3 (Catalog # MAB1976Z), αVβ5 (Catalog # MAB1961Z) α2 (Catalog # MAB1950Z), and α5 (Catalog # MAB1956Z) (Chemicon International California, U.S.A.). Plates were incubated for 24h at 37 °C in a 5% CO_2 atmosphere before fixation and staining with crystal violet.

Signalling Pathway Kinase Inhibitors Assay

In order to study the IGFBP-5-induced cell adhesion, a variety of inhibitors to various pathways were employed. The inhibitors used were LY 294002 (phosphatidylinositol-3-kinase inhibitor), PD 98059 (mitogen-activated protein kinase kinase or MEK 1 inhibitor), U 0126 (MEK 1/2 inhibitor), SB 203580 (p38 mitogen-activated protein kinase inhibitor), A 8301 (TGFβ kinase/activin receptor-like kinase (ALK 5) or Smad inhibitor), SB 4131542 (ALK R or Smad inhibitor) and Sphingosine Kinase type 1 inhibitor.

Briefly, 2 x 10^5 MCF-7 cells were seeded in the presence and absence of 10 µg/ml IGFBP-5 to 96 well plate in serum free conditions. The inhibitors were prepared at 0.1% DMSO and added to control and IGFBP-5-treated MCF-7 cells. Simultaneously, 100µl of MCF-7 cells was added to a separate 96 well plate containing 1% FCS as a start value

for cell adhesion (T=0 plate). After 24 hrs of incubation, in CO_2 incubator at 37 ^oC the cells were fixed, stained, photographed and solubilized. Before and after solubilisation values were measured at absorbance 540 nm.

Immunofluorescence staining

MCF-7 cells were cultured for 24h in the absence or presence of 10 ug/ml of IGFBP-5 in wells of a chamber slide. As a positive control, cells were also cultured with 10% FBS DMEM. After 24 h, medium was removed from the wells by inverting the chamber slides and cells were fixed with 200 µl of 2% (w/v) paraformaldehyde in PBS for 10 min at room temperature. This was followed by permeabilization with 200 µl of 0.5% Triton X-100 in PBS for 15 min at room temperature. Nonspecific staining was diminished by incubating with PBS containing 10% Heat Inactivated (HI) serum from the species in which the second antibody was produced. Cells were incubated with 200 µl of 5 ug/ml mouse anti-human E-cadherin (Catalog No. 610181, BD Biosciences, U.S.A.) and 200 µl of 1:250 dilution of rabbit anti-human paxillin as described by the manufacturer (Product No. ab32084, Abcam, Cambridge, United Kingdom) at 4^oC overnight. This was followed by 1 h incubation with respective secondary antibody and with rhodamine phalloidin (Catalog No. 610181, Biotium, U.S.A.) to stain F-actin at 37^oC. Slides were overlaid with antifade DAPI nucleic acid mountant containing 4',6-diamidino-2-phenylindole (DAPI) and mountant (Molecular Probes, Invitrogen, U.S.A). The slides were incubated in dark overnight at room temperature and visualized with a Nikon Eclipse Epifluorescence microscope. Other than specified, 400x and 1000x immunofluorescence magnified images were obtained with oil immersion technique.

Phospho-Kinase arrays:

To determine the relative levels of IGFBP-5 mediated protein phosphorylation profiles, we employed a Human Phospho-Kinase Array Kit (R&D Systems, Cat # ARY003). Exogenous IGFBP-5 treated or adenovirus-transduced-IGFBP-5 MCF-7 cells were seeded at 1,000,000 cells/ml in triplicates. After culture for 6h or 48h, cells were lysed and cellular extracts were prepared and total protein concentrations were determined using BCA protein assay kit (Pierce, Cat # 23225). Kinase array blots were incubated with 250 ug of total protein in array buffer (provided in the kit) and instructions were followed in accordance to the manufacturer's kit. All the blots were developed with an ECL plus Western blot detection system (GE healthcare) and visualised with an LAS 3000 image reader (Fuji, Dusseldorf, Germany).

Interpretation of Phospho Kinase array/Western blots:

Using Image J densitometry software, mean gray values (MGV) of each spot of the array were taken from early and late exposure blots of independent experiments. Then, MGV of duplicate spots were averaged and compared with corresponding treatments. Results were shown as mean $\pm$ standard error of the mean (SEM). In case of ILK blots, the MGV were normalised with GAPDH and plotted as percentage of gray value. Standard deviation with error bars and Student's t-test were performed using Microsoft Excel.

Statistical Analysis

All the statistical analyses of the experimental data were determined by Student's unpaired or paired t-test using Microsoft Excel software.

Chapter III

Results

Sub-Chapter 1

Expression, Production & Measurement of Recombinant IGFBP-5

Expression of Recombinant IGFBP-5

Recombinant wildtype (wt) IGFBP-5 expression was carried out employing conditions identical to those described by Allan and co-workers (Allan et al., 2002). cDNA for mouse wt IGFBP-5 without the signal peptide-encoding sequence was cloned into pGEX 6P-1 vector between BamHI and EcoRI in the multiple cloning site. 50 ng of this construct was used for the bacterial expression of IGFBP-5. A 400 ml of bacterial culture typically yielded about 1 mg protein.

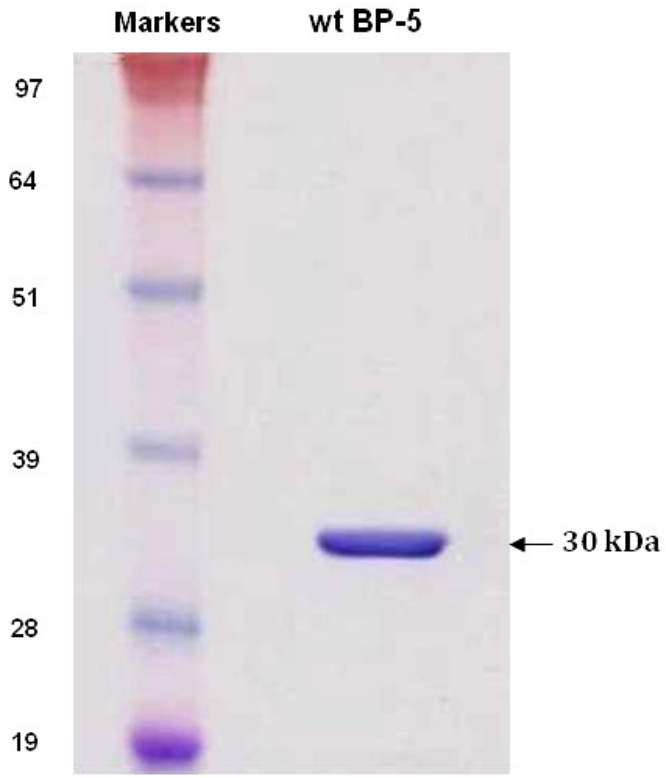


Fig 3.1.1: ***SDS PAGE image of wt-IGFBP-5 separation with molecular weight markers in kilo Daltons (kDa).***

Expression of Recombinant C-Terminus (005)

C-terminal domain of IGFBP-5, here after refererred as 005, recombinant expression was carried out using conditions identical to those described by Allan and his colleagues (Allan et al., 2002). cDNA for mouse wt IGFBP-5 without the signal peptide-encoding sequence was cloned into pGEX 6P-1 vector between BamHI and EcoRI in the multiple cloning site. 50 ng of this construct was used for the bacterial expression of IGFBP-5. A 400 ml of bacterial culture typically yielded about 500 ug protein.

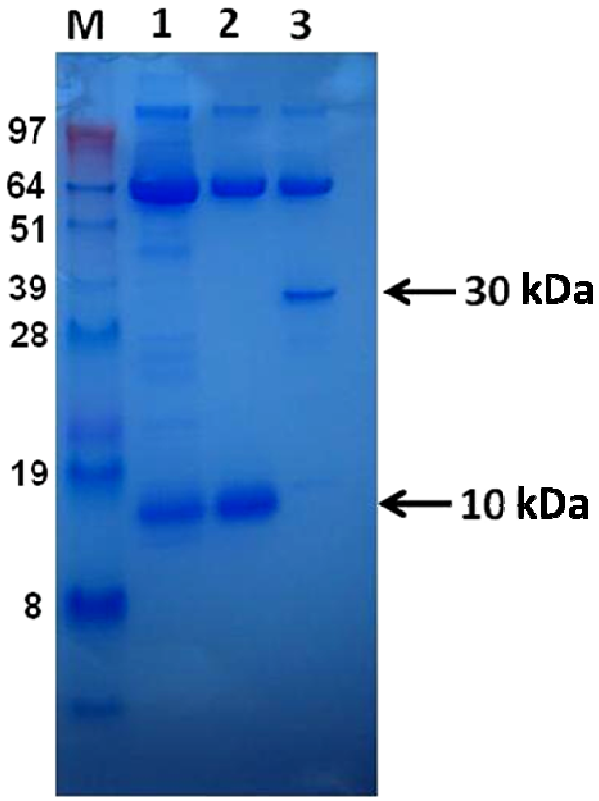


Fig 3.1.2: ***SDS PAGE image showing difference in molecular weight of wt-IGFBP-5 and 005 protein. Lane M, 1 -3: Molecular weight marker, HPLC purified 005, GST purified 005, and HPLC purified IGFBP-5.***

Studies with Adenovirus-Mediated Transduction:

β - Galactosidase Transduction Assay:

Appropriate adenoviral DNA transduction levels were optimised and achieved in MCF-7 cells and HDFs by transfecting efficiently with adenovirus containing β - galactosidase and using a transfection reagent, Gene Jammer. After transduction, the cells were stained using the X - gal staining technique. Similar patterns were also obtained in the case of MCF-7 cell lines. Results demonstrated that there is increase in the transduction from 50 to 200 m.o.i. and it's further improved by GeneJammer reagent.

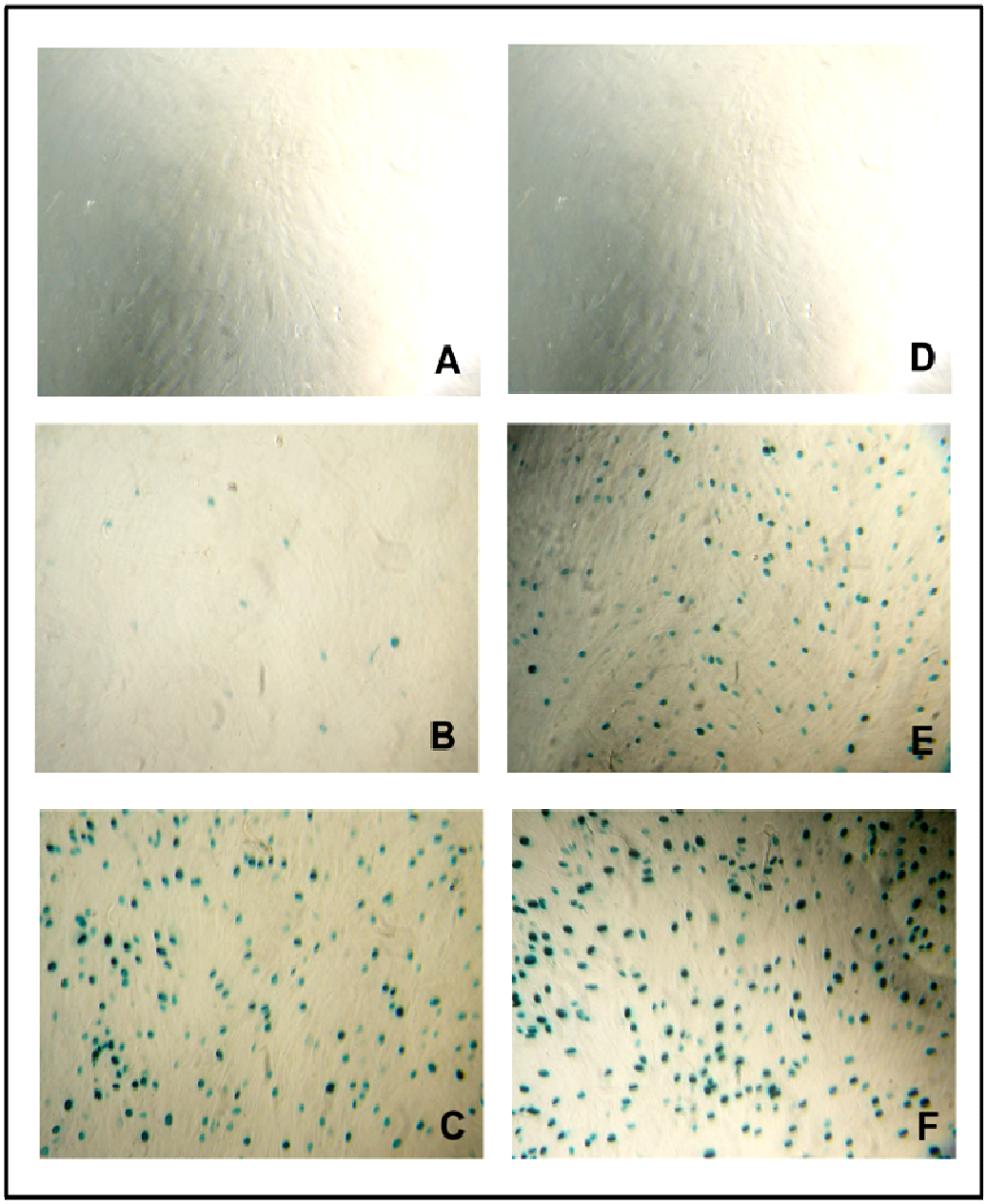


Fig 3.1.3: ***β-gal Adenovirus transduction assay in Human Dermal Fibroblasts. (A) Uninfected HDFs without gene jammer (B) & (C) β - gal adenovirus-transduced HDFs at 50 and 200 m.o.i. (D) Uninfected HDFs with Gene Jammer (E) & (F) β - gal adenovirus transduced at 50 and 200 m.o.i in the presence of Gene Jammer. n=1***

Measurement of IGFBP-5 concentration in IGFBP-5-transduced cells

The levels of IGFBP-5 were measured on a daily basis in the medium from IGFBP-5-transduced human epithelial and dermal fibroblasts employing IGFBP-5 specific ELISA. A correlation between the concentration of IGFBP-5 in the medium and multiplicity of infection was observed in IGFBP-5-transduced human epithelial and mesenchymal cells. The concentration of IGFBP-5 secreted into the conditioned medium by the IGFBP-5-transduced cells increased from 1 d post transduction to 3 d post transduction and the concentration remained unchanged until 10 d post-transduction.

The amount of IGFBP-5 in the conditioned medium samples of empty virus-transduced epithelial cells and fibroblasts was found to be nearly zero (results not shown). The IGFBP-5 concentration on 3 d was found to be highest at 200 m.o.i. and measured as 124 ng/ml and 238 ng/ml in IGFBP-5-transduced epithelial cells and fibroblasts respectively. The amount of IGFBP-5 in the conditioned medium samples of IGFBP-5-transduced epithelial cells and fibroblasts were also analysed using western blotting technique. The densitometric values obtained were found to be similar to the IGFBP-5 ELISA values. The concentrations of IGFBP-5 in culture medium after 3d in cell adhesion assays were found to be 40 ng/ml and 266.5 ng/ml in IGFBP-5-transduced MCF-7 cells and 31.2 ng/ml and 889.4 ng/ml in IGFBP-5-transduced MCF-10A cells in serum-free and high-serum concentrations respectively (Figures. 3.1.7 & 3.1.8).

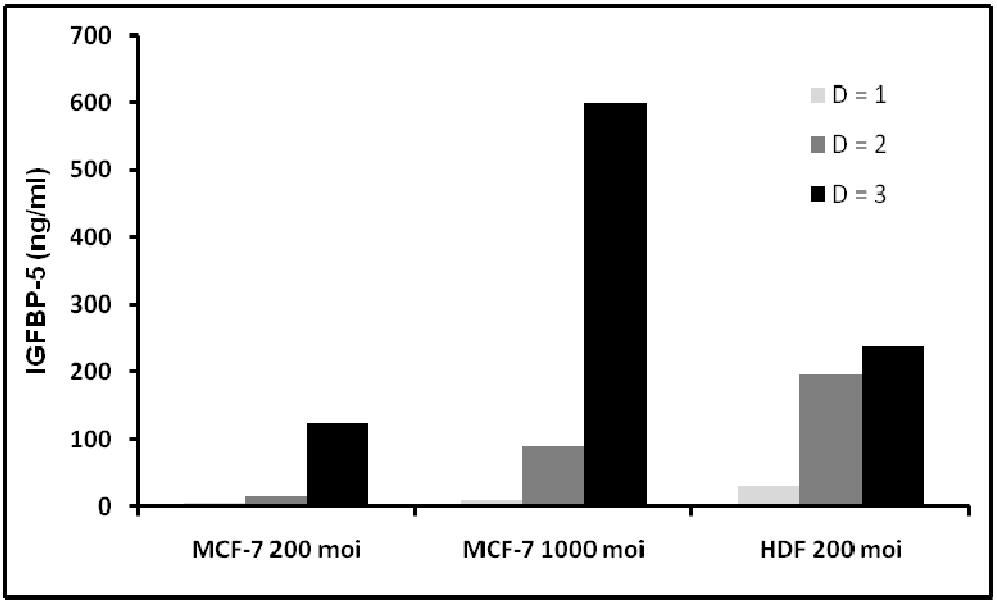


Fig 3.1.4: ***The levels of secreted IGFBP-5 in conditioned cultured medium by adenovirus-transduced epithelial and mesenchymal cells. n=1***

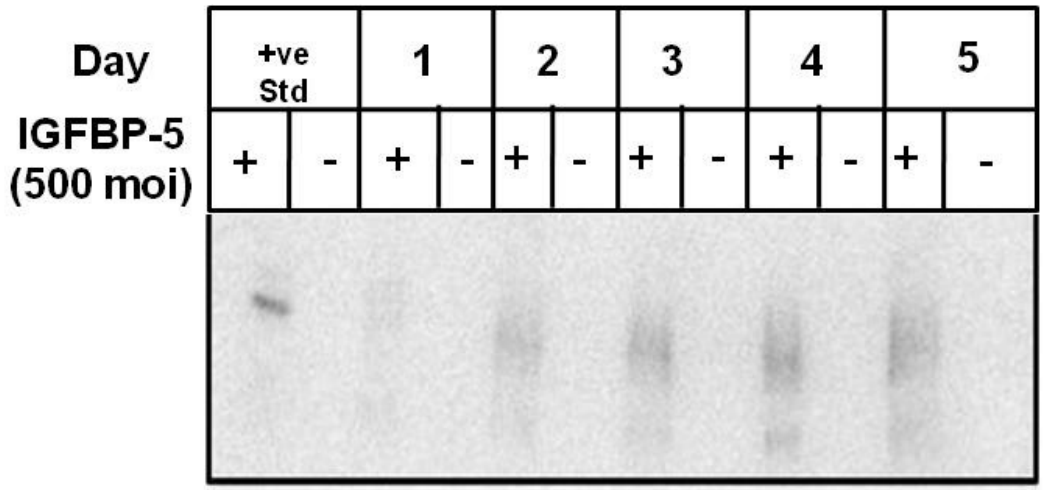


Fig 3.1.5: ***IGFBP-5 immunoblot of 15µl conditioned medium 1 - 5 d of IGFBP-5-transduced MCF-7 cells. Where +ve Std: Positive Standard at 100 ng. n=2***

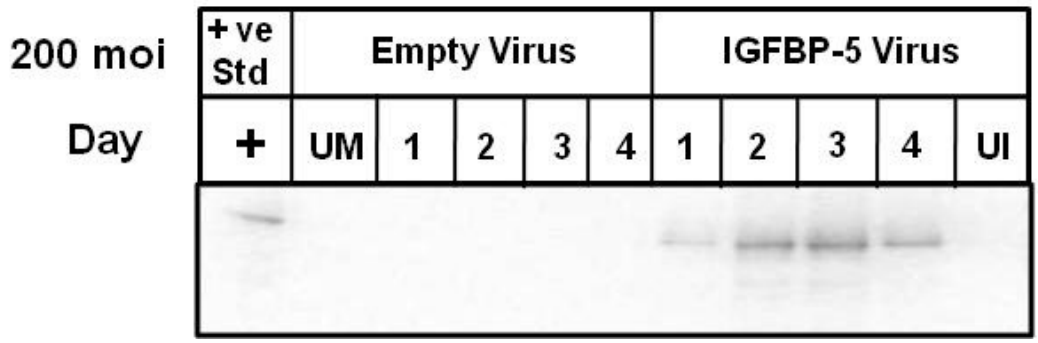


Fig 3.1.6: ***IGFBP-5 immunoblot of 15µl conditioned medium 1 - 4 d of IGFBP-5-transduced HDFs. Where +ve Std: Positive Standard at 100 ng; UM: Unconditioned HDFs medium; UI: Unifected. n=2***

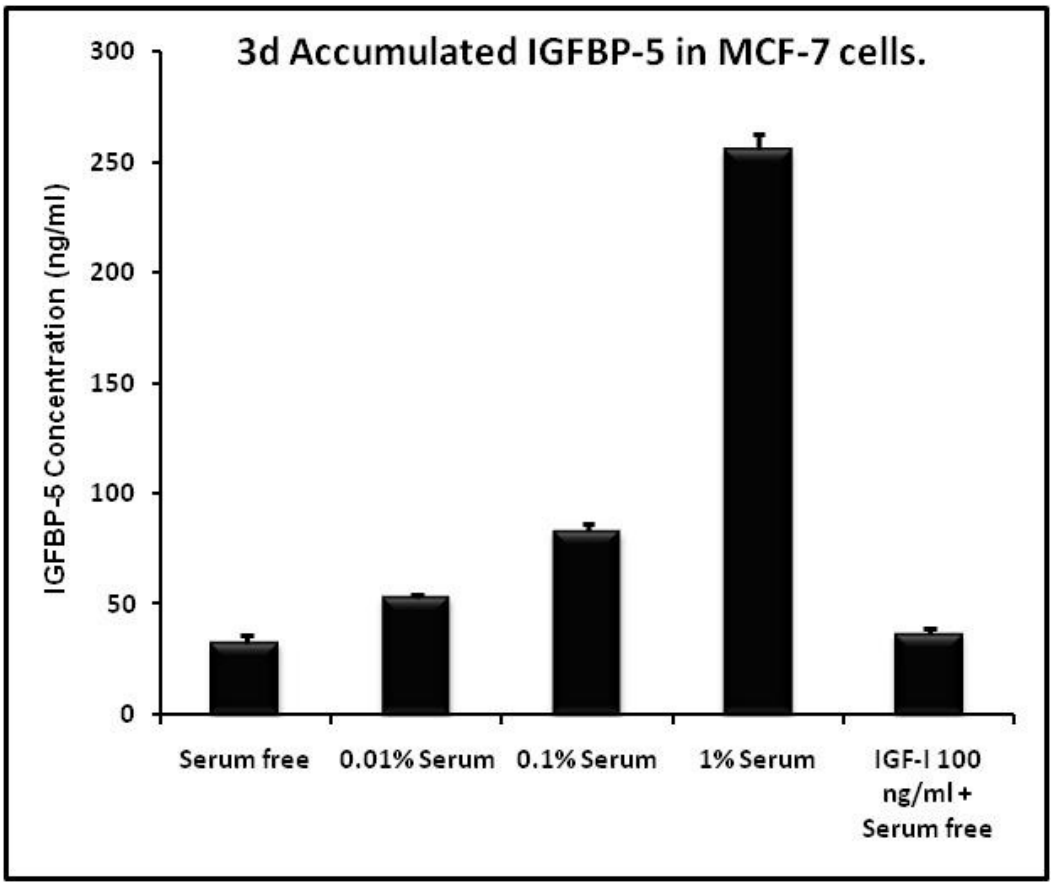


Fig 3.1.7: ***The graph showing the concentration of IGFBP-5 in IGFBP-5-transduced MCF-7 cells 3d post-transduction. Values represent mean ± SEM of 4 observations of two independent experiments. n=2.***

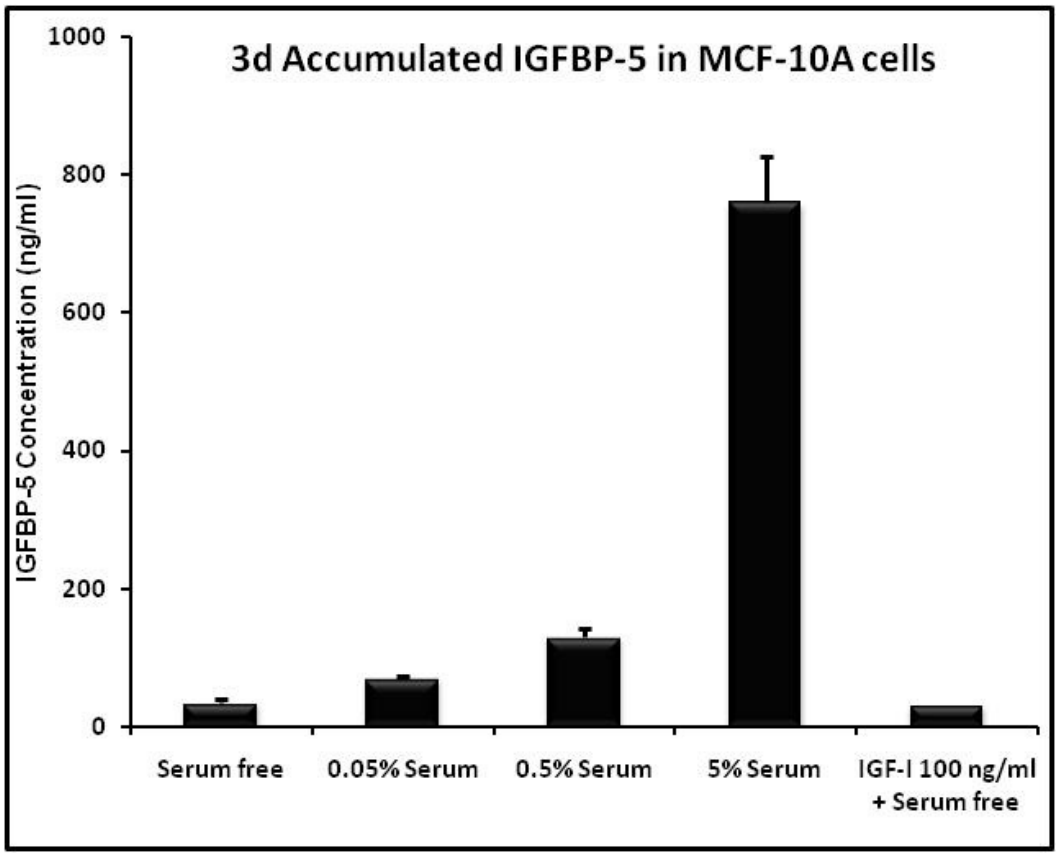


Fig 3.1.8: ***The graph showing the concentration of IGFBP-5 in IGFBP-5-transduced MCF-10A cells 3d post-transduction. Values represent mean ± SEM of 4 observations of two independent experiments. n=2.***

Discussion:

My first objective was to determine optimal conditions for the bacterial expression and purification of recombinant IGFBP-5. Expression of recombinant IGFBP-5 protein from the mouse cDNA was achieved successfully by bacterial system. Using the purification system of PreScission protease and HPLC, Origami B bacterial cultures typically yielded 1 mg of purified IGFBP-5 protein. This native recombinant IGFBP-5 was found to be approximately 30 kDa in molecular weight.

To study the biological properties of IGFBP-5 more precisely, attempts were made to determine optimal conditions for the purification of recombinant C-terminal domain. Expression of recombinant 005 protein was achieved successfully using the same E coli expression system as used for wild-type iGFBP-5 cell system. The precission protease and HPLC purification system typically yielded 500 µg of purified 005 protein. This C-terminal domain of IGFBP-5 was found to be approximately 10 k Da in molecular weight as predicted from its theoretical molecular weight.

I also demonstrated that adenoviral-mediated transduction was an efficient method of gene delivery to study the role of IGFBP-5 and its potential functions in altered cellular responses including fibrosis and metastasis. Different levels of mock transduction were assessed and, in order to improve the efficiency of viral-mediated transduction of cells, Gene Jammer was used (Bosch. P. et al., 2006; Fouletier-Dilling et al., 2005). Addition

of Gene Jammer to either MCF-7 or HDF cells during the adenoviral-mediated transduction led to significant increase in total number of transduced cells.

The success of adenoviral transduction was assessed using a specific ELISA for IGFBP-5 and western blotting. The assay was sufficiently sensitive to allow the concentration of IGFBP-5 to be measured on a daily basis secreted into the conditioned medium of both human epithelial cells and dermal fibroblasts transduced with IGFBP-5. The concentration of IGFBP-5 on day 3 was found to be highest at 200 m.o.i. and measured as 124 ng/ml and 238 ng/ml in IGFBP-5-transduced epithelial cells and fibroblasts respectively (Fig. 3.1.4). The 3 d accumulated IGFBP-5 in conditioned medium of IGFBP-5-transduced MCF-7 and MCF-10A cells were also measured at low serum concentrations (1% and 0.5%) and found to be 255.6 ng/ml and 130.8 ng/ml respectively. Densitometric analysis of IGFBP-5 immunoblots from the same conditioned medium samples achieved was similar (results not shown) The concentrations of IGFBP-5 in the medium were within the physiological range for the mammary gland (Tonner et al., 2002) and indeed were significantly lower than those observed, during the involution of rodent mammary gland *in vivo* when values of 100 µg/ml are attained.

The concentration of IGFBP-5 was also determined in the medium from the 3d cell adhesion assays of MCF-7 and MCF-10A cells and was found to be 40 ng/ml and 266 ng/ml in IGFBP-5-transduced MCF-7 cells and 31 ng/ml and 889 ng/ml in IGFBP-5-transduced MCF-10A cells in serum-free and 1% serum, respectively.

IGFBP-5 gene-expression at the mRNA level was increased in a dose-dependent fashion. Approximately, 35 fold increase of human IGFBP-5 gene-expression has been demonstrated at the level of mRNA in IGFBP-5-transduced MCF-7 cells. This 35 fold increase in endogenous expression of IGFBP-5 was physiologically relevant to the upregulation of IGFBP-5 in mammary epithelial cells *in vivo* during involution of the rodent mammary gland (Tonner et al., 2002).

These initial studies successfully achieved an expression system for the production of recombinant IGFBP-5 and an adenoviral IGFBP-5 expression system which could produce sustained production and secretion of IGFBP-5 at physiological concentrations. These studies thereby laid the foundations of studies aimed at addressing the mechanism of action of IGFBP-5.

Sub-Chapter 2
Biological Effects of IGFBP-5

Effects of IGFBP-5 on Cell: Substratum Adhesion

The effect of adenovirus expressing IGFBP-5 (Ad-IGFBP-5) on the cell: substratum adhesion in non-invasive mammary tumour cells, MCF-7 and normal mammary epithelial cells, MCF-10A cells was investigated by performing 3d adenoviral-transduced cell adhesion assays.

Cell Adhesion Assay studies in MCF-7 and MCF-10A cells

In serum-free conditions, IGFBP-5-transduced MCF-7 cells showed a 3 fold increase in cell: substratum adhesion and the degree of cell adhesion have been significantly increased with the increase in concentrations of serum. In the presence of IGF-I, IGFBP-5-transduced MCF-7 cells showing cell adhesion to substratum is still evident. In contrast, IGFBP-5-transduced MCF-10A cells showed 3 fold decrease in cell adhesion to substratum in serum-free conditions and the degree of cell adhesion was relatively unaffected with the increase in serum concentration.

The crystal-violet staining images (fig 3.2.1) showed that IGFBP-5-transduced MCF-7 cells in serum-free conditions illustrating the property of cell adhesion on to the substratum whereas, empty virus-transduced MCF-7 cells didn't show any cell adhesion to substratum. Upon exogenous treatment of IGF-I at 0.1 µg/ml concentration to IGFBP-5-transduced MCF-7 cells, IGFBP-5 considerably decreased the cell: cell adhesion effect of IGF-I where as empty virus-transduced MCF-7 cells didn't show any cell: substratum adhesion but rather showing cell: cell adhesion. In contrast, IGFBP-5-

transduced MCF 10A cells showed a decrease in cell adhesion to substratum even in the presence of serum (fig. 3.2.3).

The results were quantified and the spectrophotometric values infer that IGFBP-5 in case of MCF-7 cells showed cell spreading on to the substratum in serum-free culture medium and the property gradually increases with the increase in serum concentration. In case of IGF-I treatment, empty vector transduced cells showed large multicellular spheroids whereas, IGFBP-5-transduced cells showed relatively less cell adhesion and considerably decreased the effect of IGF-I (fig. 3.2.1). Whereas, IGFBP-5 in MCF-10A cells showed de-adhesion in the presence of both serum-free and serum concentrations and resulted in anoikis (fig. 3.2.3). The deadhesion effect of IGFBP-5 in MCF-10A cells might be due to the differerence in activation or deactivation of cell type specific surface receptors or cell: cell communication and the machinery present in the extracellular matrix. These opposing effects observed were possibly due to IGFBP-5 cell-type specificity and context dependency.

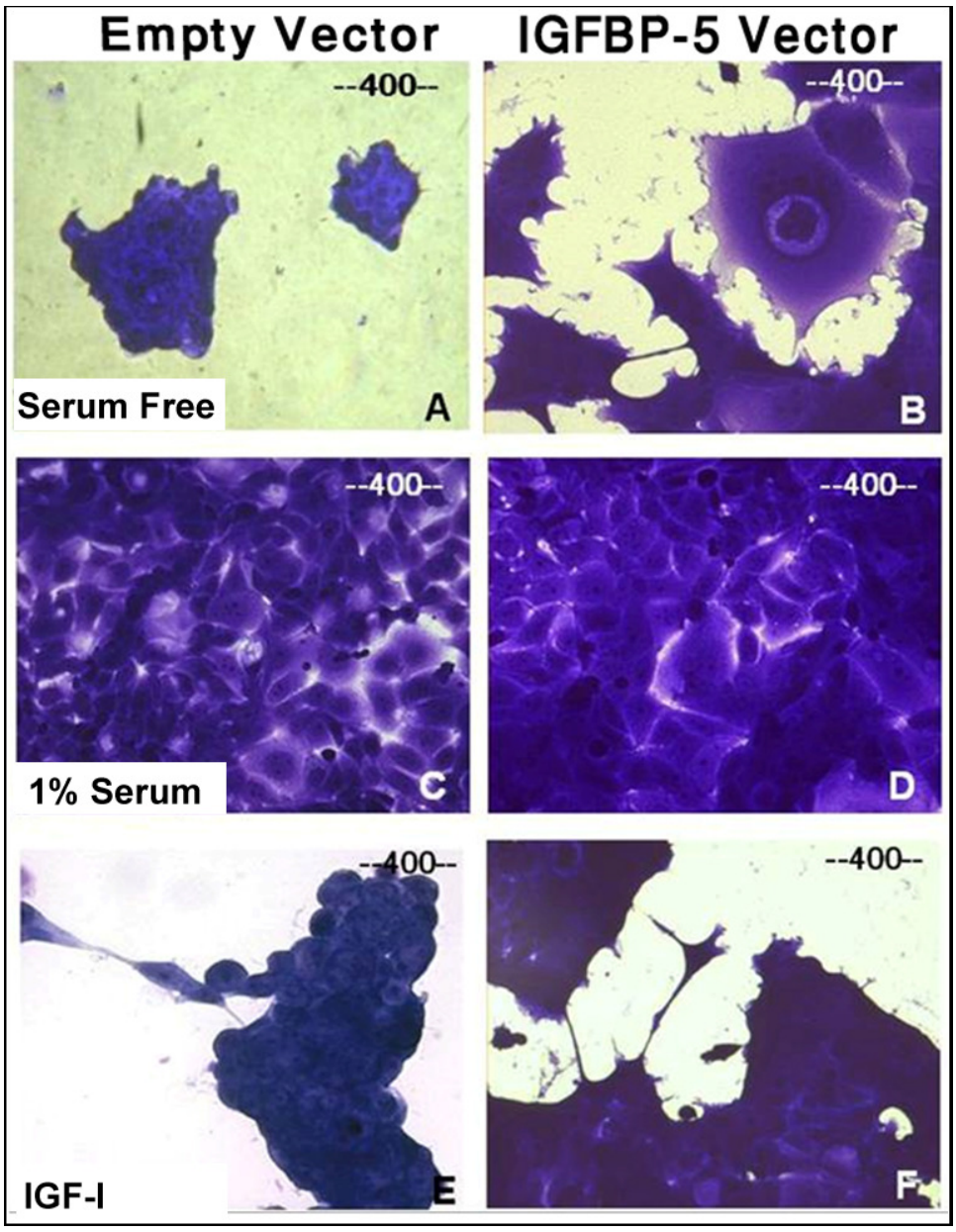


Fig 3.2.1: ***Crystal violet images of IGFBP-5-transduced MCF-7 cell adhesion assay post transduction 3 d. In serum-free conditions, (A) Empty virus-transduced MCF-7 cells showing cell: cell adhesion. (B) IGFBP-5-transduced MCF-7 cells showing increased cell: substratum adhesion. At relatively high serum (1%) concentrations (C) & (D) both empty virus-transduced MCF-7 cells and IGFBP-5-transduced MCF-7 cells showing cell: substratum adhesion. Exogenous treatment of IGF-I at 0.1 µg/ml concentration to (E) empty virus-transduced MCF-7 cells were showing cell: cell adhesion. (F) IGFBP-5-transduced MCF-7 cells were showing cell: substratum adhesion.***

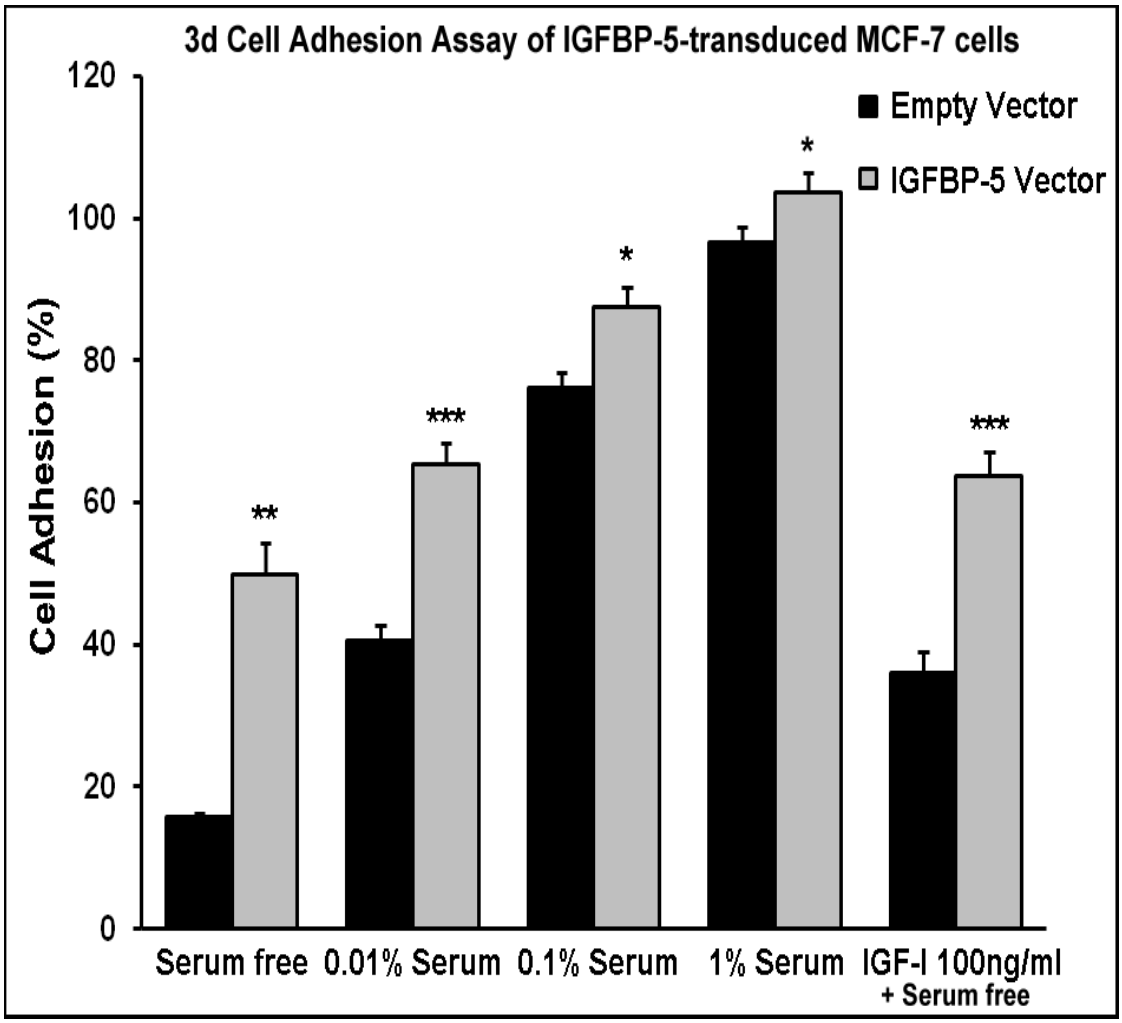


Fig 3.2.2: ***IGFBP-5 increases cell adhesion even in the presence of serum in MCF-7 cells. Values are mean ± SEM of 12 observations where * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 compared with control as determined by Students unpaired t-test. n=4***

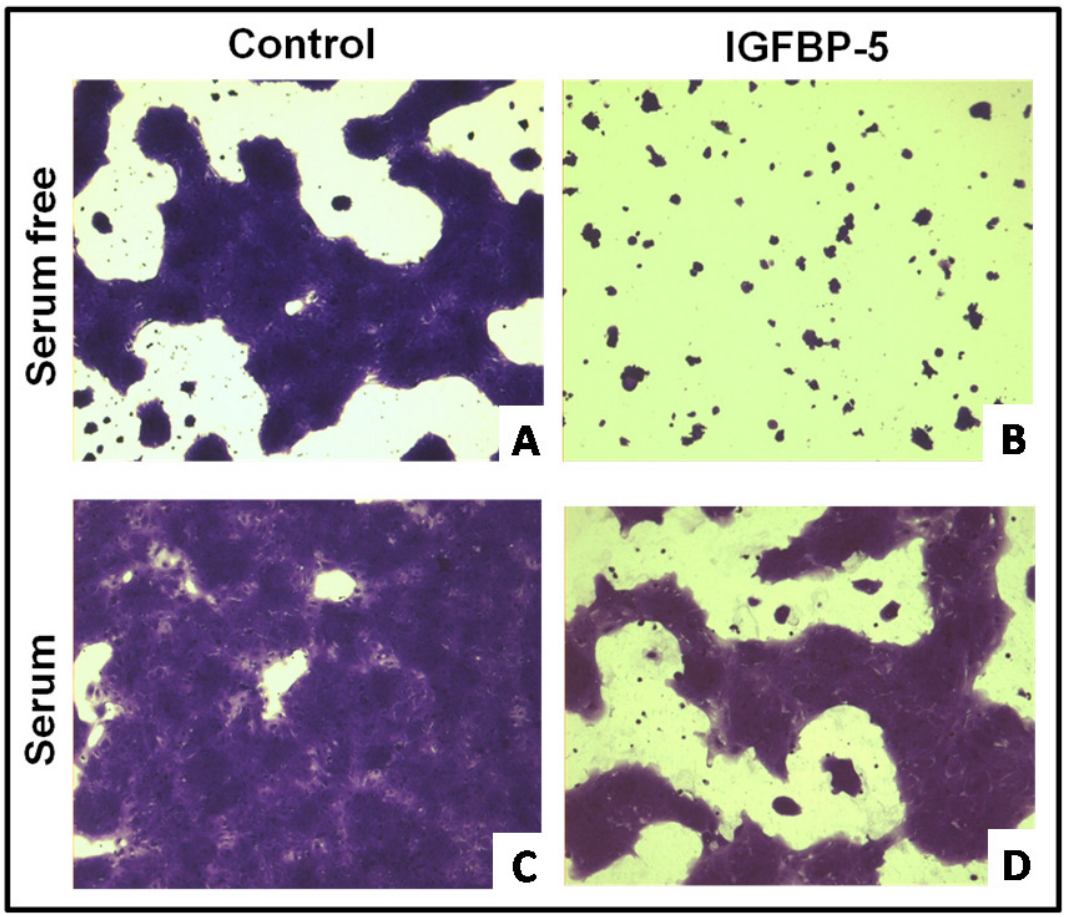


Fig 3.2.3: ***Crystal violet images of IGFBP-5-transduced MCF-10A cell adhesion assay post transduction 3 d. In serum-free conditions (A) Empty virus-transduced MCF-10A cells showing cell: substratum adhesion and (B) IGFBP-5 induces de-adhesion in IGFBP-5-transduced MCF-10A cells. At high serum (5%) concentrations (C) Empty-virus-transduced MCF-10A cells showing increased cell: substratum adhesion as compared to IGFBP-5-transduced MCF-10A cells. (D) IGFBP-5 decreases cell: substratum adhesion even in the presence of serum in IGFBP-5-transduced MCF-10A cells. Images were photographed at 100x magnification.***

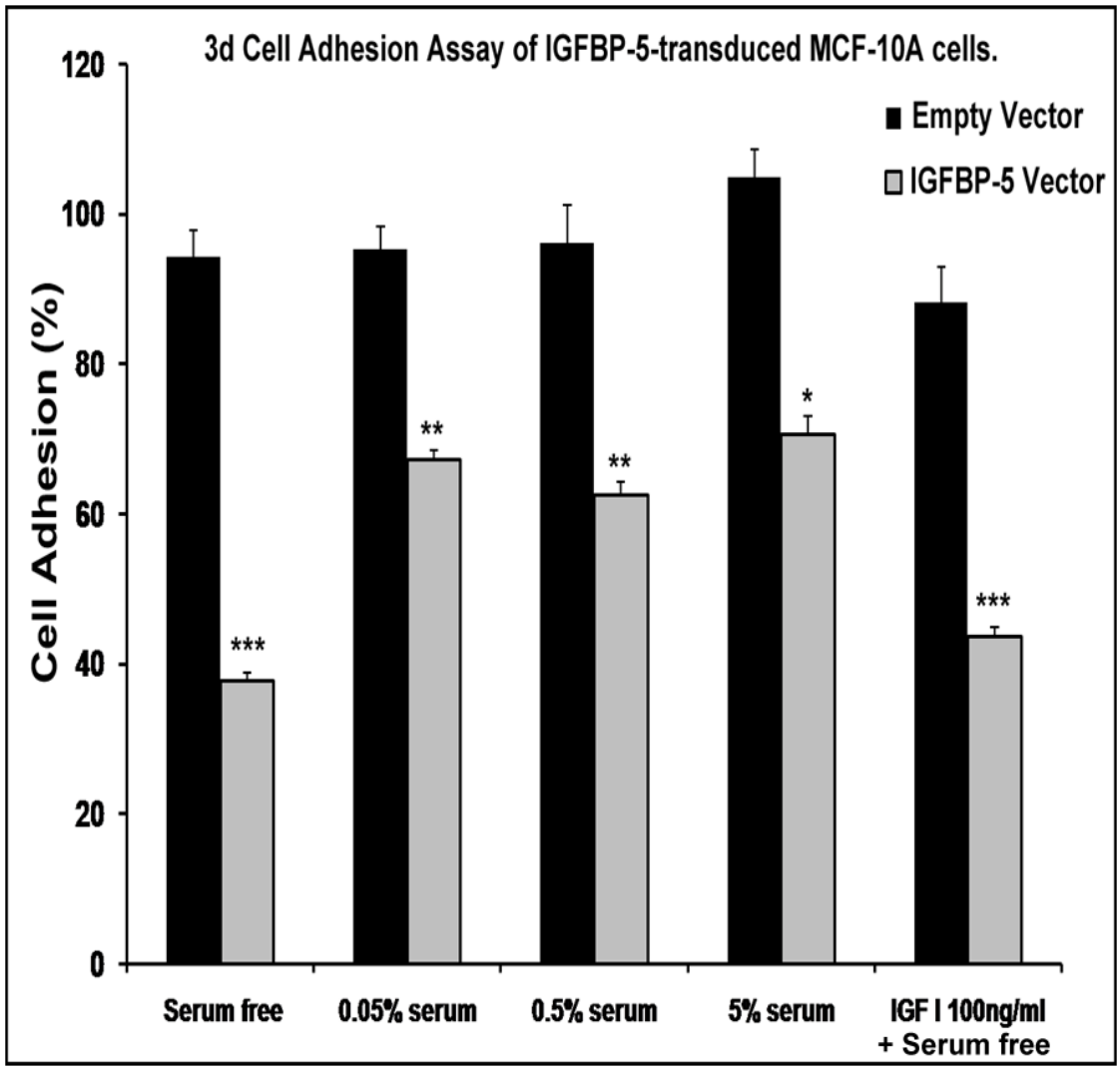


Fig 3.2.4: ***IGFBP-5 induces de-adhesion even in the presence of serum in IGFBP-5-transduced MCF-10A cells. Values are mean ± SEM of 9 observations where * indicated as p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 compared with control as determined by Students unpaired t-test. n=3***

Exogenous IGFBP-5 Cell Adhesion Assay in MDA-MB-231 cells

In order to study the effect of IGFBP-5 in highly invasive mammary tumour cells, MDA MB 231 cells were used. In serum-free and serum concentrations, MDA MB 231 cells were seeded at 200,000 cells/ml in 96 well plate and treated with recombinant IGFBP-5 at 10 µg/ml and cultured in 5% CO_2 incubator at 37 ^{O}C for 24 h. Similar to the effect in MCF-7 cells, IGFBP-5 also induced cell adhesion to substratum in MDA MB 231 cells. IGFBP-5 failed to show additional cell adhesion to substratum in the presence of serum (Fig 3.2.5 & 3.2.6).

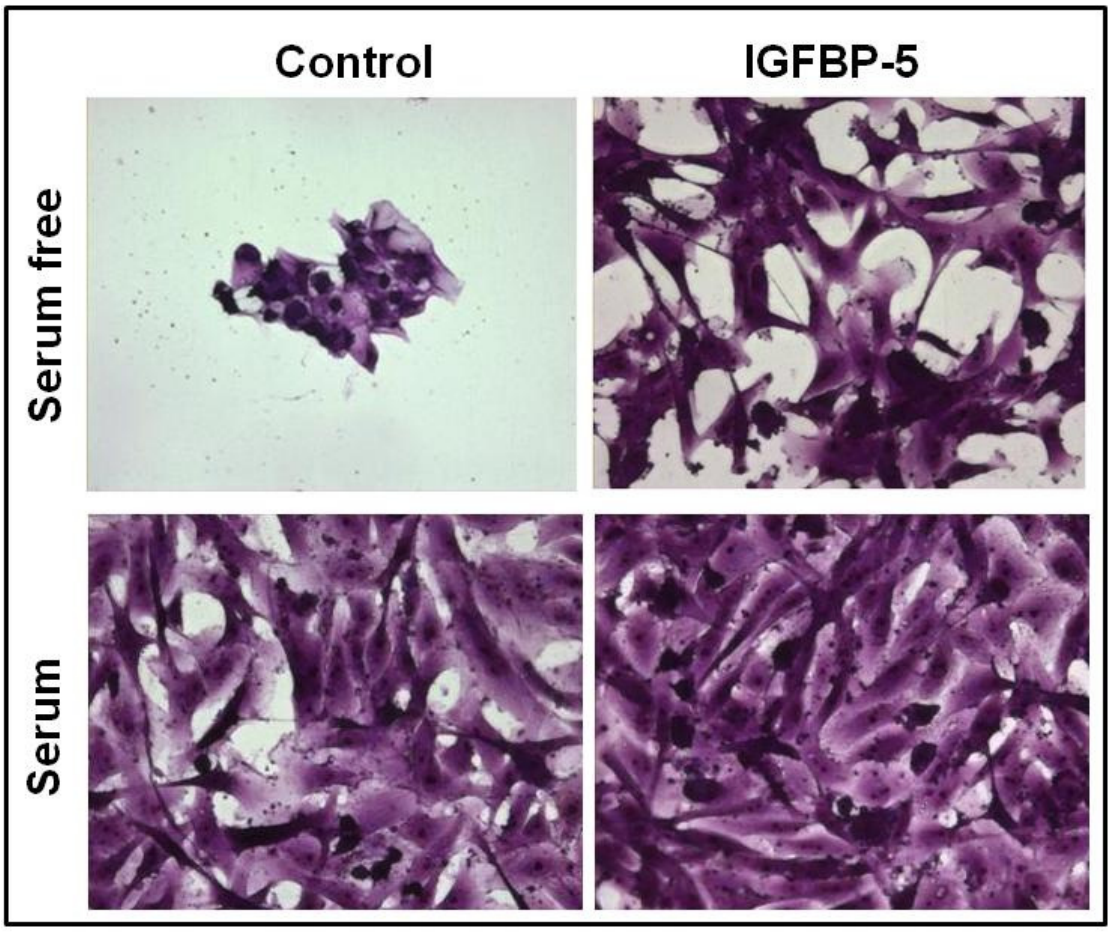


Fig 3.2.5: ***Crystal violet stained images of IGFBP-5-induced cell adhesion of MDA-MB-231 cells. IGFBP-5 treated MDA-MB-231 cells showed increased cell: substratum adhesion in serum-free environments. In the presence of serum, IGFBP-5 treated MDA-MB-231 cells showed negligible increase in cell: substratum adhesion.***

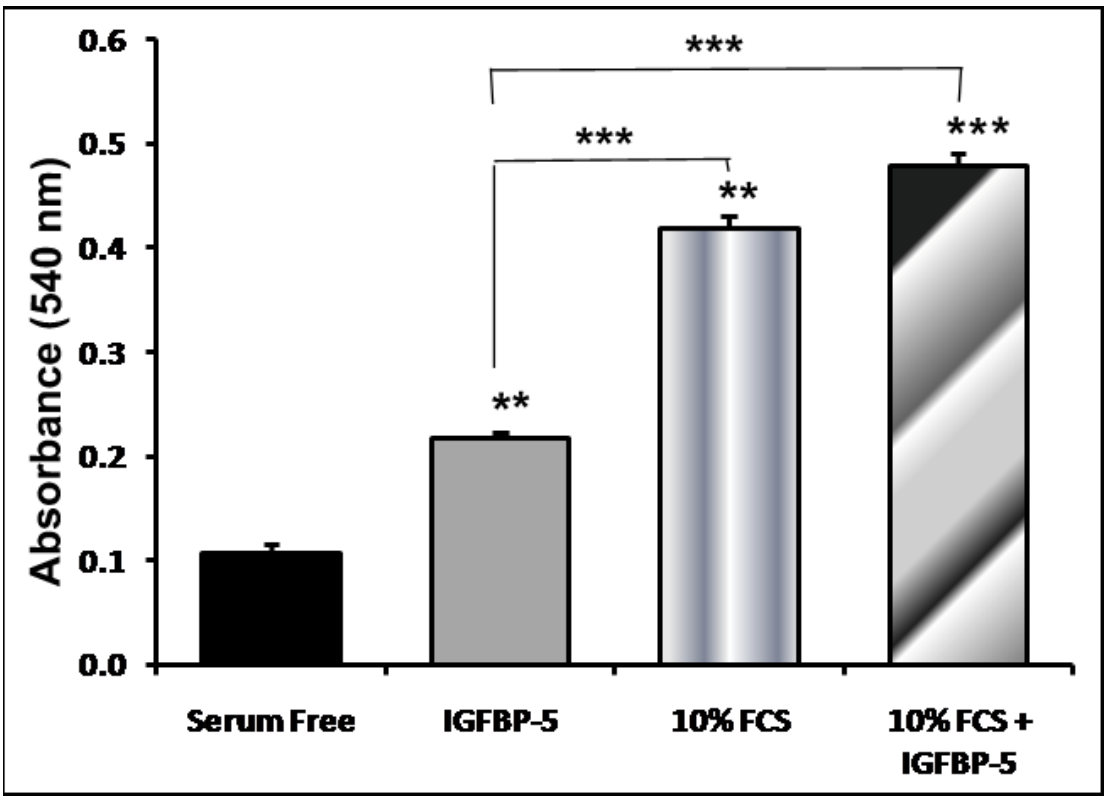


Fig. 3.2.6: ***Quantified data showing IGFBP-5-induced MDA-MB-231 cell adhesion in serum free conditions and serum concentrations and in combination. Values are mean ± SEM of 6 observations where ** indicates p<0.01; *** indicates p < 0.001 as compared with control and determined by Student's unpaired t-test. n=2***

Activation of Fibroblasts

The effect of IGFBP-5 on fibroblast activation was assessed through the use of adenoviral transduction and also the difference in the expression of fibronectin using RT PCR. Briefly, 5 x 10^5 cells/ml HDFs were transduced with empty virus and IGFBP-5 virus at 200 m.o.i on a 24 well plate in serum-free conditions. The transduction was carried out in serum free conditions for 3h at 37 ^oC and the cells were topped up with serum (2%). After 24 hours of transduction, the cells were trypsinised and seeded at 25 x 10^4 cells/well on to a 96 well plate and cultured for 24 h in serum-free and serum concentrations. After 24 h of incubation, the cells were fixed and stained and observed under microscopy at 5x, 20x, and 40x objective to examine the effect of IGFBP-5 on the morphological appearance of IGFBP-5-transduced HDFs.

IGFBP-5-tranduced HDFs were observed to be more elongated, spindle shaped and aligned in a streamy like fashion compared with the more flattened appearance and rounded empty virus-transduced HDFs. This appearance suggests that IGFBP-5 is allowing fibroblasts more spindle shaped and streamy-like morphology. However, the effect was found to be not evident at high serum concentrations (Fig. 3.2.6).

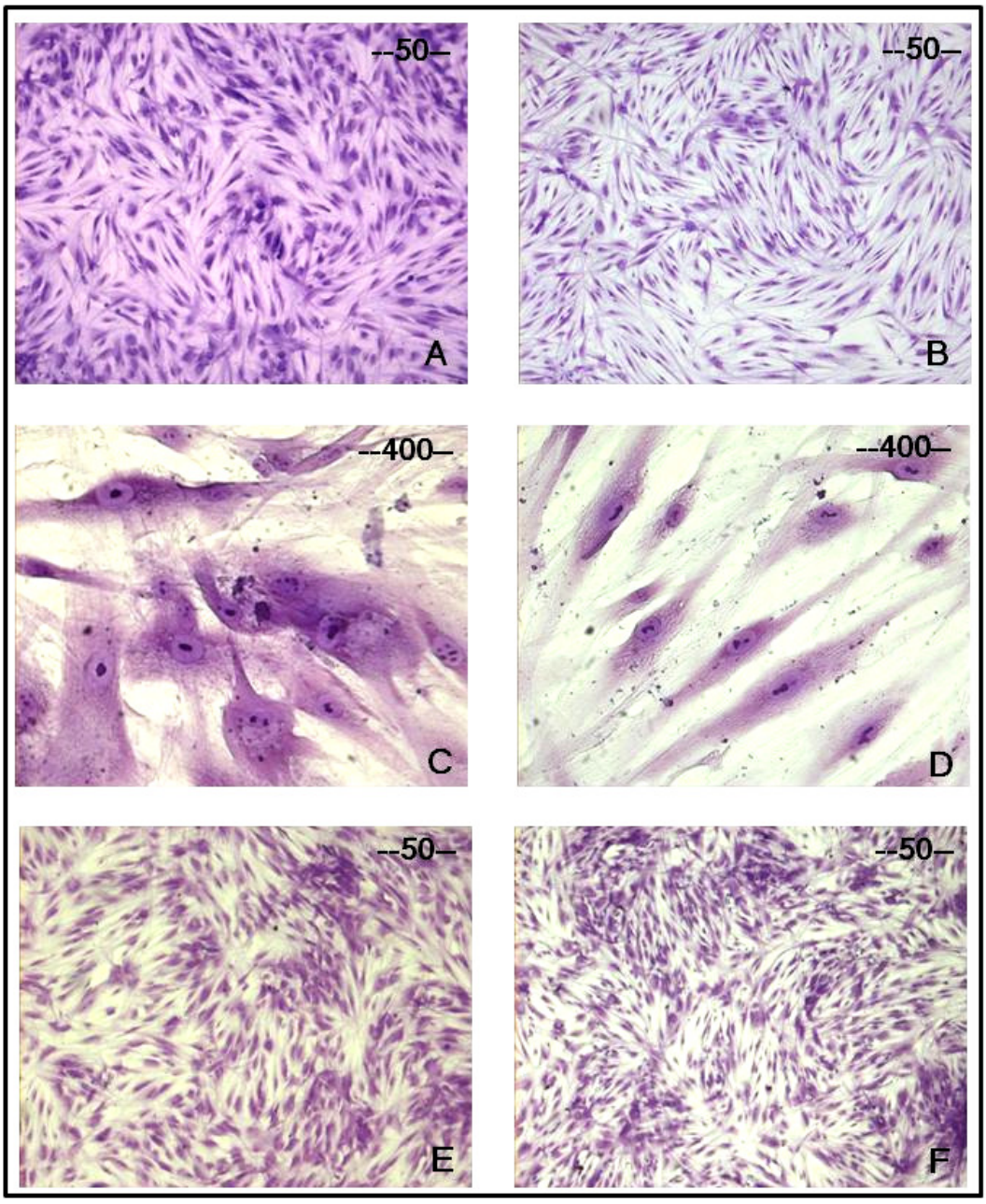


Fig 3.2.7: ***Crystal violet images showing Adenoviral transduction of human dermal fibroblasts. In serum-free conditions, at lower magnification (A) Empty virus-transduced HDFs with bulgy nucleus (B) IGFBP-5-transduced HDFs with elongated shaped nucleus and cytoplasmic tails and showing more streaming effect. At higher magnification (C) & (D) Empty virus-transduced HDFs and IGFBP-5-transduced HDFs. In high serum conditions, at lower magnification (E) & (F) both empty virus-transduced and IGFBP-5-transduced HDFs failed to show difference in morphological appearance.***

Effect of IGFBP-5 on Senescence associated Human Dermal Fibroblasts.

Senescence Associated β- Galoctosidase Assay

To examine the effect of IGFBP-5 on cellular senescence, senescence-associated beta galactosidase activity assay was undertaken. HDFs were regularly subcultured at 5 day time intervals until passage number 25 - 30 was achieved and hereafter referred as old HDFs. Old HDFs were confirmed by B-galactosidase activity at pH 6 and also by morphological appearance (Fig. 3.2.8). Whereas, HDFs with passage number 3 - 5 were hereafter referred as young HDFs. Briefly, 1,000,000 cells/flask of young and old HDFs were transduced with IGFBP-5 virus and empty virus at 200 m.o.i. Following adenovirus transduction, HDFs were lysed with RW buffer and total RNA was isolated. Total RNA was assessed for RNA integrity using the Bio-Rad Experion System. The RNA concentration was determined by RNA Quality Indicator (RQI) number and found to be 10 (highest) in all the four samples. Once RNA integrity was confirmed, gene expression of IGFBP-5 in adenovirally-transduced HDFs was assessed by employing comparative threshold cycle method. Results indicated that there is 0.4 fold decrease in overexpression of IGFBP-5 in old HDFs (Table 3.2.1). This data suggests that old HDFs express less IGFBP-5 as compared to young HDFs.

Table 3.2.1: ***shows relative fold difference in expression of IGFBP-5 transduced young and old HDFs using comparative C_T method.***

Sample (m.o.i)	Old HDFs	Young HDFs	Relative Fold Difference
IGFBP-5 (200)	0.0095850	0.0217929	0.4
EV (200)	0.0164018	0.0720429	0.2

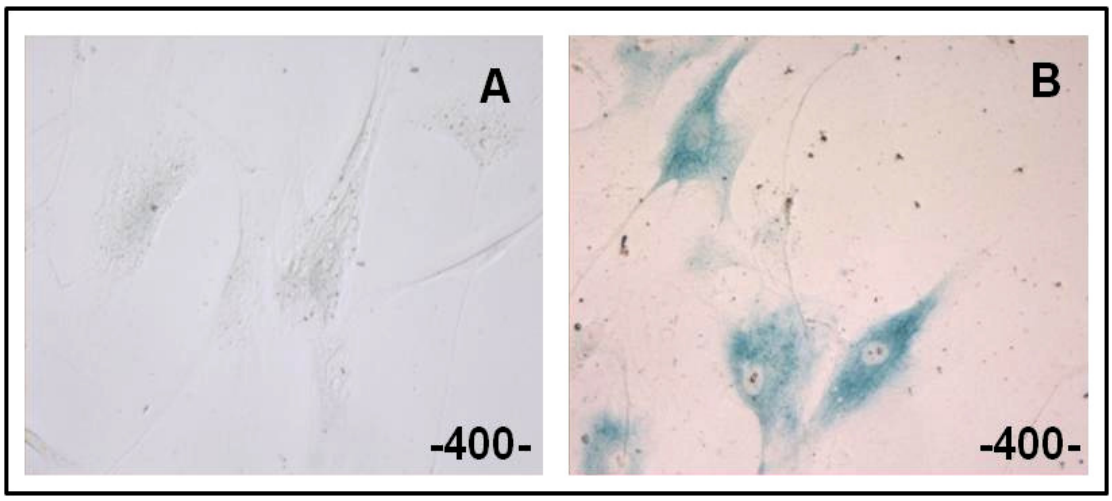


Fig 3.2.8: ***Images showing β-galactosidase activity (pH 6) of young and old HDFs. (A) Young HDFs not showing any β-galactosidase activity at pH 6. (B) Old HDFs stained with X-gal and showing β-galactosidase activity at pH 6.***

Effects of IGFBP-5 on fibronectin synthesis and secretion

IGFBP-5 Immunoblot analysis of IGFBP-5-transduced MCF-7 cells:

In order to examine the effect of IGFBP-5 on the production of fibronectin, MCF-7 cells were transduced with adenoviral constructs of IGFBP-5 at two m.o.i (200 and 1000) and cells were lysed 4 d post-transduction. Total protein concentration was estimated from cell lysates and 10 µg of total protein together with 100 ng of IGFBP-5 standard and 15 µl of medium samples were loaded under reducing conditions for polyacrylamide gel electrophoresis. After immunoblotting, both soluble medium and cell-associated preparations of IGFBP-5-transduced MCF-7 demonstrated a dose-dependent increase in the amount of IGFBP-5 (fig 3.2.9).

The same immunoblot was stripped and probed with an antibody to fibronectin antibody to study the effect of IGFBP-5 on the production of fibronectin (deposited secreted in to the medium and cell-associated) has been depicted (Fig. 3.2.10). Densitometric values, indicate that IGFBP-5 decreased the amount of fibronectin within the cell compartment and but increased it in the soluble medium compartment (Fig .3.2.11).

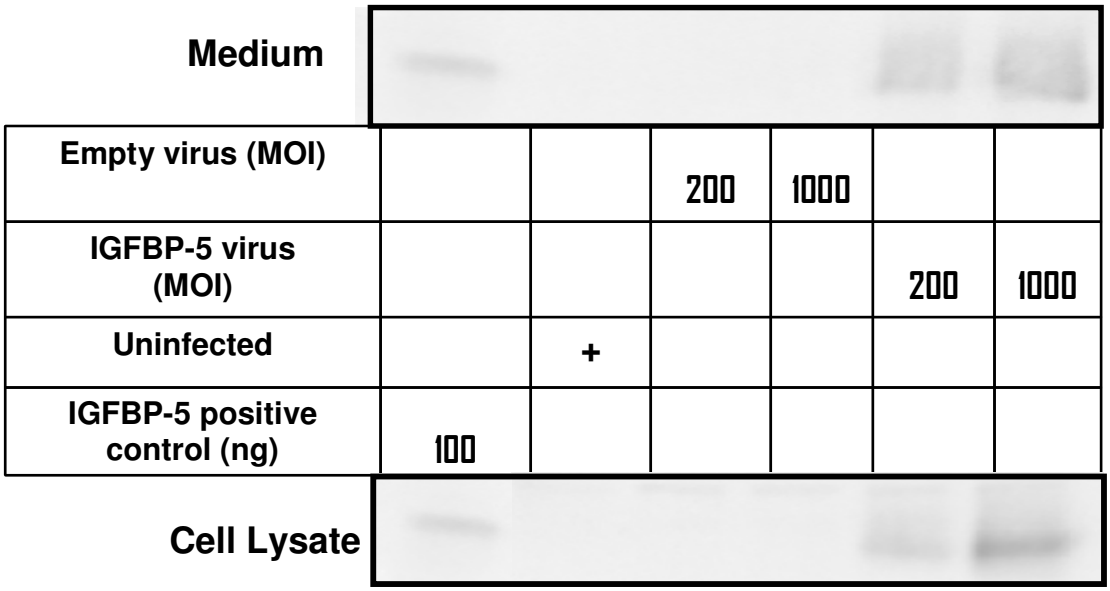
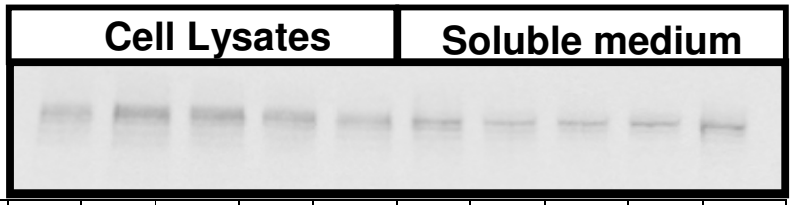


Fig 3.2.9: ***Immunoblot analysis of medium and cell homogenates from IGFBP-5-transduced MCF-7 cells along with recombinant IGFBP-5 positive control. Cell homogenates were obtained after cells were harvested in lysis buffer and were loaded at 10 ug/ml. Conditioned medium samples were loaded at volume 15 ul and immunoblotted with α-IGFBP-5. n= 1***



Uninfected	+									
Empty Virus (MOI)		200	1000			+	200	1000		
IGFBP-5 Virus (MOI)				200	1000				200	1000

Fig 3.2.10: ***Fibronectin Immunoblot of IGFBP-5-transduced MCF-7 cells of soluble medium and cell-associated preparation. Cell homogenates were obtained after cells were harvested in lysis buffer and were loaded at 10 ug/ml. Conditioned medium samples were loaded at 15 ul and immunoblotted with α-fibronectin. n=1***

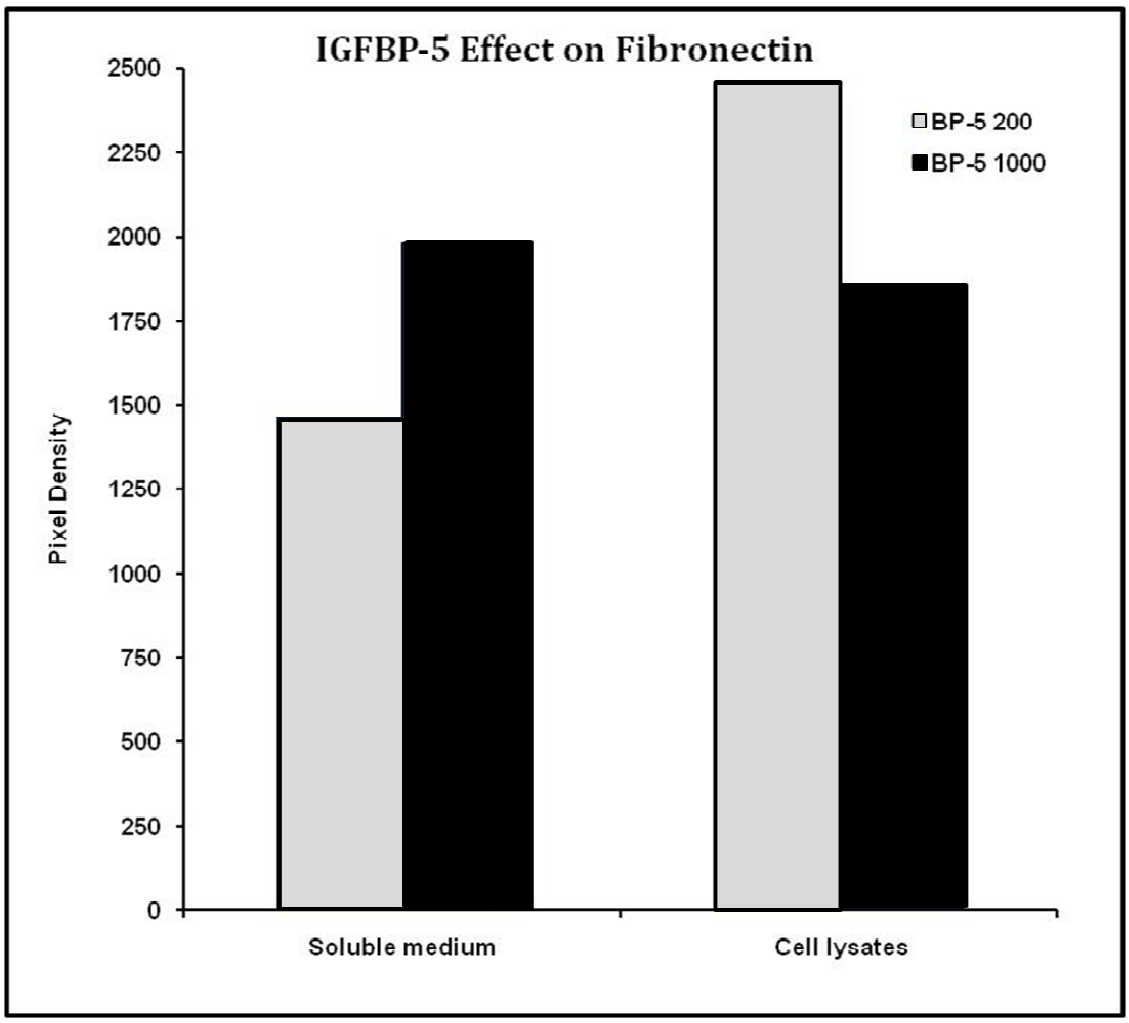


Fig. 3.2.11: ***Densitometric values of fibronectin immunoblot of IGFBP-5-transduced MCF-7 cells. n=1***

Gene Expression Studies of IGFBP-5-Adenoviral-mediated Transduction

In order to examine the effect of IGFBP-5 on fibronection at the levels of gene expression, Real-Time PCR approach was employed. The gene expression analysis of IGFBP-5-transduced cells was studied by employing Quantitative Real-Time PCR. Quantitative gene expression analysis of IGFBP-5-transduced MCF-7 cells was studied by employing comparative threshold cycle value (CT) method which is a widely used method to present relative gene expression. For this purpose, total RNA was isolated from uninfected, empty virus-transduced and IGFBP-5 virus-transduced MCF-7 & HDFs. A ratio of A260/A280 to all the transduced samples was measured for RNA purity and found to be more than 1.8. Total RNA was taken as starting template for complementary DNA synthesis. Amplification of cDNA was carried out following the manufacturer's instructions and quantification of individual genes (mouse IGFBP-5 and human IGFBP-5) was normalised using an internal control gene, human cyclophilin. Endogenous human IGFBP-5 expression levels were also measured in IGFBP-5-transduced MCF-7 cells and compared with mouse IGFBP-5 expression. The values indicate zero fold difference in case of control sample and approximately 23 and 35 fold increase in case of IGFBP-5-transduced MCF-7 cells at 200 & 1000 m.o.i (Figures 3.2.12 & 3.2.13). The IGFBP-5-transduced HDFs were run against fibronectin gene along with empty virus-transduced HDFs and normalised to cyclophilin for a relative gene expression. Results demonstrated that there is 4 fold increase of fibronectin gene in IGFBP-5-transduced HDFs at 200 m.o.i (Tables 3.2.2 & 3.2.3).

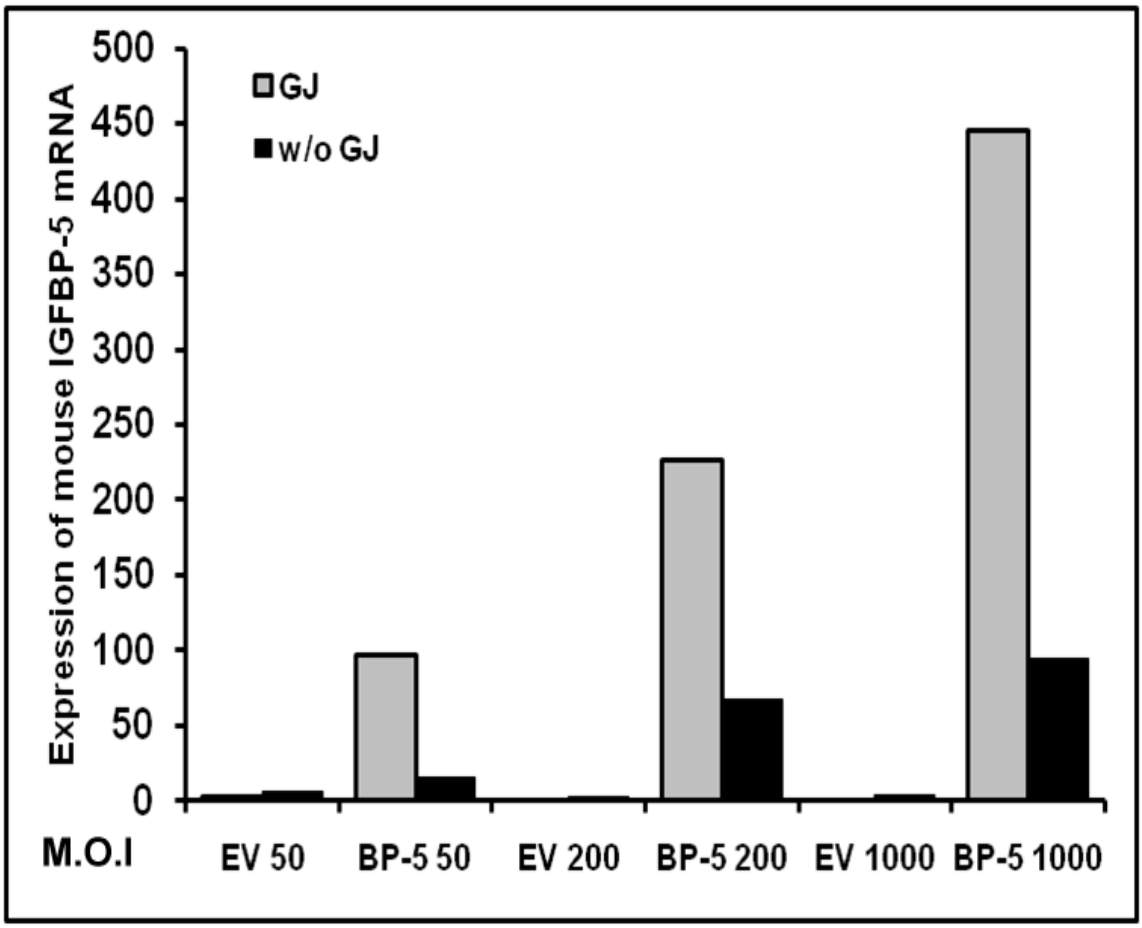


Fig 3.2.12: ***Graph showing the measurement of mouse IGFBP-5 gene expression normalised to cyclophilin in mouse IGFBP-5-transduced with and without Gene Jammer MCF-7 cells. (GJ: Gene Jammer; w/o GJ: Without Gene Jammmer). n=1***

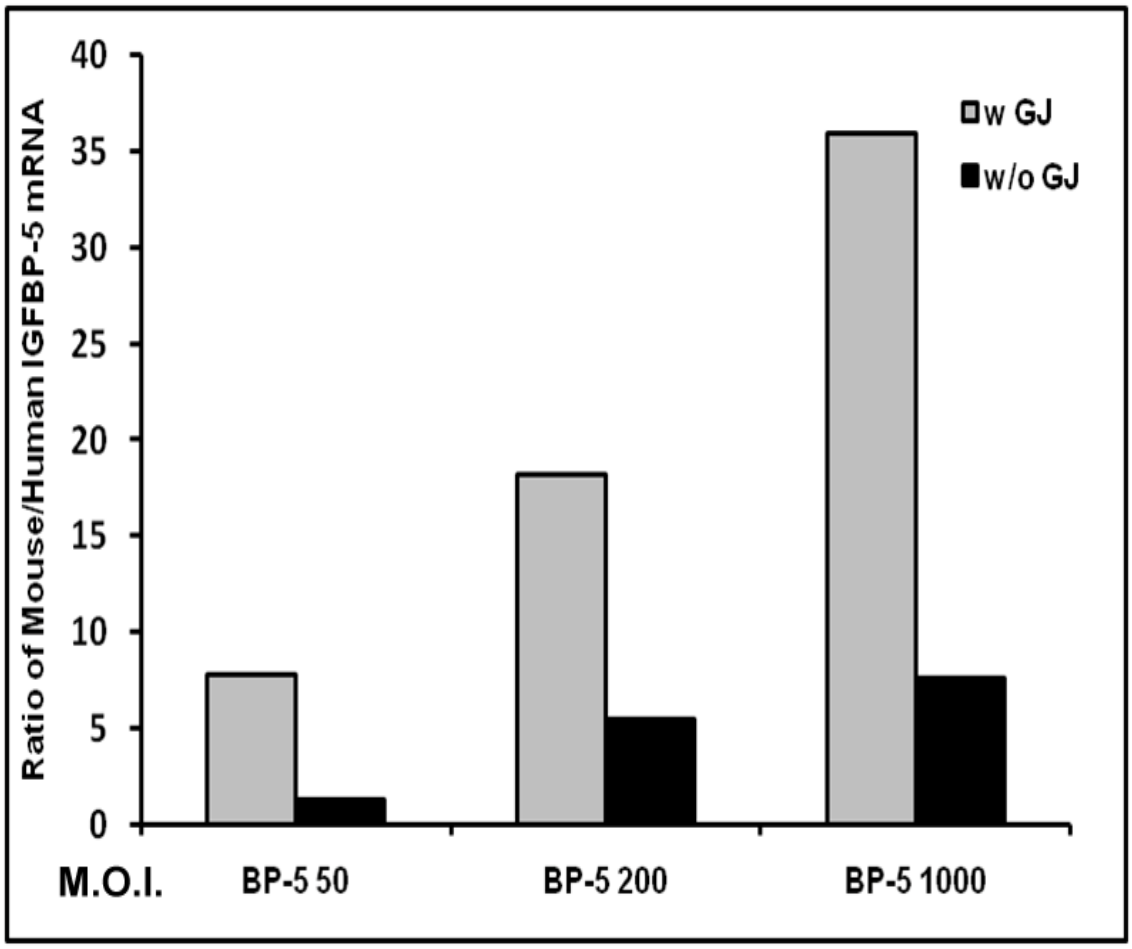


Fig 3.2.13: ***Graph showing the ratio of mouse to human IGFBP-5 gene expression normalised to cyclophilin in mouse IGFBP-5-transduced MCF-7 cells at 50, 200 & 1000 multiplicity of infections. (w GJ: With Gene Jammer; w/o GJ: Without Gene Jammmer) n=1***

Table 3.2.2: shows ***the relative fold difference in expression of fibronectin gene obtained by comparative C_T method in IGFBP-5-transduced MCF-7 cells.***

Relative Gene Expression of Fibronectin			
MOI	**Empty Virus**	**IGFBP-5 Virus**	**Fold Change**
200	**1.82**	**1.4**	**0.8**
1000	**1.82**	**1.15**	**0.6**

Table 3.2.3: shows ***the relative fold difference in expression of fibronectin gene obtained by comparative C_T method in IGFBP-5-transduced HDFs.***

Relative Gene Expression of Fibronectin			
MOI	**Empty Virus**	**IGFBP-5 Virus**	**Fold Change**
50	**564.1**	**910.2**	**1.6**
200	**136.2**	**522.2**	**4.0**

IGFBP-5 Cell Survival Assay Studies

Exogenous IGFBP-5 MCF-7 Cell Survival Assay

The effect of IGFBP-5 on cell survival characteristics of MCF-7 cells was studied at a variety of nutrient and nutrient-deprived environments with and without the presence of recombinant IGFBP-5 or Ad-IGFBP-5 at high and low seeding densities on a daily basis.

In serum-free conditions, MCF-7 cells were seeded at 500,000 cells/ml in a 96 well plate and treated with 10 µg/ml recombinant IGFBP-5 exogenously on a daily basis. Control cells were treated with serum-free medium to maintain the same volume of medium. Depending upon the response of viability and cell to substratum adhesion both treated and untreated cells were fixed and stained at time intervals. The results showed that IGFBP-5 treated MCF-7 cells were adhesive to substratum until day 9 whereas, the control MCF-7 cells detached from day 5 onwards (Figures. 3.2.14 & 3.2.15).

In serum, MCF-7 cells were seeded at 50,000 cells/ml in a 96 well plate and treated with 10 µg/ml recombinant IGFBP-5 exogenously on a daily basis without any medium change. The results showed the cells achieved confluency on day 5 irrespective of the IGFBP-5 treatment. However, the IGFBP-5 treated MCF-7 cells were metabolically active even after 18 days of culture without any change of medium whereas the control cells showed de-adhesion and also metabolic activity was decreased after 8 days of culture without change of medium (Figures 3.2.16 & 3.2.17).

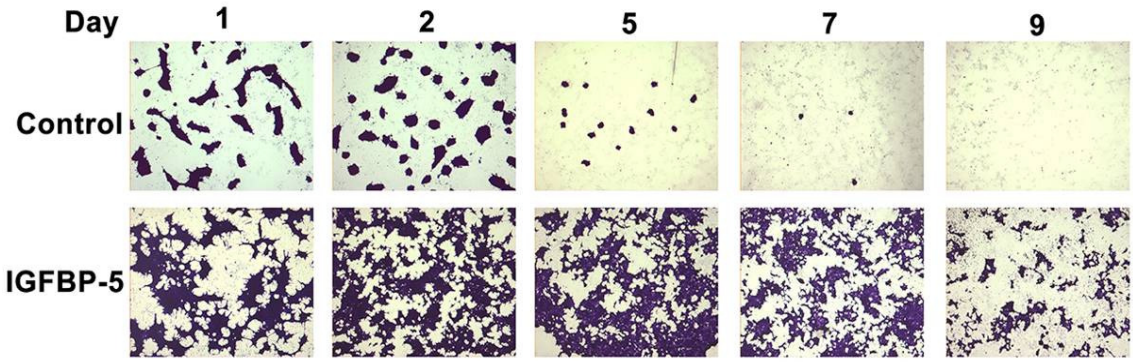


Fig 3.2.14: ***Crystal violet images of exogenous IGFBP-5 survival assay at high seeding density in serum-free conditions. MCF-7 cells showing cell: substratum adhesion was still evident even after 9 days in nutrient-deprived environments. Images are photographed at 100x magnification.***

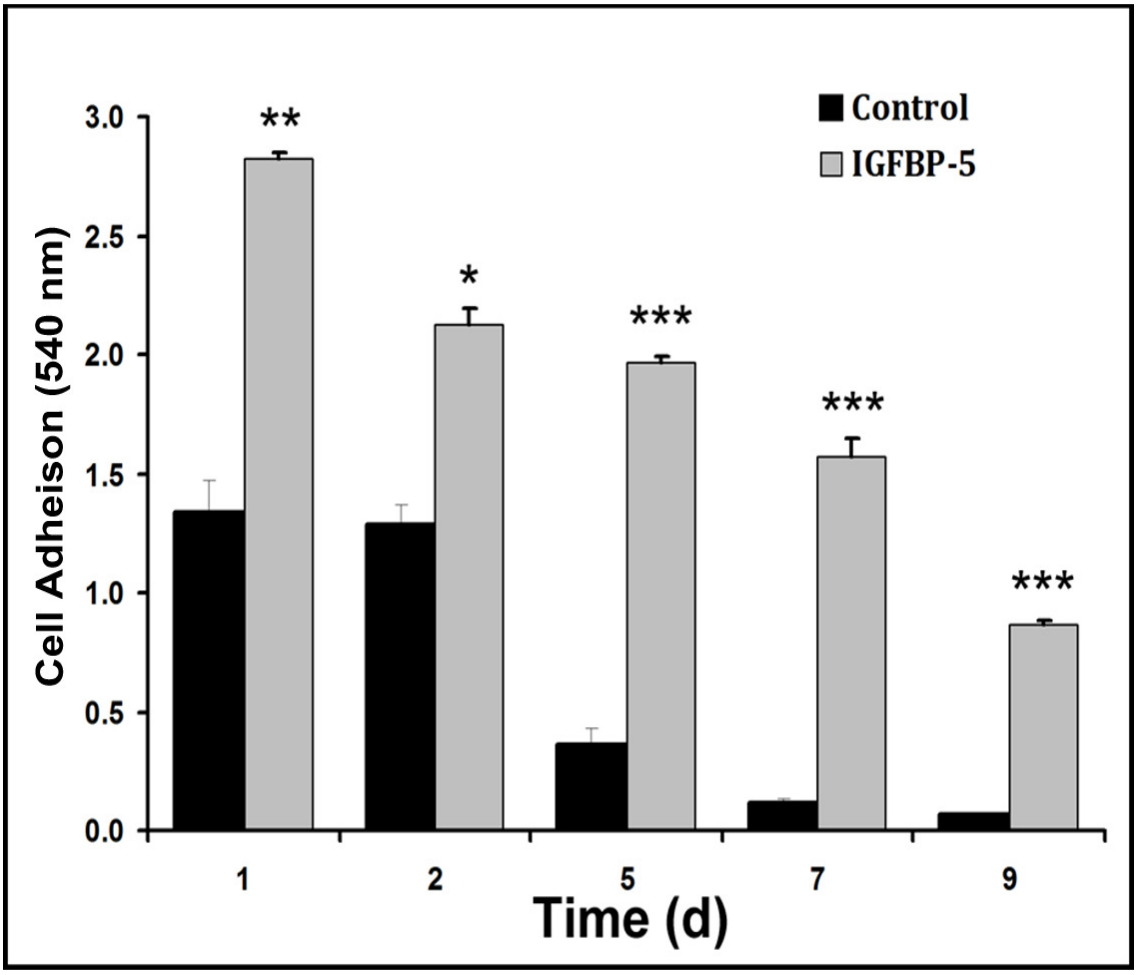


Fig 3.2.15: ***Quantified data showing exogenously added IGFBP-5 cell survival assay at serum-free conditions and high seeding density of MCF-7 cells. Values represent mean ± SEM of 6 observations.*** **Where * indicates p < 0.05; ** indicates p < 0.01;** ****** indicates p < 0.001 compared with control as determined by Students unpaired t-test. n=2***

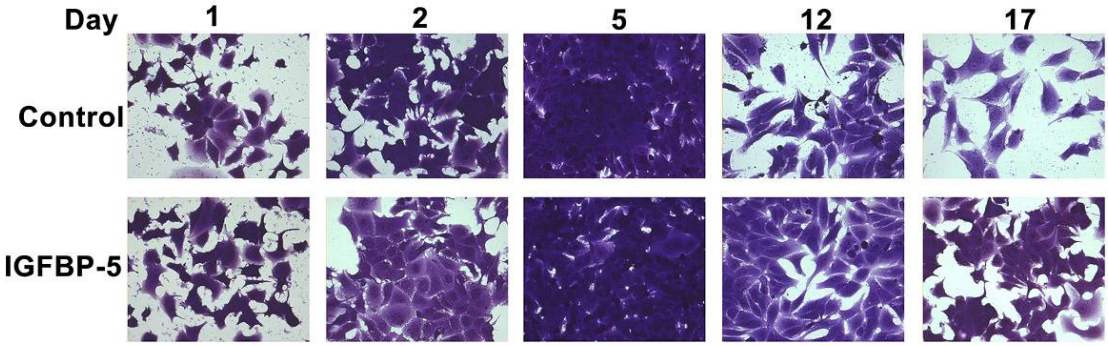


Fig 3.2.16: ***Crystal violet images of exogenous IGFBP-5 Cell Survival Assay at low seeding density in nutrient environments. MCF-7 cells appear to be metabolically active even after 18 days without any medium change in nutrient-rich environments. Images are photographed at 400x magnification.***

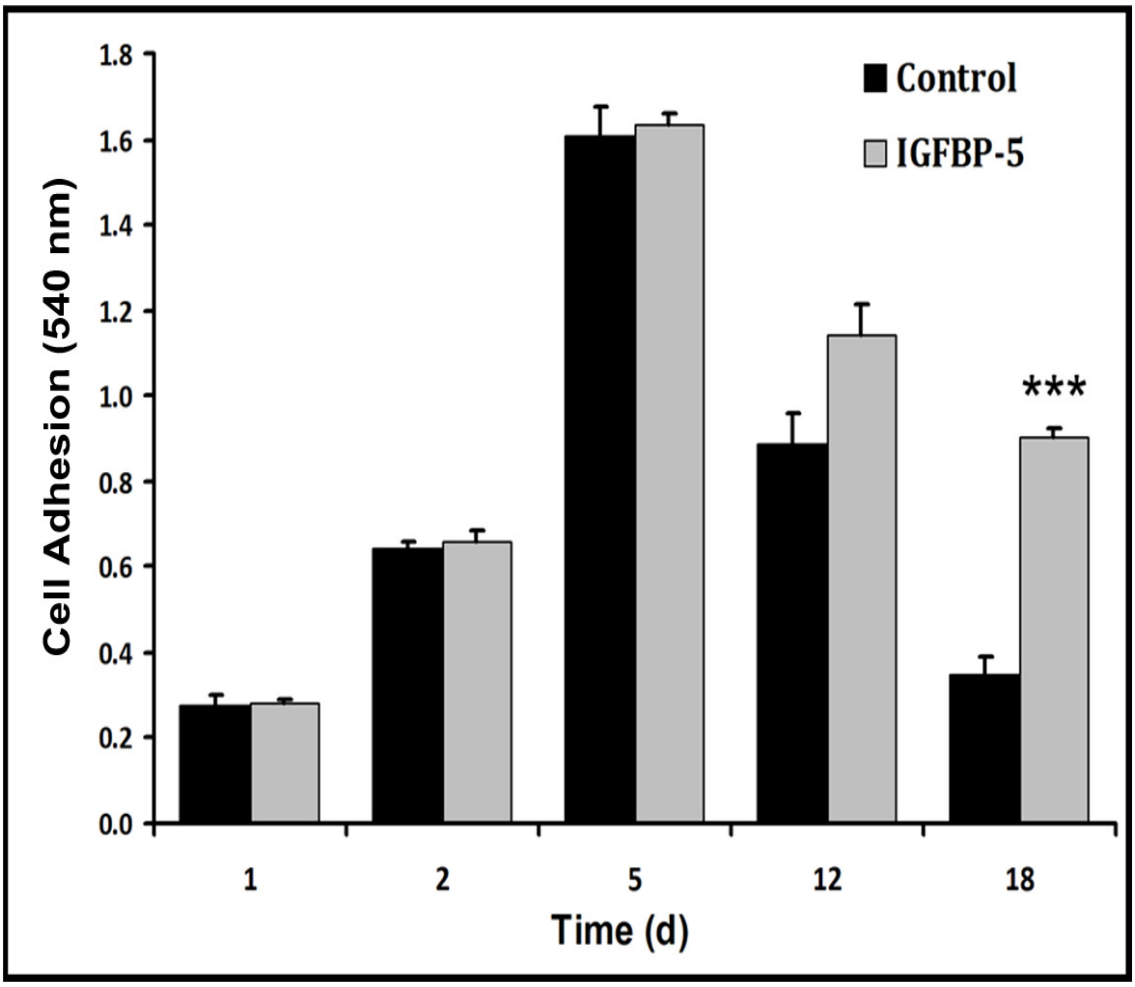


Fig 3.2.17: ***Quantified results of exogenously added IGFBP-5 cell survival assay at 10% serum and low seeding density of MCF-7 cells. Values are mean ± SEM of 6 observations where *** indicates p < 0.001 compared with control as determined by Students unpaired t-test. n=2***

IGFBP-5 Cell Survival Assay employing Adenovirus Transduction

In order to examine the effect of adenovirus expressing IGFBP-5 on MC7-cell survival, long term cell survival assays were carried out at high and low seeding densities at a range of nutrient-deprived to nutrient-rich environments experiments were carried out.

Briefly, 1,500,000 MCF-7 cells/ml were transduced with empty virus and IGFBP-5 virus at 200 m.o.i in serum-free conditions for 3 h at 37 ^{o}C in 5% CO_2 incubator. Then, both empty virus- and IGFBP-5 virus-transduced cells were treated with 10% serum. Next day, the cells were trypsinised and seeded at high seeding density, 500, 000 cells/ml at a variety of serum-free to serum concentrations. On the basis of cell survival response, cells were fixed and stained. Results showed that IGFBP-5-transduced MCF-7 cells enhanced cell survival in serum-free or 1% serum concentrations. In the presence of 10% serum concentrations, both empty virus- and IGFBP-5 virus-transduced MCF-7 cells started to detach after 8 days and possibly due to lack of nutrients demanding by the high seeding densities (Fig. 3.2.18).

Similarly, adenovirus-transduced MCF-7 cells were seeded into 96 well plate at low seeding density i.e., 5, 000 cells/ml at a variety of serum-free to serum concentrations. IGFBP-5-transduced MCF-7 cells had enhanced cell survival in 1% serum concentrations whereas, in the presence of 10% serum, IGFBP-5-transduced MCF-7 cells achieved confluency at day 5, maintained confluency until day 13 and exhibited the cell survival characteristics until day 18 (Fig. 3.2.19).

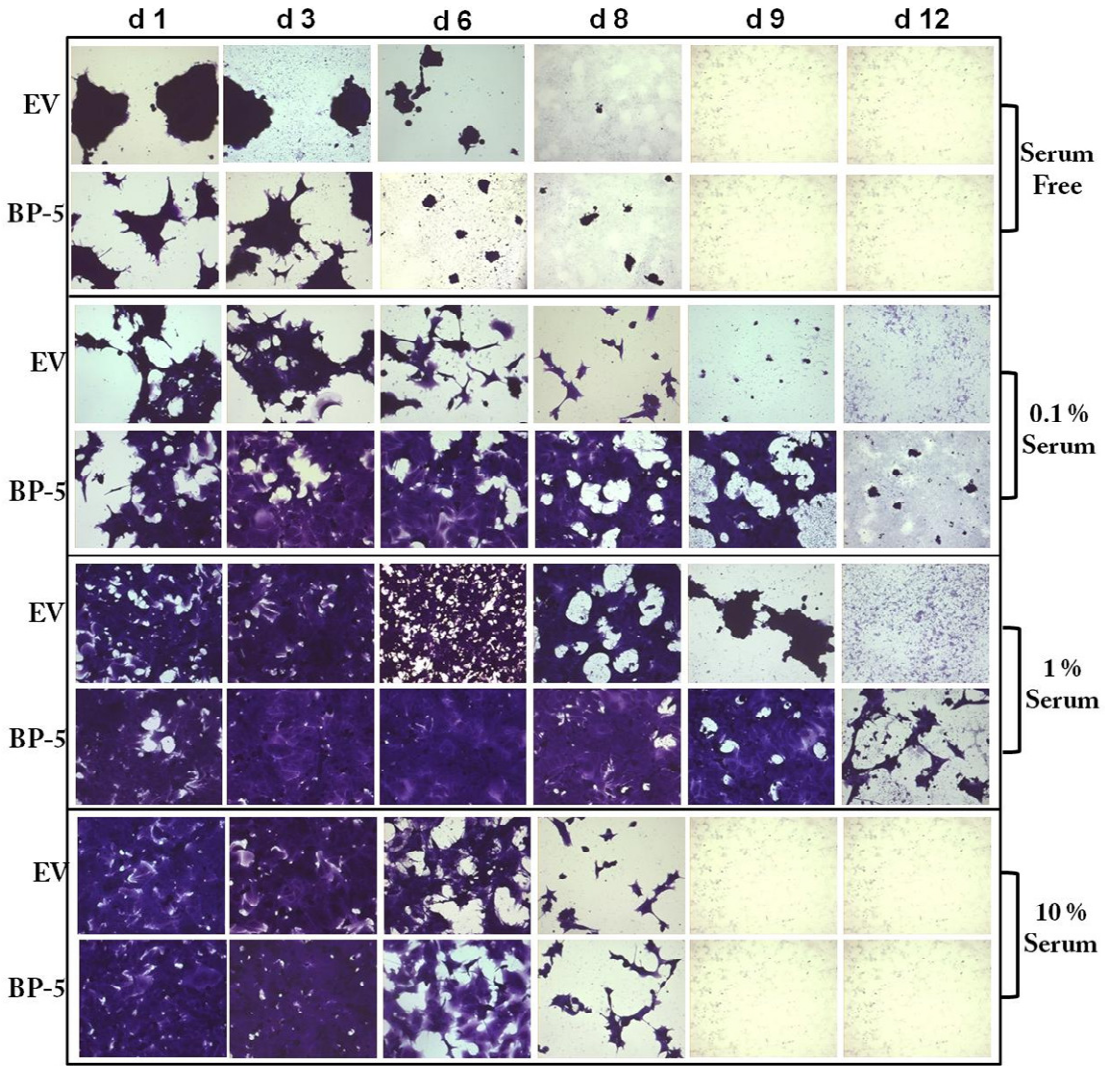


Fig 3.2.18: ***Crystal violet images of IGFBP-5 cell survival assay at high seeding density via adenovirus transduction. IGFBP-5-transduced MCF-7 cells showed increased cell survival at a variety of nutrient-deprived to nutrient-rich environments without any medium change. In case of 10% serum concentrations, both empty virus-transduced and IGFBP-5-transduced MCF-7 cells were detached by day 8 due to high seeding densities. EV: Empty virus-tranduced MCF-7 cells; BP-5: IGFBP-5-transduced MCF-7 cells.***

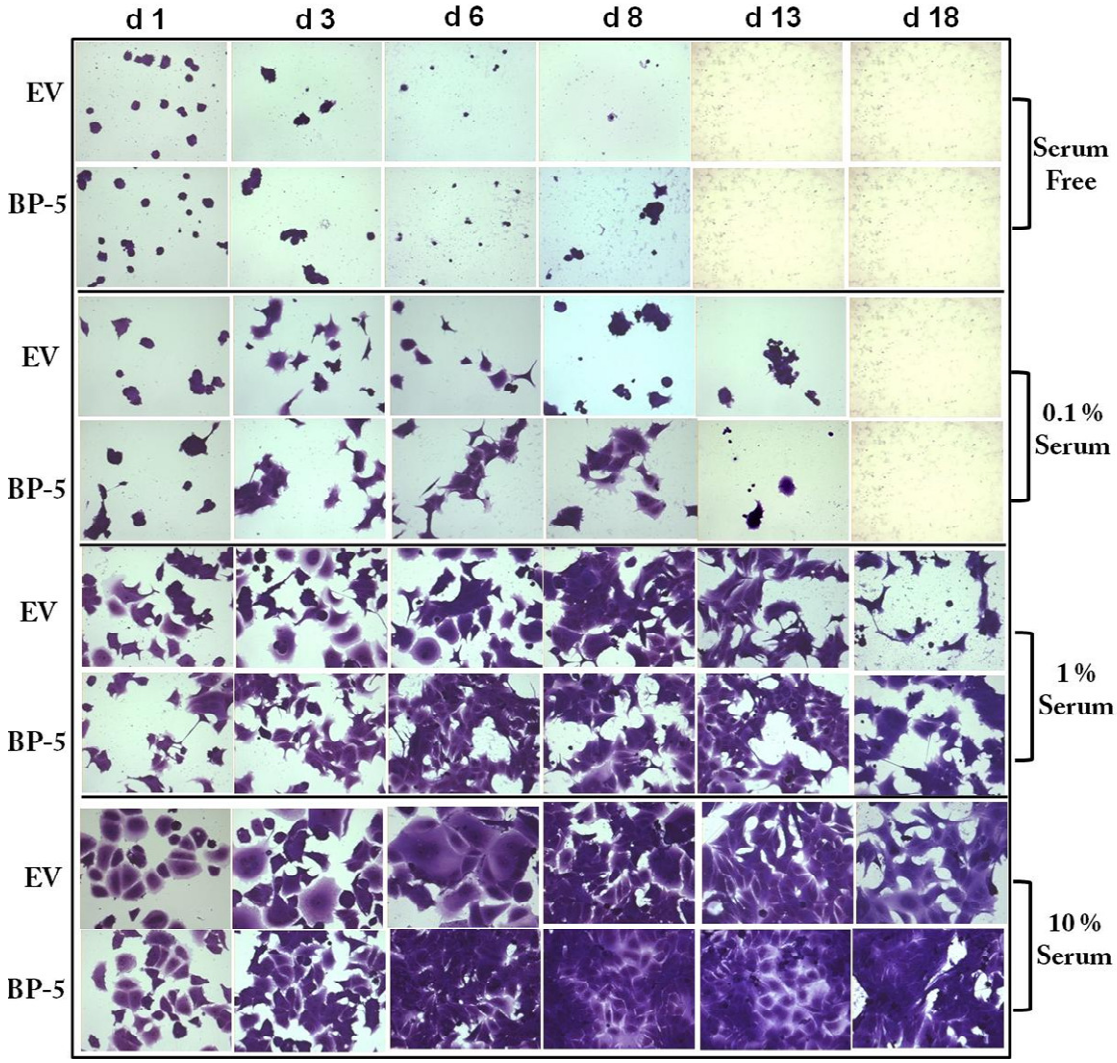


Fig 3.2.19: ***Crystal violet images of IGFBP-5 cell survival assay at low seeding density via adenovirus transduction. IGFBP-5-transduced MCF-7 cells showed increased cell survival at a variety of nutrient-deprived to nutrient-rich environments without any medium change. In the presence of 10% serum, IGFBP-5-transduced cells achieved confluency on day 6 and maintained until day 13. EV: Empty-virus-tranduced MCF-7 cells; BP-5: IGFBP-5-transduced MCF-7 cells.***

IGFBP-5 Live Dead Cell Staining Assay

To further prove the survival activity of IGFBP-5, live and dead cell staining assays were carried out using adenovirally-transduced MCF-7 cells. Briefly, 100ul of adenovirally transduced MCF-7 cells were seeded onto 96 well plates at 1% (low) and 10% (high) serum concentrations and cultured at 37 ^{o} C in 5% CO_2 atmosphere. Metabolic values were also measured to study the metabolic activity at regular time intervals in adenovirally-transduced MCF-7 cells. Results showed that, at low serum concentrations, live cell values of empty vector transduced MCF-7 cells were lower than IGFBP-5-transduced MCF-7 cells. Dead cell values of empty vector transduced MCF-7 cells steadily increased until day 12 time interval whereas dead cell values of IGFBP-5 transduced cells increased more slowly (Figures. 3.2.20 & 3.2.22).

At high serum concentrations, live cell values of IGFBP-5-transduced MCF-7 cells were maintained until day 12 whereas, empty vector transduced cells steadily decreased and reached nearly zero value by day 10. Whereas, dead cell values of empty vector transduced MCF-7 cells were considerably increased by day 8 the dead cell values of IGFBP-5-transduced MCF-7 cells increased much more slowly up to day 14 (Figures. 3.2.21 & 3.2.23).

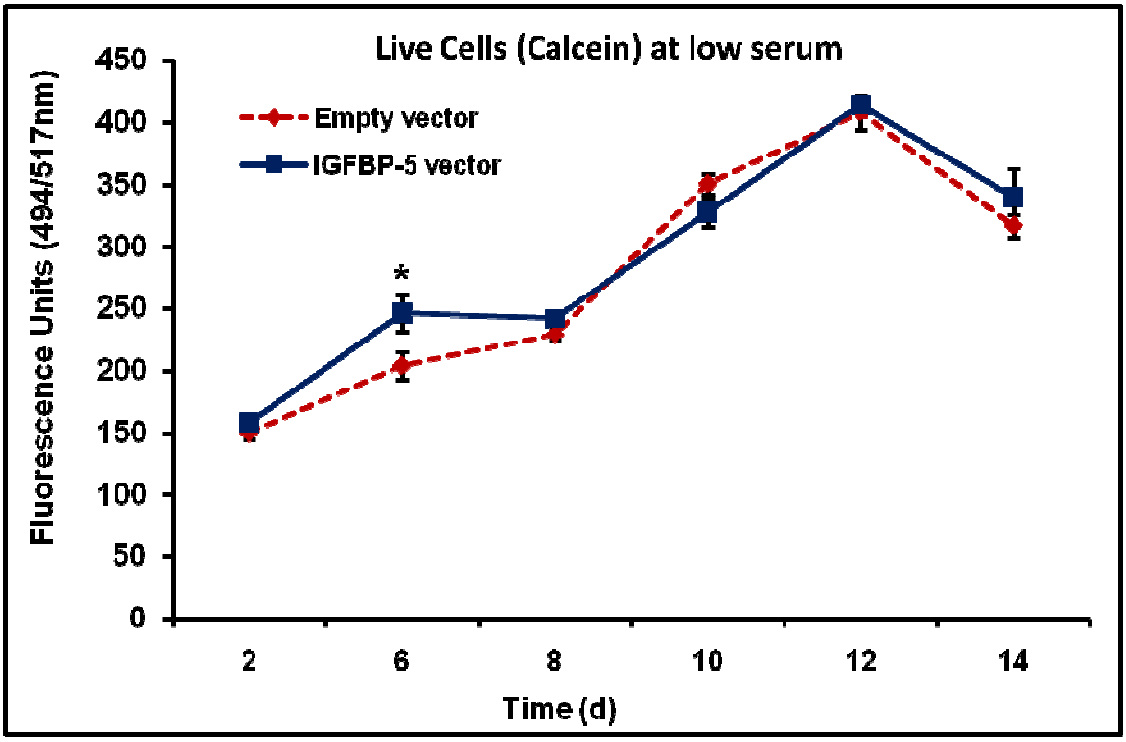


Fig 3.2.20: ***Quantified data showing live cell fluorescence of adenovirally-transduced MCF-7 cells at 1% (low) serum concentrations. Values represent mean ± SEM of 6 observations. Where * indicates as p<0.1 as compared with control and determined by Student's unpaired t-test. n=2***

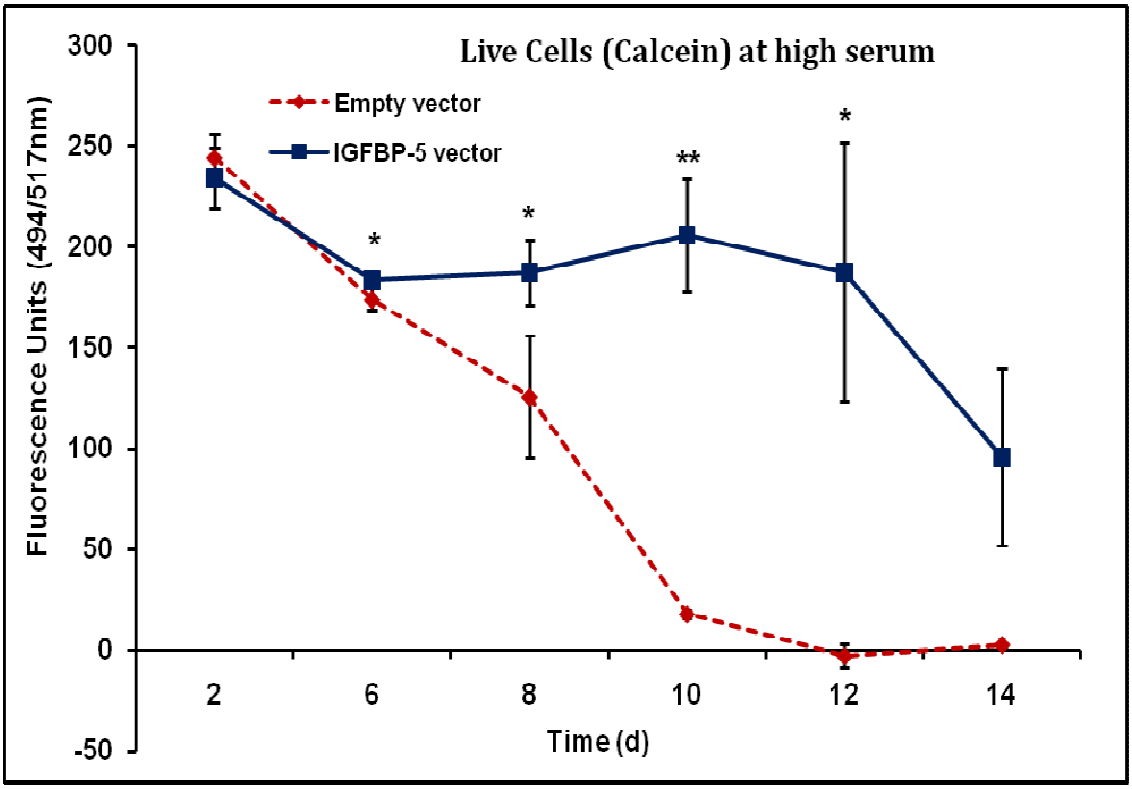


Fig. 3.2.21: ***Quantified data showing live cell fluorescence of adenovirally-transduced MCF-7 cells at 10% serum concentrations. IGFBP-5-transduced MCF-7 cells showed a steady increase until day 8 and a 20 fold increase at day 10 time interval. Values represent mean ± SEM of 6 observations where * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 compared with control as determined by Students unpaired t-test. n=2***

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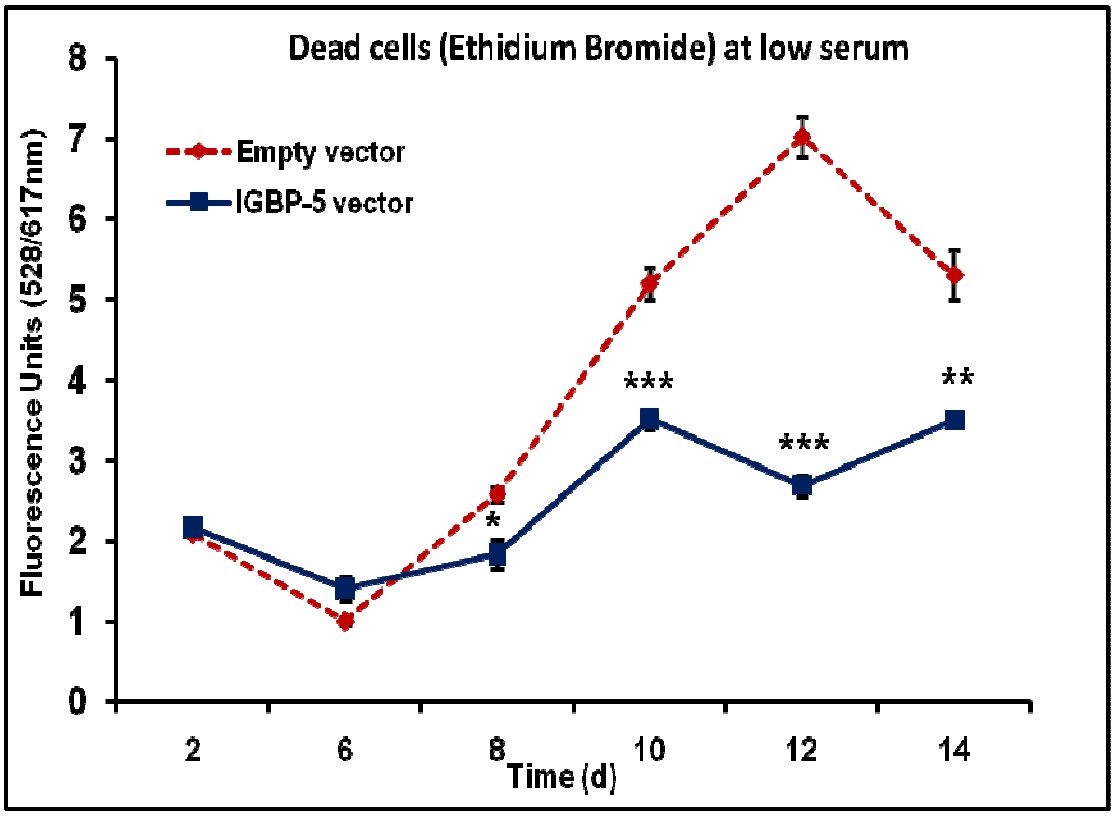

Fig. 3.2.22: ***Quantified data showing dead cell fluorescence of adenovirally-transduced MCF-7 cells at low (1%) serum concentrations. IGFBP-5-transduced MCF-7 cells showed two fold decrease in dead cell staining at day 12 time interval. Values represent mean + SEM of 6 observations where * indicates p<0.05, ** p<0.01 and *** p<0.001 as compared with control and determined by Student's unpaired t-test. n=2***

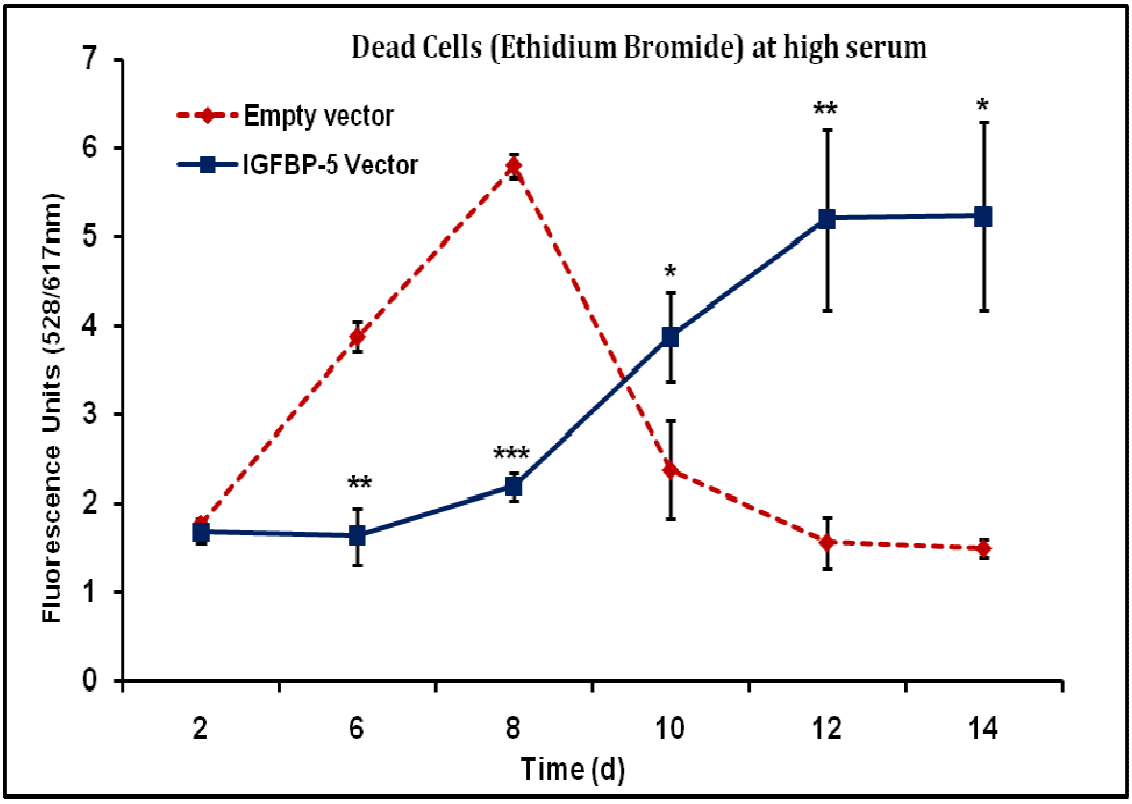

Fig 3.2.23: ***Quantified data showing dead cell fluorescence of adenovirally-transduced MCF-7 cells at high serum concentrations. Empty vector-transduced MCF-7 cells showed a dramatic increase in dead cell staining until day 8 and thereafter cells continued to detach until day 10 by which time most cells were lost. Values represent mean + SEM of 6 observations where * indicates p < 0.05; ** indicates p < 0.01; *** indicates p < 0.001 compared with control as determined by Students unpaired t-test. n=2***

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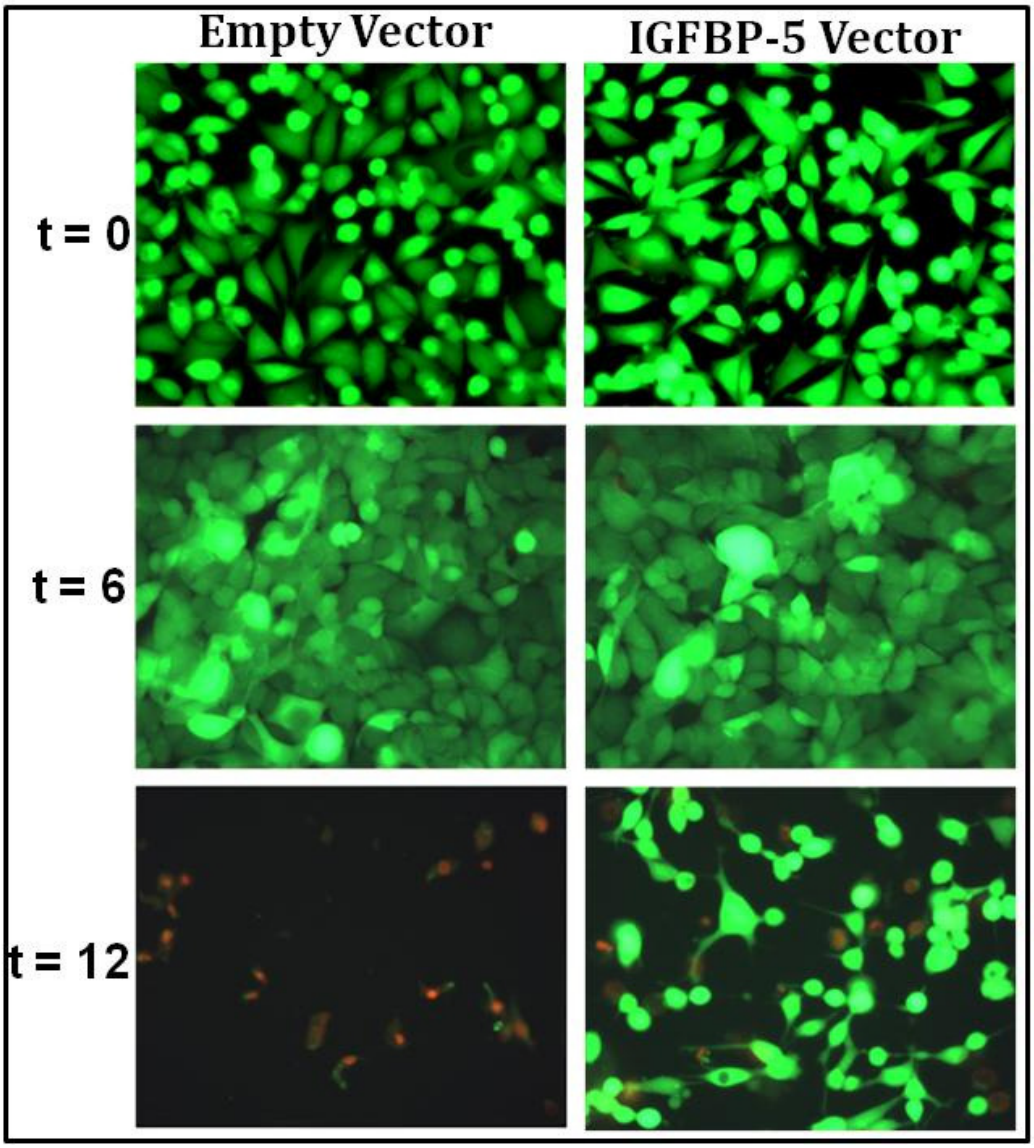


Fig 3.2.24: ***Fluorescence images showing IGFBP-5-transduced MCF-7 cells were viable (green) until d12 time interval in nutrient-deprived conditions. Empty vector-transduced MCF-7 cells reached confluence by d6 but live cells detached completely by d12 with few remaining dead cells (orange).***

IGFBP-5 Metabolic Activity Assay

In order to examine the metabolic rates in nutrient-deprived adenovirally transduced MCF-7 cells, a metabolic activity assay was carried out. Metabolic values were determined from adenovirally-transduced MCF-7 cells at low and high serum concentrations at regular time intervals. IGFBP-5-transduced MCF-7 cells showed higher metabolic activity on a regular basis at both low serum concentrations until day 14 time interval (Fig. 3.2.25). At high serum concentrations, the metabolic values of empty vector transduced MCF-7 cells significantly decreased until day 8 time interval and then remained low until day 14. In contrast, metabolic values of IGFBP-5-transduced MCF-7 cells were maintained high until day 10 time interval, then decreased but remained slightly above control cells from day 12 to day 14 time intervals (Fig. 3.2.26).

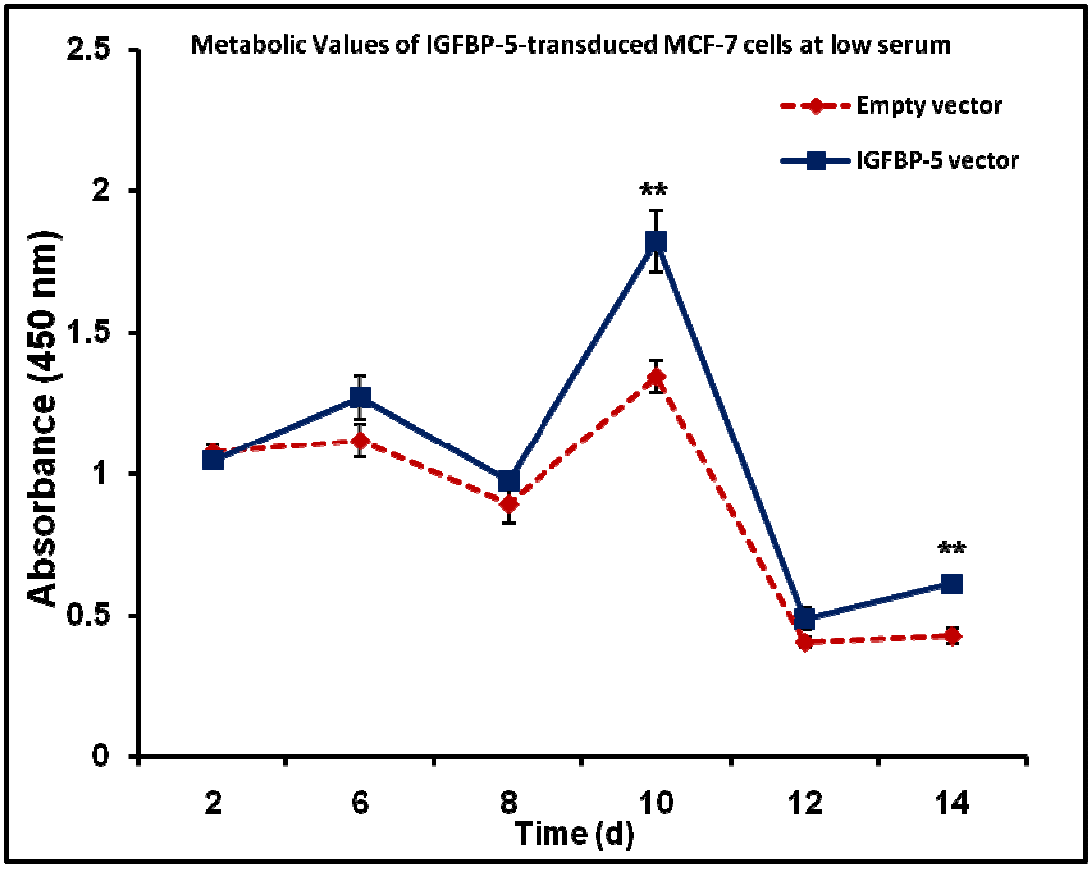


Fig 3.2.25: ***Graph showing metabolic values of adenovirally transduced MCF-7 cells at 1% serum on a time scale. IGFBP-5-transduced MCF-7 cells showed a slight increase in metabolic activity until day 14 time interval. Values represent mean ± SEM of 3-4 observations where ** indicates p<0.01 compared with control as determined by Student's unpaired t-test. n=2***

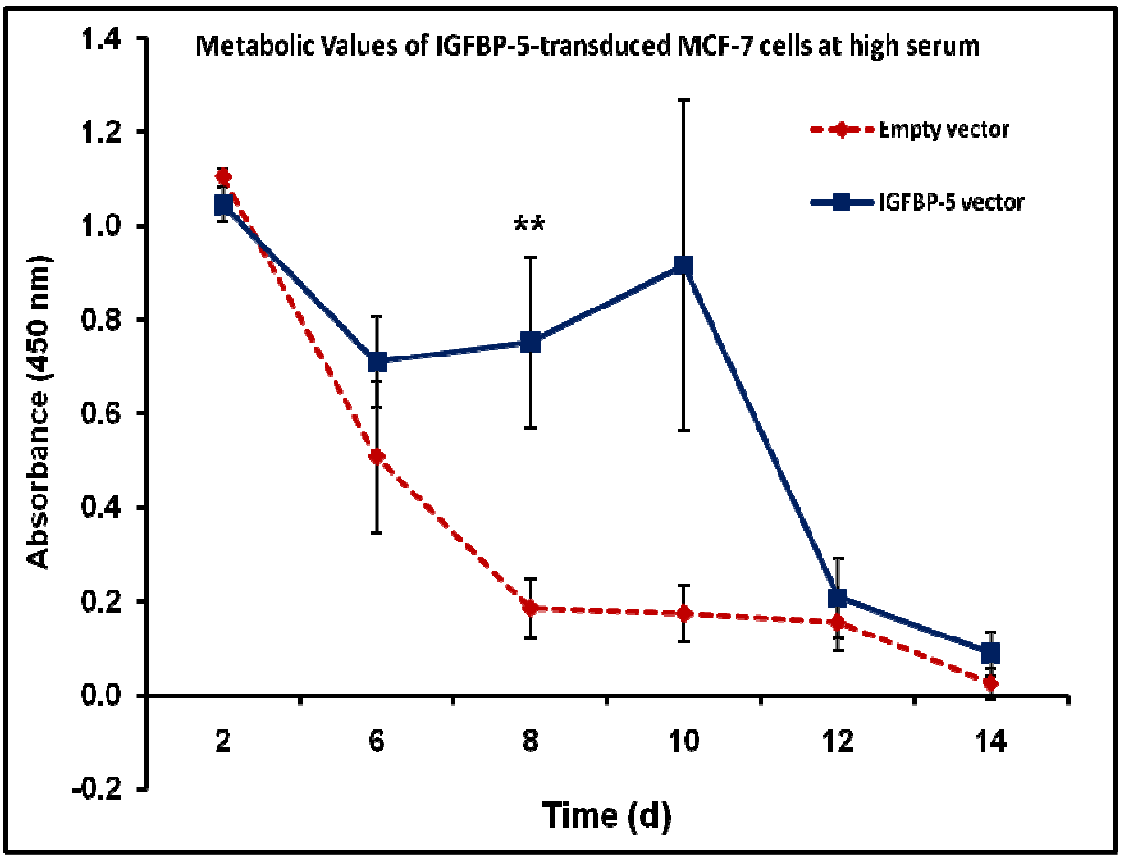


Fig 3.2.26: ***Graph showing metabolic values of adenovirally-transduced MCF-7 cells at 10% serum concentrations on a time scale. IGFBP-5-transduced MCF-7 cells showed increase in metabolic activity at all time intervals and a 5 fold increase in metabolic activity at day 10 time interval. Values represent mean ± SEM of 6 observations where ** indicates p<0.01 compared with control as determined by Student's unpaired t-test. n=3***

IGFBP-5 Cell Migration Assays

Cell migration is a key process in the natural development as well as cancer metastasis. To this end, I have attempted to examine the effect of IGFBP-5 on cell migration in mammary tumour cell lines. Cell migration assays were carried out using Ibidi cell culture inserts^{TM} to study the response of IGFBP-5 towards cell migration.

Briefly, 50 µl (50,000 MCF-7 cells) were seeded into culture inserts in 10% serum DMEM and incubated overnight at 37 ^{o}C in 5% CO_2 atmosphere. During incubation, treated cells were pre-incubated with 10 µg/ml of recombinant IGFBP-5. Next day, cell culture inserts were removed and IGFBP-5 at 10 µg/ml was added along with 100 ng/ml IGF-I, 100ng/ml EGF or 10% FCS medium. Control, IGF-I and EGF treated cells were maintained in 1% serum to keep cells alive. Results showed that IGFBP-5 inhibited MCF-7 cell migration in all treatments. After 24 h of treatment, cells were fixed and stained. Results were quantified after inverting the images using Image J software and plotted against mean gray value. IGFBP-5 treated MCF-7 cells showed a threefold decrease in cell migration in the presence of 10% serum. (Figures. 3.2.27 & 3.2.28).

Similarly, the effect of IGFBP-5 on cell migration in highly invasive mammary tumour cells, MDA-MB 231 cell lines was studied. Briefly, 50 µl of 50,000 cells/chamber were seeded into cell culture inserts in serum-free and 10% serum concentrations and incubated overnight at 37 ^{o}C in 5% CO_2 atmosphere. Treatments were carried out similar to MCF-7 cells. Results showed that IGFBP-5 also inhibited the migration of MDA-MB 231 cells (Figures. 3.2.29 & 3.2.30).

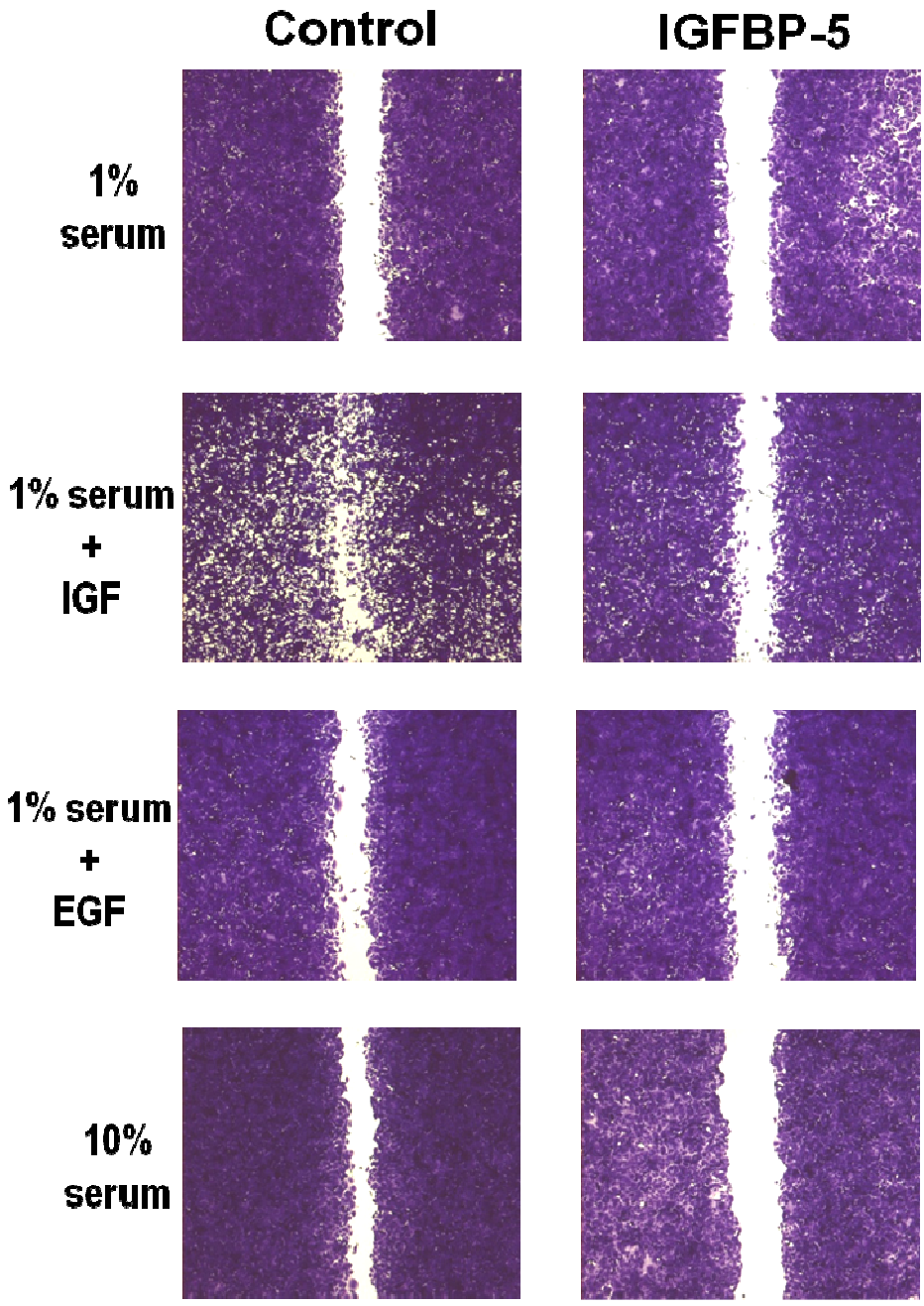


Fig. 3.2.27: ***Crystal violet staining images of IGFBP-5 cell migration assay of MCF-7 cells. IGFBP-5 treated MCF-7 cells showing inhibition of migration in response to IGF, EGF and serum. Images were photographed at 40x magnification.***

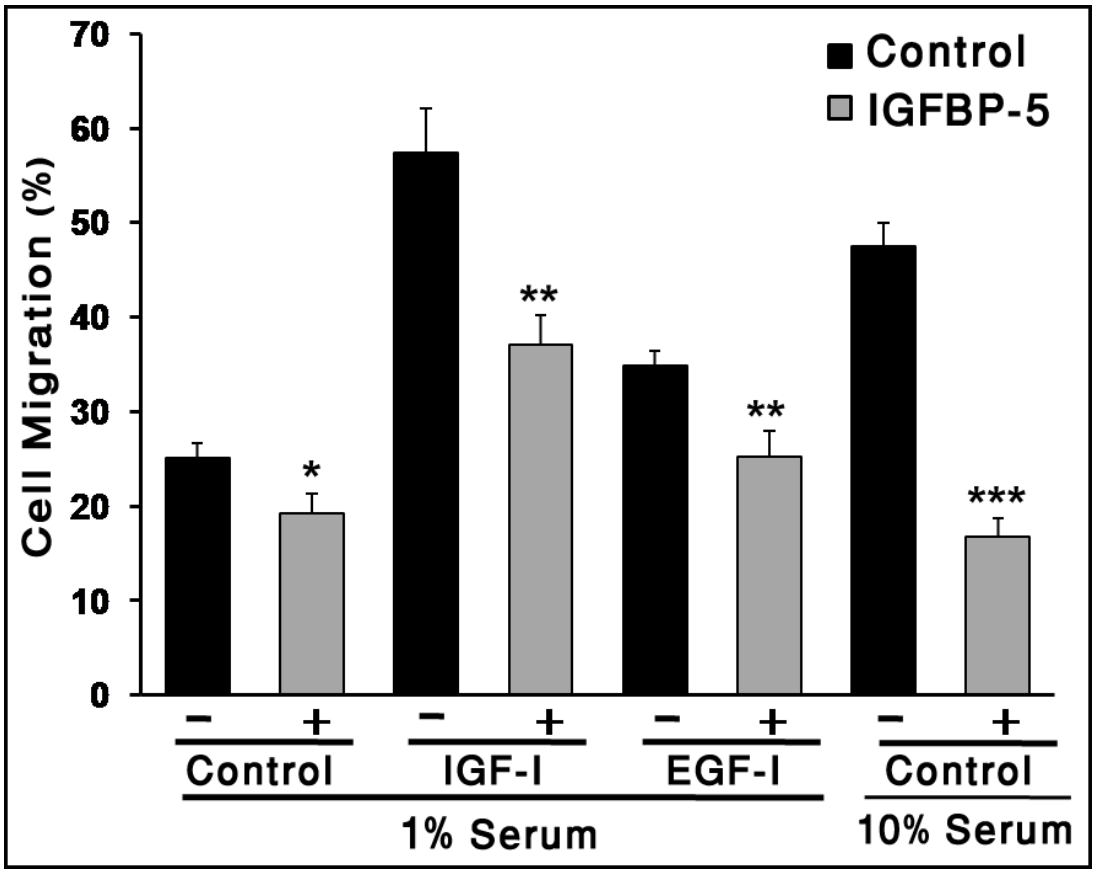


Fig. 3.2.28: ***Quantified results of IGFBP-5 Cell Migration Assay in MCF-7 cells. IGFBP-5 showing inhibition of cell migration in response to IGF, EGF and serum. Values are mean + SEM from 3-4 observations where * p < 0.05; ** p < 0.01, *** p < 0.001 as compared with control and determined by Student's unpaired t-test. n=3***

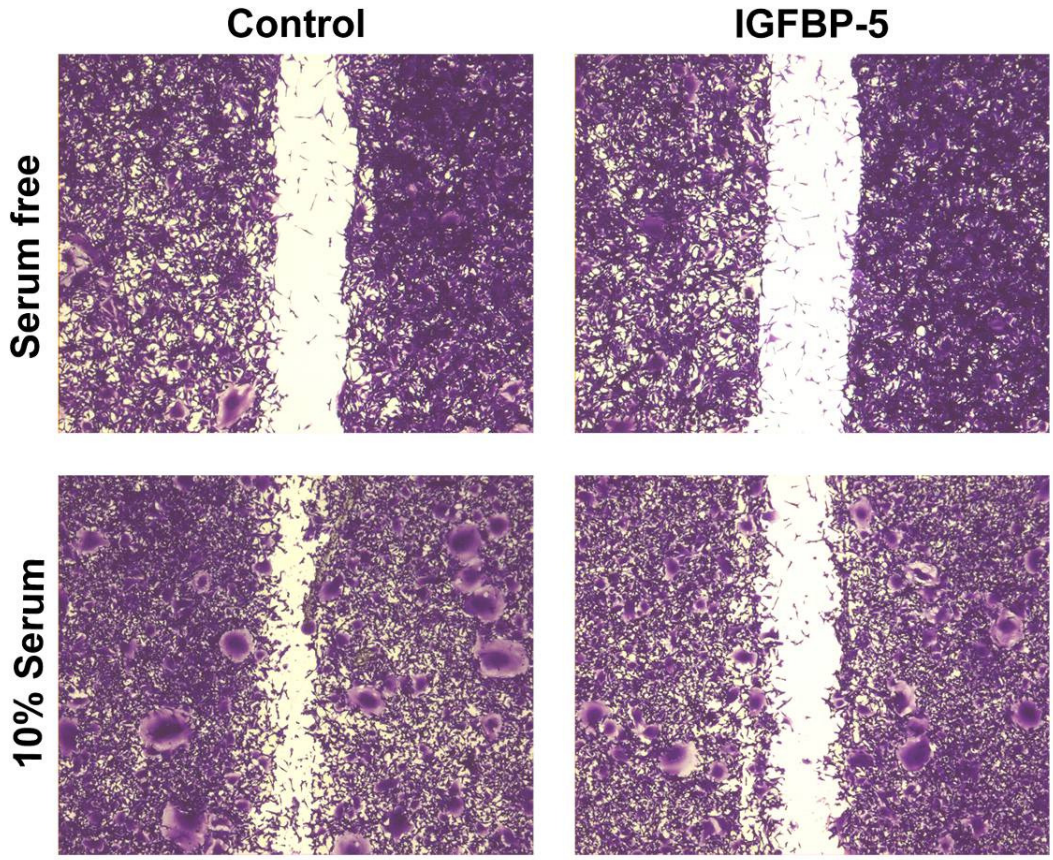


Fig. 3.2.29. ***Crystal violet images of IGFBP-5 cell migration assay of MDA-MB-231 cells. IGFBP-5 treated MDA-MB-231 cells showed inhibition of cell migration in response to serum. Images were photographed at 40x magnification.***

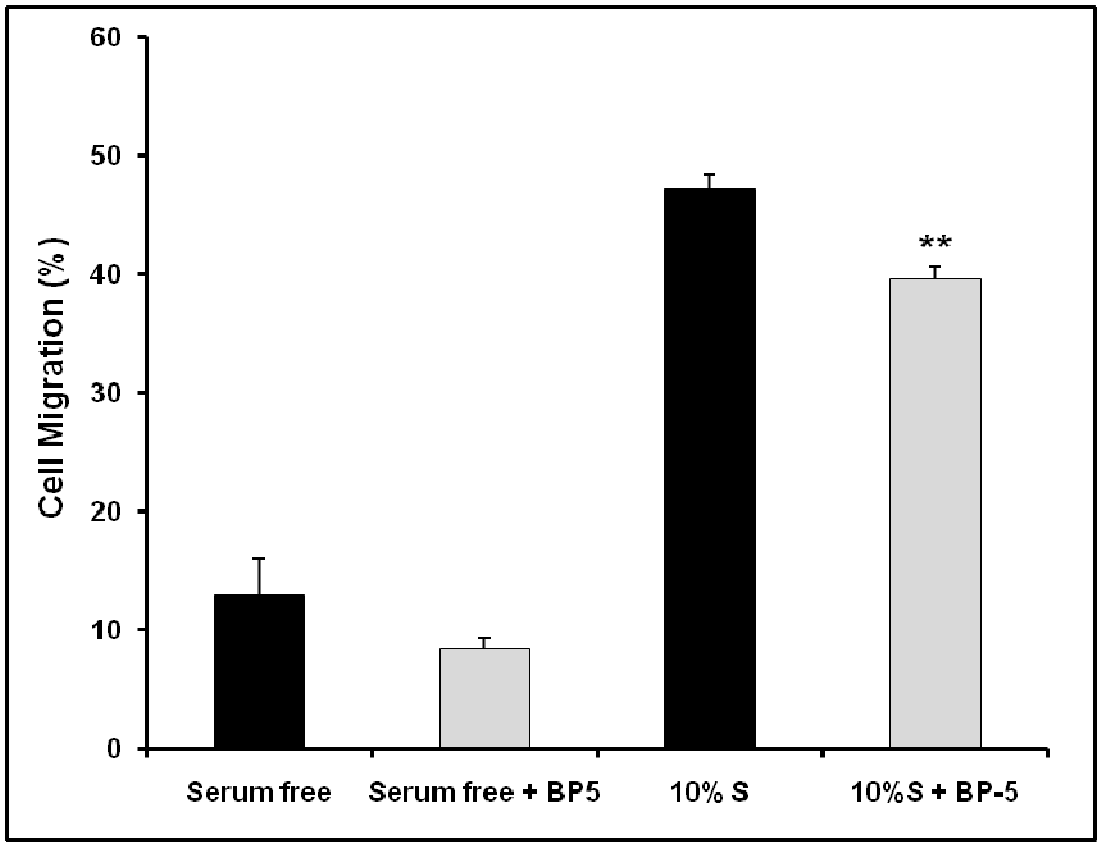


Fig. 3.2.30: ***Quantified results of IGFBP-5 Cell Migration Assay in MDA-MB-231 cells. IGFBP-5 showed inhibition of cell migration in response to serum. Values are mean ± SEM from 3-4 observations where ** indicates p < 0.01 compared with control as determined by Student's unpaired t-test. n=3***

β-Galactosidase Activity Assay

Increased cell spreading and cell survival abilities of IGFBP-5 in MCF-7 cells at nutrient-deprived conditions infer that IGFBP-5 may play a role in cellular senescence. In order to examine whether or not IGFBP-5-transduced MCF-7 cells were undergoing the process of cell senescence, β-galactosidase activity assays were employed at pH 4 (lysosomal) and pH 6 (senescence-associated). Expression of β-galactosidase activity in the cells at pH 4 would indicate lysosomal degration and pH 6 would indicate cell senescence as a gold standard. Adenovirally-transduced MCF-7 cells were seeded at 200,000 cells/ml into a 96 well plate at low and high serum concentrations. Without any medium change, the cells were cultured on a long term basis and stopped on a regular time basis until the point where cells were lifted off. The results indicated that at pH 4.0 IGFBP-5 decreased β-galactosidase activity at both low and high serum concentrations (Figures. 3.2.31 & 3.2.32).

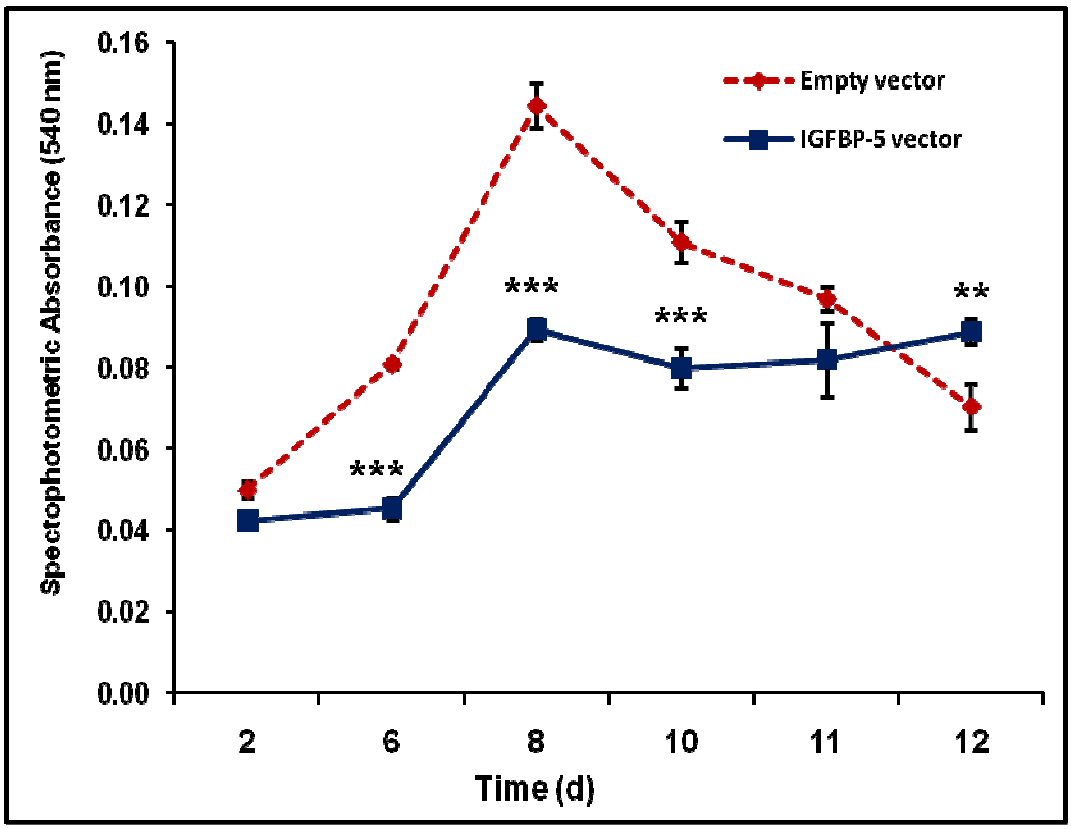


Fig 3.2.31: ***Graph showing β-galactosidase activity values of adenovirally transduced MCF-7 cells at low pH 4.0 and at low serum concentrations on a time scale. IGFBP-5-transduced MCF-7 cells showed a slight decrease in β-galactosidase activity until day 11 time interval. Error bars mean ± SEM of 6 observations where ** indicates p < 0.01; *** indicates p < 0.001 compared with control as determined by Students unpaired t-test. n=2***

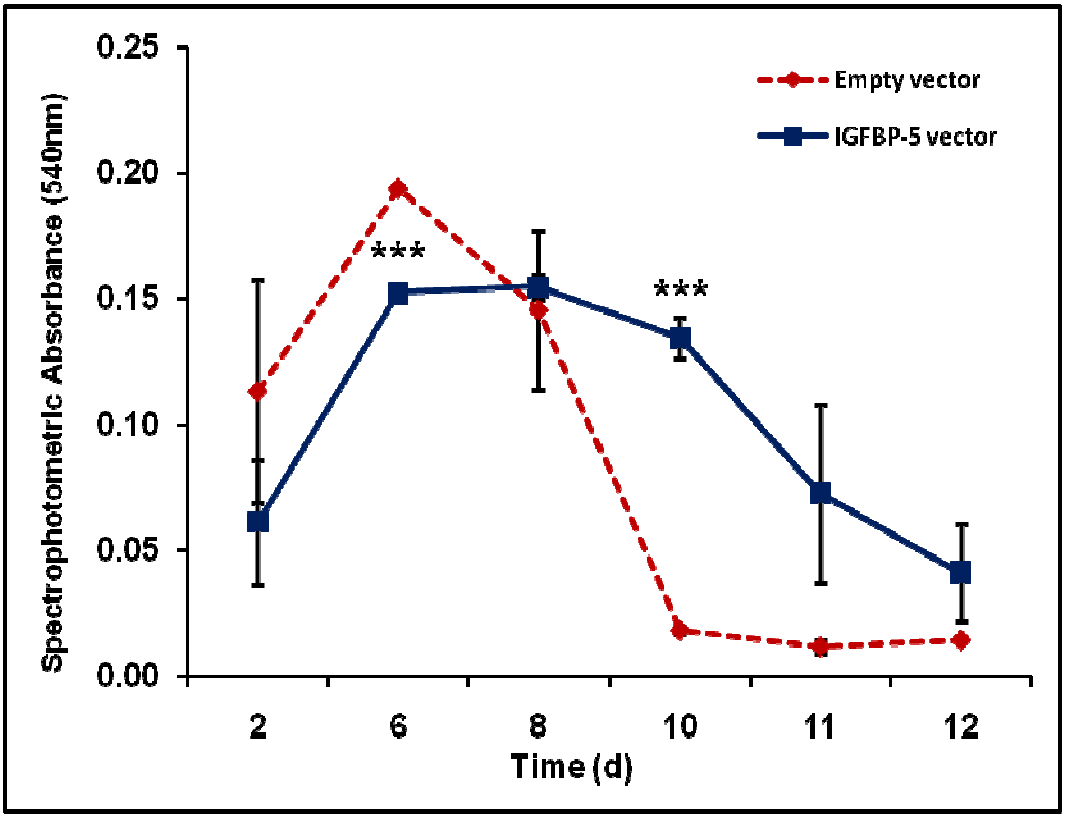


Fig 3.2.32: ***Graph showing β-galactosidase activity values of adenovirally-transduced MCF-7 cells at low pH 4.0 and at high serum concentrations. IGFBP-5-transduced MCF-7 cells showing delayed β-galactosidase activity until day 12 time interval. In contrast, empty vector treated MCF-7 cells showed β-galactosidase activity until day 8 after which there was little activity due to cell detachment. Error bars represent mean ± SEM of 6 observations where *** indicates p<0.001 compared with control as determined by Student's unpaired t-test. n=2***

Biological Effects of C-Terminus of IGFBP-5

To characterize the mechanism and biological effects of full length IGFBP-5 in human breast cancer cells, C-terminal domain of IGFBP-5 biological assays were carried out. In serum-free conditions, recombinant C-terminus was treated with MCF-7 cells in the presence or absence of full length IGFBP-5. Results indicated that the C-terminus partially mimics the cell adhesion to substratum property of full length IGFBP-5 (Figures. 3.2.33 & 3.2.34).

To investigate whether or not C-terminus of IGFBP-5 is required for the inhibitory effect of IGFBP-5 on MCF-7 cell migration, cell migration experiments were undertaken with the C-terminus of IGFBP-5. Results showed that C-terminus has no or little effect in inhibiting the MCF-7 cell migration (Figures. 3.2.35; 3.2.36 & 3.2.37).

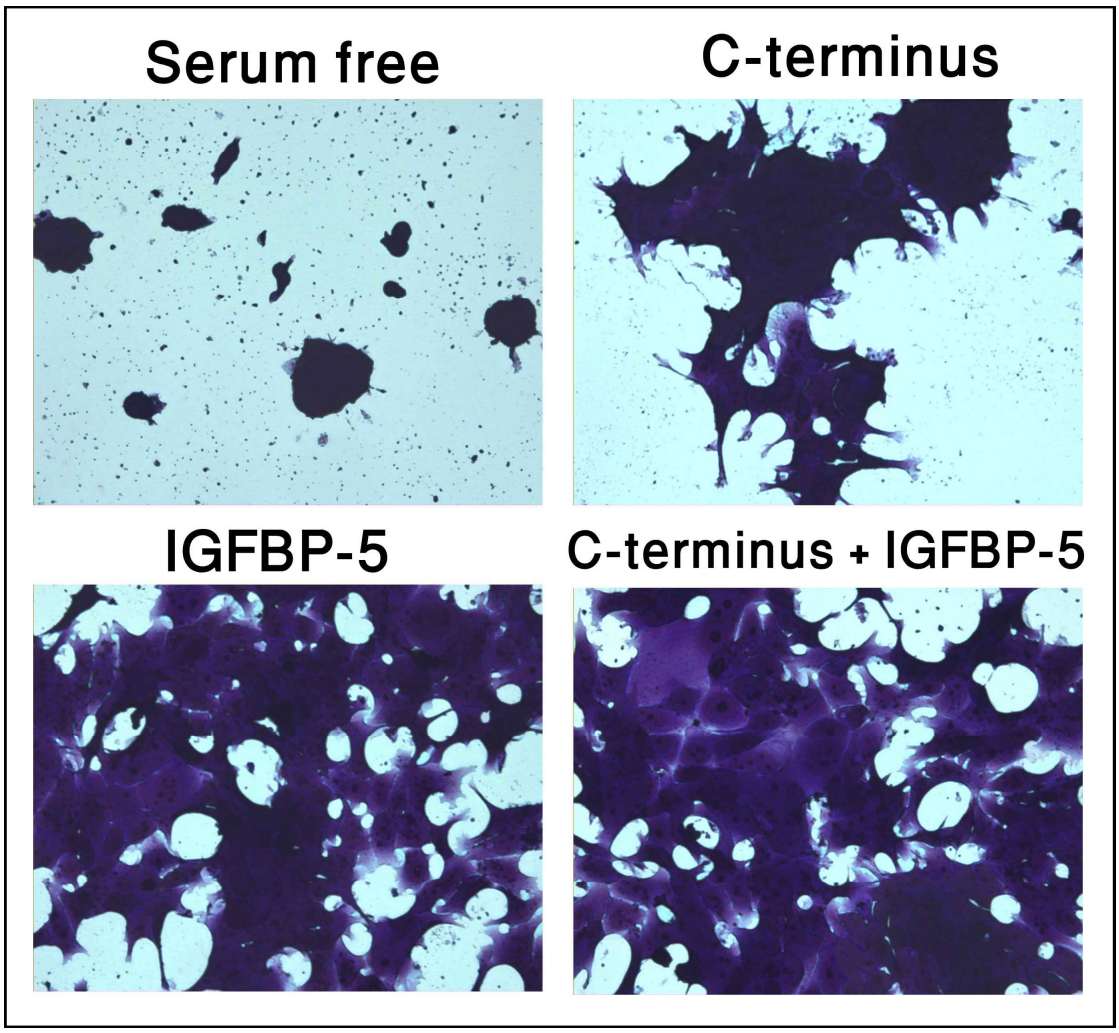


Fig 3.2.33: ***Crystal violet stained images showing the cell: substratum adhesion property by C-terminus treated MCF-7 cells in serum-free conditions. Combination treatment of IGFBP-5 and 005 showing increased cell to substratum adhesion in MCF-7 cells. Images were photographed at 400x magnification.***

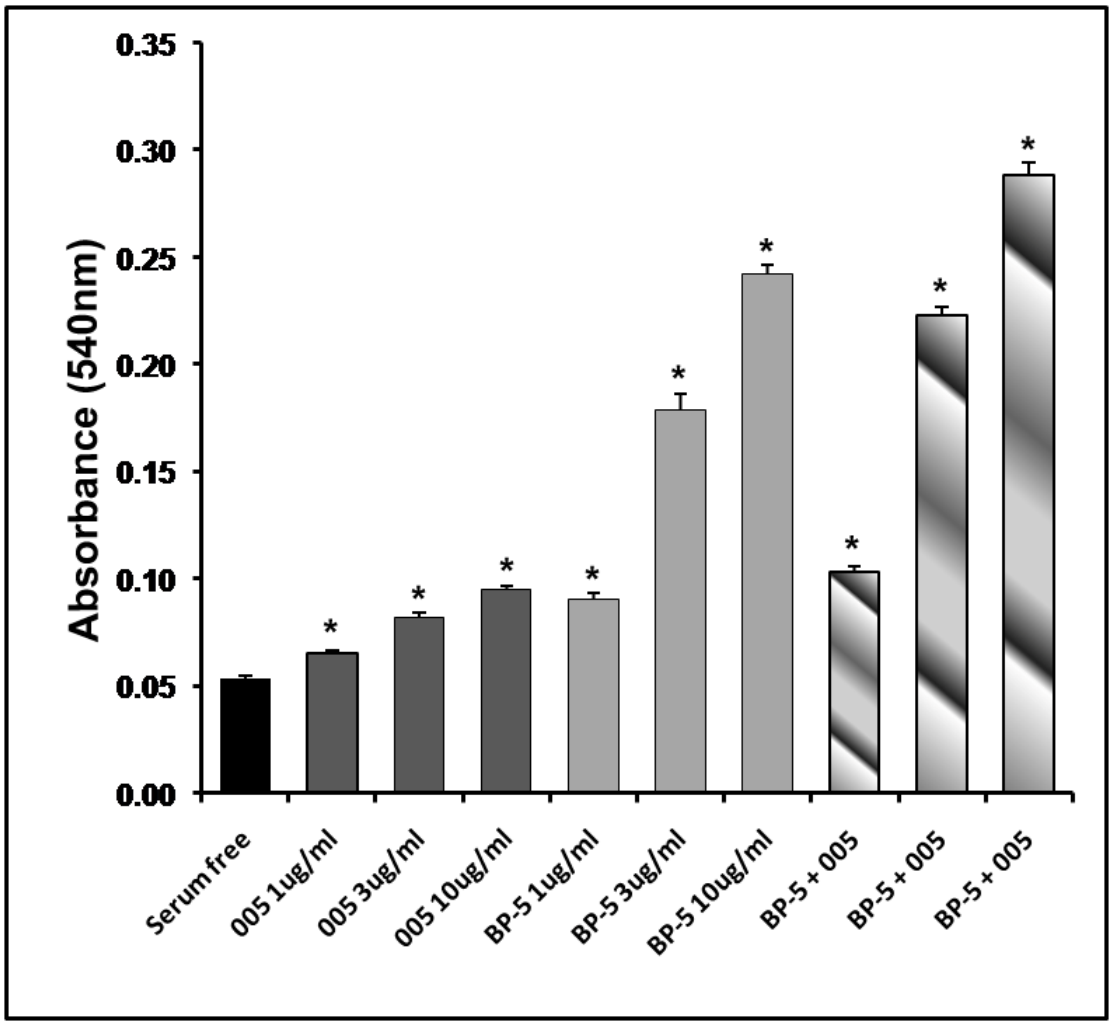


Fig 3.2.34: ***Graph showing the quantified data of C-terminus exogenous addition to MCF-7 cells. The spectrophotometric absorbance has been found to be increased with the combination treatment. Values are means ± SEM of 6 observations. * p < 0.01 compared with control as determined by Student's unpaired t-test. n=2***

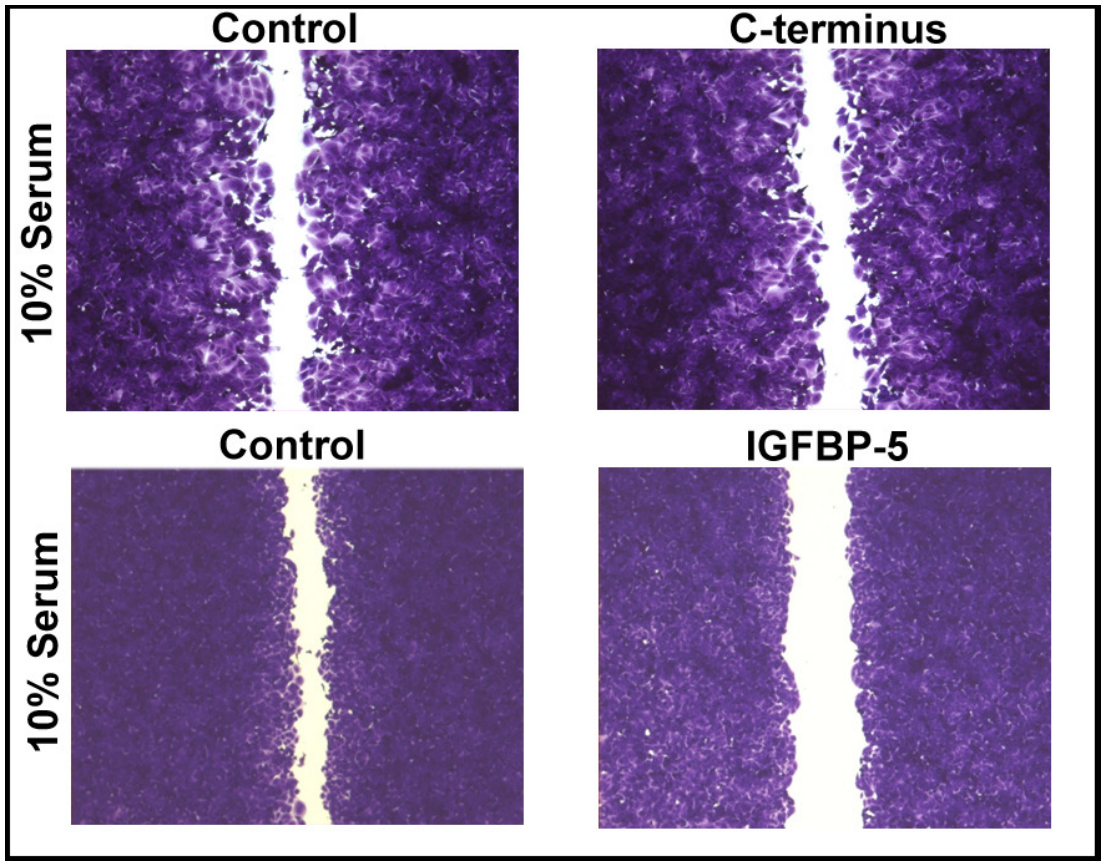


Fig 3.2.35: ***Crystal violet images of C-terminal domain of IGFBP-5 showing little effect on MCF-7 cell migration in response to serum. Full length IGFBP-5 treated MCF-7 cells showing inhibition of MCF-7 cell migration in response to serum. Images were photographed at 40x magnification.***

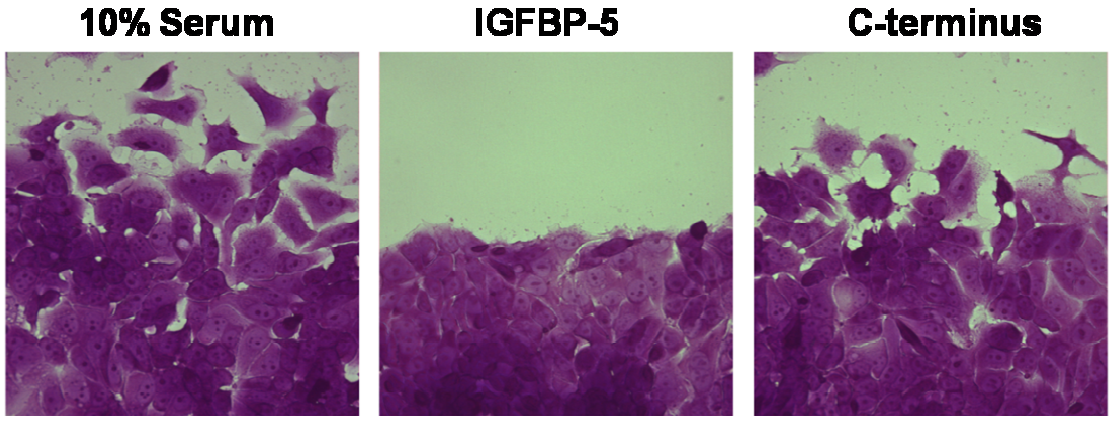


Fig 3.2.36: ***Crystal violet image showing the inhibitory effect of C-terminal domain of IGFBP-5 on MCF-7 cells. IGFBP-5 inhibits MCF-7 cells at 10% FCS treatment. C-terminus has little effect in inhibition of MCF-7 cell migration. Images were photographed at 400x magnification.***

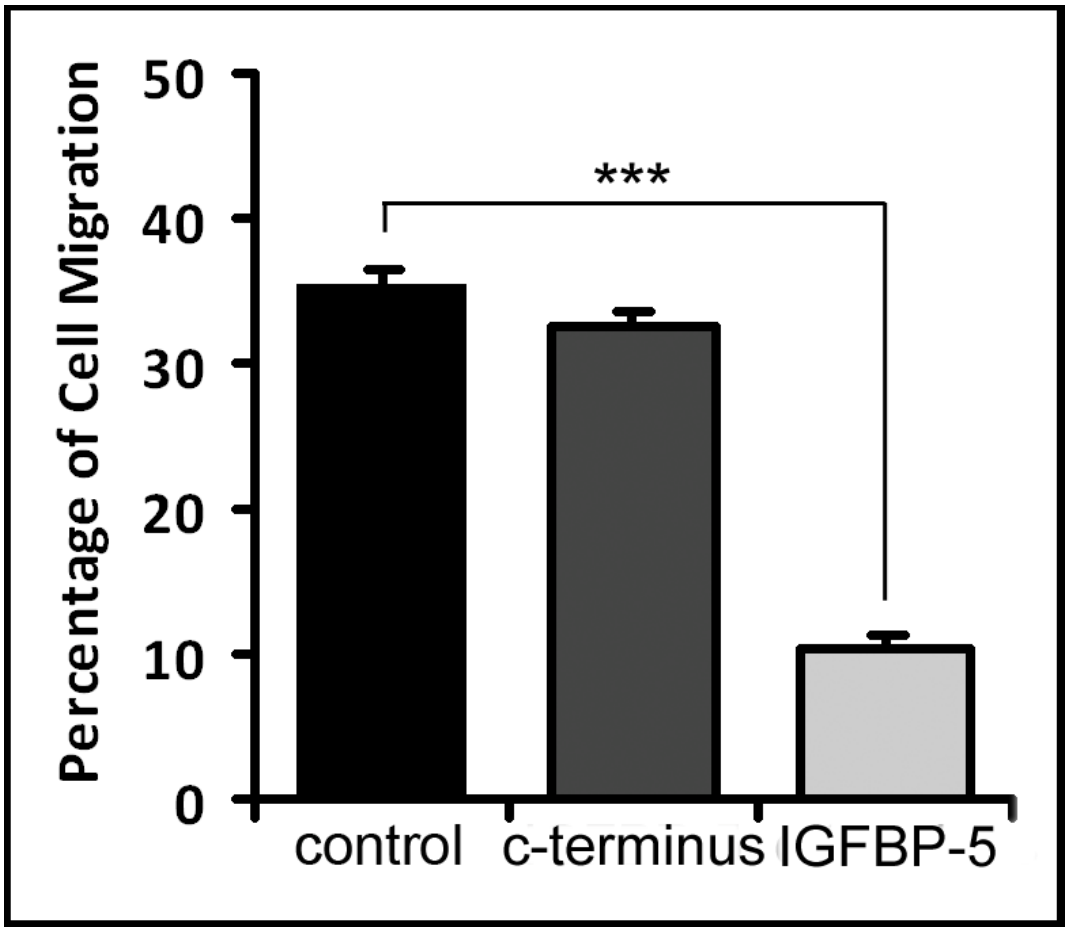


Fig. 3.2.37: ***Graph showing the quantitative analysis of C-terminus inhibition of MCF-7 cell migration assay. Values are mean + SEM from 3-4 observations where *** indicates p < 0.001; as compared with control and determined by Student's unpaired t-test. n=3***

Discussion:

Effects of IGFBP-5 on cell: substratum adhesion

Cell adhesion, by definition, is controlled by interactions between adhesion receptors and their ligands. The relationship between the cell adhesion to extracellular matrix and metastasis formation has been extensively addressed with respect to signal transduction mechanisms in recent years (Yang et al., 2003; Zeisberg et al., 2002). This study examined the role of IGFBP-5 induced cell adhesion in non-invasive mammary tumour epithelial cells, MCF-7 cells and highly invasive mammary tumour epithelial cells, MDA-MB-231 cells which as such represent weakly metastatic and highly metastatic cells respectively. In addition to this, non-tumorigenic mammary epithelial cells, MCF-10A cells were also employed.

The results of MCF-7 and MCF-10A cell adhesion assays showed that adenovirus expressing IGFBP-5 induced a phenotypic epithelial to mesenchymal transition (EMT) and increased cell: substratum adhesion in MCF-7 breast tumour cells but induced de-adhesion in MCF-10A cells even in the presence of serum. Of relevance to this, IGFBP-5 has been shown to induce apoptosis of normal mammary epithelial cells both *in vitro* and *in vivo* (Marshman et al., 2003; Tonner et al., 2002). This IGFBP-5-induced cell adhesion and increased cell: substratum adhesion in MCF-7 cells has been referred to as phenotypic EMT and may well play a key role in chronic degenerative fibrosis and cancer metastasis (Feghali and Wright, 1999; Yasuoka et al., 2006a).

In addition to cell: substratum adhesion in MCF-7 cells, I have also demonstrated that recombinant IGFBP-5 induces cell: substratum adhesion in MDA-MB-231 cells both in

serum free conditions and serum concentrations. The conclusion which I draw from the cell adhesion assays is that IGFBP-5 induces cell adhesion to substratum (ECM) in non-invasive MCF-7 cells and in highly invasive MDA-MB-231 cells but induces de-adhesion in normal mammary epithelial cells, MCF-10A cells.

Effect of IGFBP-5 on fibronectin secretion

Fibronectin is an extracellular matrix protein which plays numerous important functions in biological processes and normal development. It is involved in cell adhesion, growth, migration and differentiation (Mosher et.al., 1989; Carsons et.al., 1989; Hynes et.al., 1990, Yamada and Clark, 1996; Pankov and Yamada, 2002). Its dysregulation and age-dependent increase in plasma and tissues has been implicated in many cancerous diseases (Choate and Moscher, 1983; Labat-Robert J, 2002). Studies implicating IGFBP-5 as a pro-fibrotic agent suggested that IGFBP-5 could increase fibronectin secretion. In order to examine the effect of IGFBP-5 on fibronectin synthesis and secretion, studies at the levels of both protein and mRNA were carried out. Fibronectin production and secretion were assessed using Western blotting.

In this present study, IGFBP-5 altered fibronectin in both the medium (soluble) and cell-associated compartments. In preliminary experiments, IGFBP-5-transduced MCF-7 cells had decreased fibronectin levels in cell homogenates and increased levels of fibronectin in conditioned medium. This inferred that IGFBP-5, a secreted protein, increased soluble fibronectin secretion form epithelial cells which could serve to act distally and mediate fibroblast adhesion and spreading (Webb et. al., 2000). Thereby, IGFBP-5 may regulate

fibroblast adhesion and spreading during the epithelial injury, although these findings need to be confirmed by additional experiments.

In order to examine the effect of IGFBP-5 on fibronectin at the mRNA level, quantitative real time PCR method was employed. The IGFBP-5-transduced MCF-7 samples were assessed for fibronectin gene-expression and preliminary results revealed no effect of IGFBP-5. In contrast, IGFBP-5-transduced HDFs demonstrated a 4-fold increase in fibronectin mRNA levels. This increase in fibronectin expression in fibroblasts treated with IGFBP-5 is consistent with a role for IGFBP-5 in the assembly of extracellular matrix which has important roles in the processes of metastasis, fibrosis and wound healing.

Effect of IGFBP-5 on Cell Survival

Recombinant IGFBP-5 showed enhanced cell survival properties in a variety of nutrient-deprived conditions including both high seeding density and serum-free conditions and at low seeding density and high serum concentrations. Further to this, Ad-IGFBP-5 improved cell survival over a range of serum concentrations at high and low seeding densities. In addition, live and dead cell staining values of IGFBP-5-transduced MCF-7 cells showed increase in live cells at both low and high serum concentrations whereas, the proportion of dead cells from empty vector-transduced MCF-7 cells were dramatically increased and detached from the substratum at high serum concentrations. The cell viability readings measured from these experiments indicated that the IGFBP-5-transduced MCF-7 cells were metabolically active and correlated well with live cell fluorescence values of IGFBP-5-transduced MCF-7 cells. The dead cell fluorescence

values were found to be not in accordance with live cell fluorescence values at low serum concentrations. Although seeded at right density, this could possibly have occurred due to less number of dead cells as compared to live cells which thus lead to two fold difference in dead cell fluorescence of adenovirally transduced MCF-7 cells. Differences in less number of dead cells would be exaggerated since a much smaller total number of cells are anlaysed at low serum concentrations when compared with the assay for live cells. These results thus demonstrate that IGFBP-5 promotes cell survival in MCF-7 cells.

Tumour cell migration through integrin-dependent signalling is pivotal to the process of metastasis *in vivo* (Lin et al., 2008; Zijlstra et al., 2008). Cell Migration assays demonstrated that IGFBP-5 inhibited cell migration in MCF-7 cells in response to three potent stimuli namely IGF-I, EGF and serum. Similar to this effect, IGFBP-5 also inhibited migration in response to serum in MDA-MB-231 cells.

Although IGFBP-5 significantly inhibited, rather than stimulated, migration of MCF-7 cells in response to 3 potent stimuli, IGF-I, EGF and serum. In contrast, the c-terminus of IGFBP-5, which also induced cell adhesion, failed to influence cell migration, suggesting that the adhesive and anti-migratory actions of IGFBP-5 reside in different domains of the molecule. In order to examine the role of IGFBP-5 in the process of cell senescence, β-galactosidase was assessed in nutrient-deprived IGFBP-5-transduced MCF-7 cells. Interestingly, IGFBP-5 delayed β-galactosidase activity at low pH (4) as compared to control cells. β-galactosidase activity at low pH is indicative of autophagy (Young et al., 2009). This observation suggests a role of IGFBP-5 in the lysosomal dependent protein degradation process.

Sub-Chapter 3

Mechanism of Action of IGFBP-5

Mechanism of Action of IGFBP-5

Effect of IGFBP-5-induced cell adhesion on ECM

In order to examine the cell: substratum adhesion characteristics of IGFBP-5, MCF-7 cells were seeded at 2 x 10^5 cells/ ml onto a 96 well plates a pre-coated with collagen (100 ng/ml), fibronectin (500 ng/ml) and or laminin (500 ng/ml) with and without the treatment of exogenous IGFBP-5 at 10µg/ml. After overnight incubation culture at 37 ^oC CO_2 incubator, the cells in the wells were fixed, and stained, and solubilised and read spectrophotometrically at 540 nm . Interestingly, IGFBP-5 induced cell adhesion was increased in the presence of collagen and fibronectin basal conditions. But IGFBP-5 induced cell adhesion was not evident in the presence of laminin (Fig. 3.3.1).

Of relevance to this, the cell: substratum adhesion characteristic of IGFBP-5 was also examined in metastatic cell lines HS 578T and MDA-MB-231 cell lines at similar seeding density and basal concentrations. Intriguingly, IGFBP-5-induced cell adhesion was not evident in the presence of collagen and fibronectin but increased in the presence of laminin. Whereas, in case with MDA-MB-231 cells, IGFBP-5 induced cell: substratum adhesion in all the three major ECM basal components (Figures. 3.3.2 & 3.3.3).

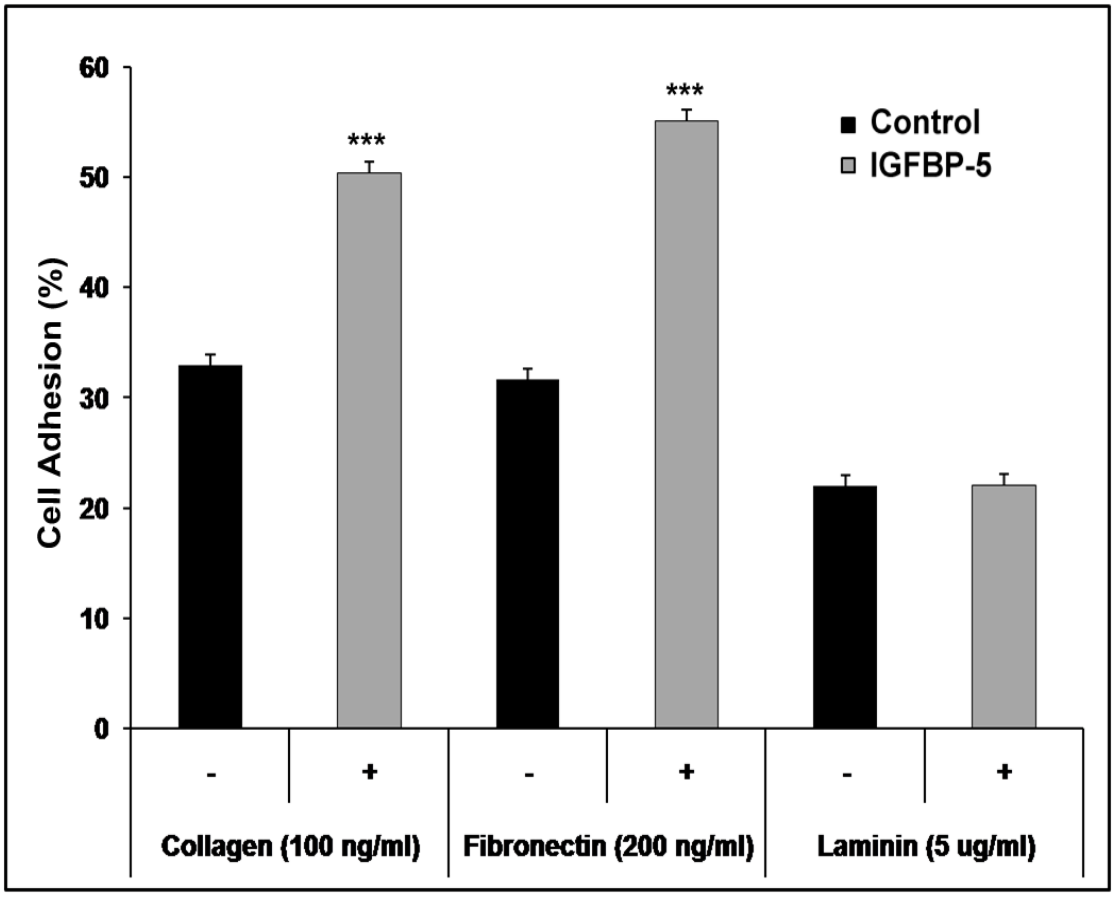


Fig 3.3.1: ***Graph showing the IGFBP-5-induced MCF-7 cell adhesion on ECM molecules. IGFBP-5 showing increased cell: substratum adhesion in the presence of collagen and fibronectin but not in the presence of laminin. Values are mean ± SEM of 9 observations where *** p < 0.001 compared with control as determined by Student's unpaired t-test. n=3***

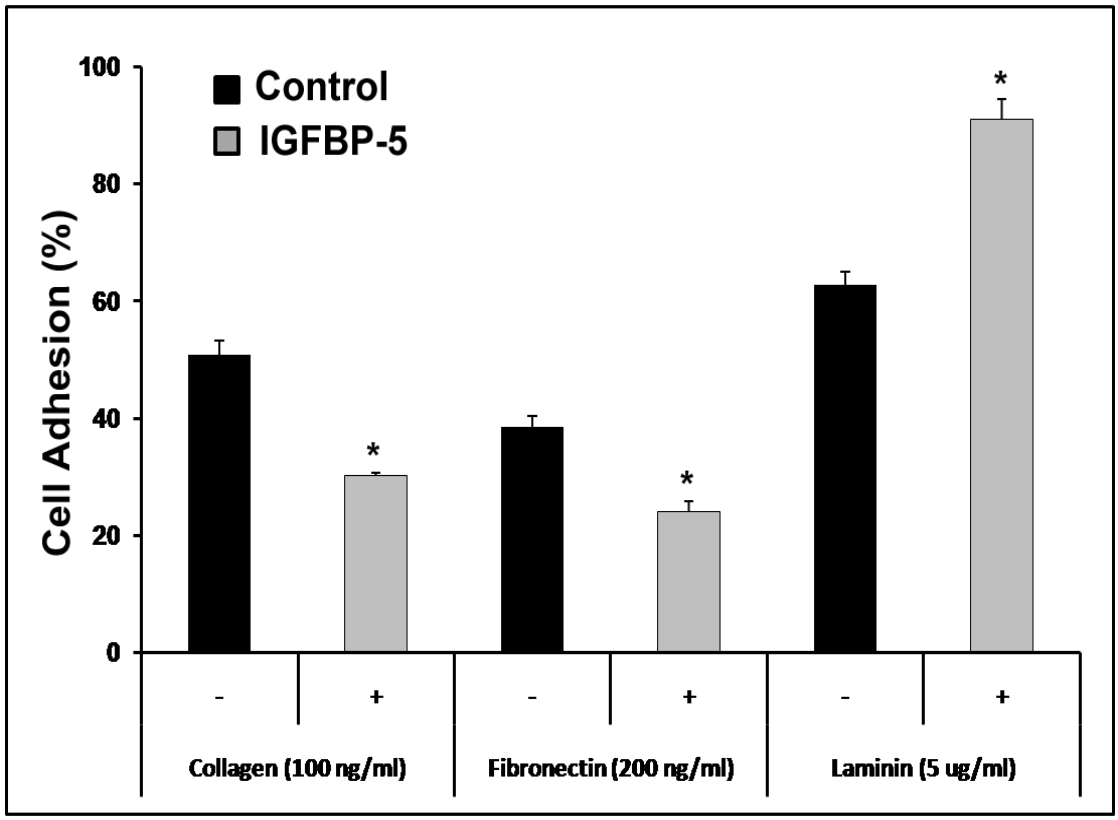


Fig. 3.3.2: ***Graph showing the IGFBP-5-induced HS 578T cell adhesion on ECM molecules. IGFBP-5 promoting cell: substratum adhesion in the presence of laminin but not in the presence of collagen and fibronectin in HS 578T cells. Values are mean $\pm$ SEM of 9 observations where * $p < 0.01$ compared with control as determined by Student's unpaired t-test. n=3***

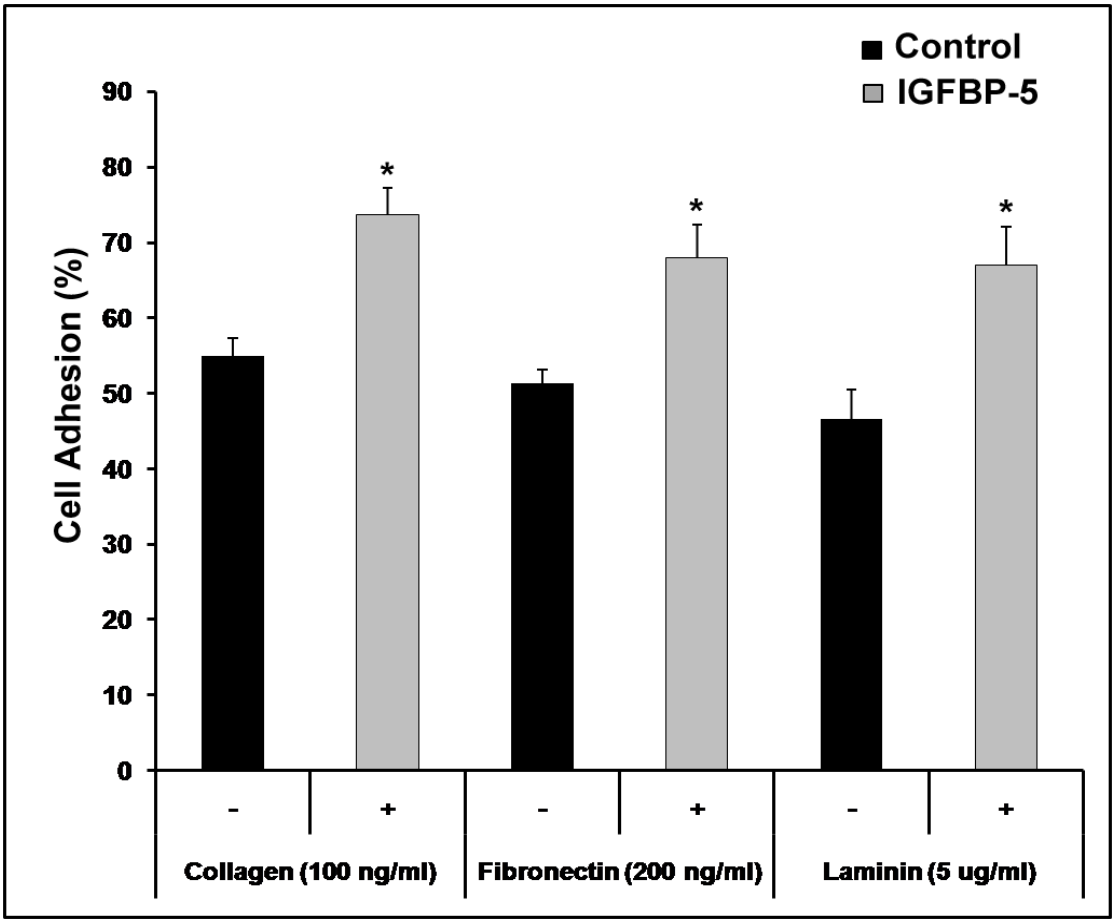


Fig 3.3.3.: ***Graph showing the IGFBP-5-induced MDA-MB-231 cell adhesion on ECM molecules. IGFBP-5-induced cell: substratum adhesion is increased in the presence of all the three basal substrata in MDA-MB-231 cells. Values are mean ± SEM of 9 observations where * p < 0.01 compared with control as determined by Student's unpaired t-test. n=3***

Effect of Function Blocking Integrin Antibodies on Cell Adhesion

In order to study the action of IGFBP-5 cell: substratum adhesion more effectively, a series of function-blocking antibodies to various integrins were employed. Integrins are major adhesion molecules and we therefore examined the effect of IGFBP-5 on cell: extracellular matrix adhesion using function blocking integrin antibodies. 1mg/ml of integrins antibodies were added in the presence and absence of 10ug/ml of IGFBP-5. MCF-7 Cells were trypsinised, counted and adjusted to 300,000 cells/ml. Then 100 µl of MCF-7 cells were added to each well. Plates were then incubated for 24h at 37 °C in a 5% CO2 atmosphere before fixation and staining. Results showed that the actions of IGFBP-5 were inhibited by antibodies to $\alpha 2$ and $\beta 1$ integrins as well as (to a lesser extent) by $\alpha v \beta 6$ (Fig. 3.3.4).

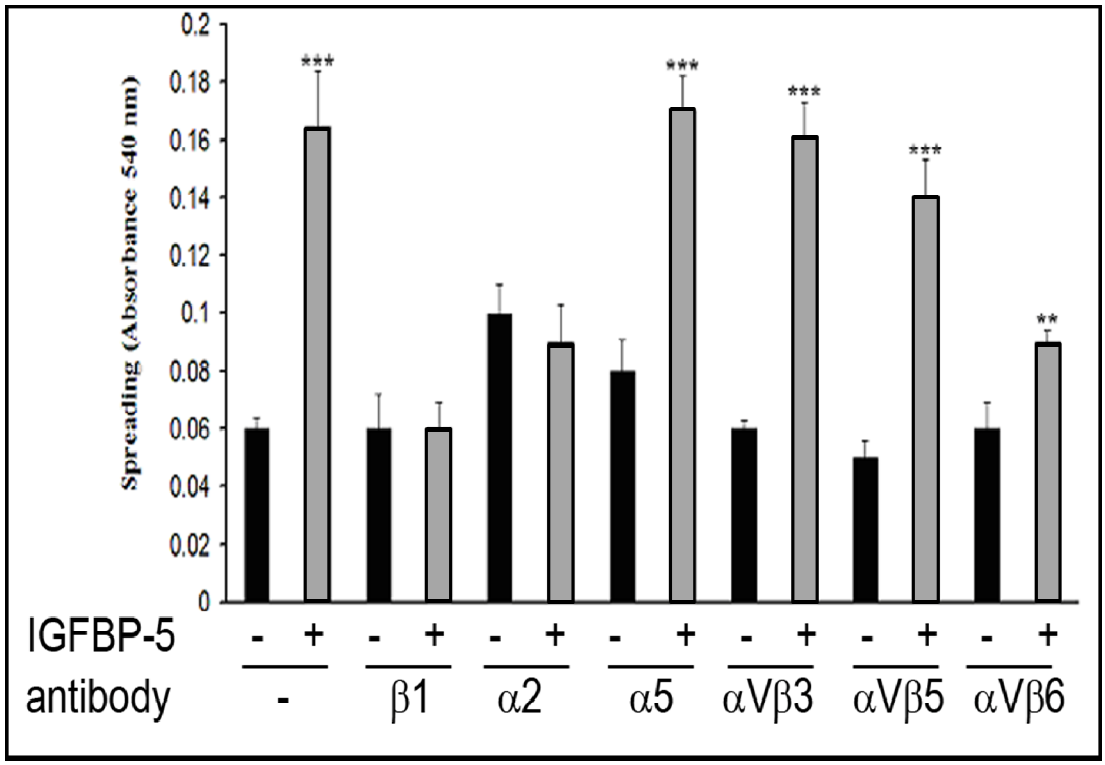


Fig 3.3.4: ***Graph showing the adhesion effect of IGFBP-5 significantly blocked by α2, β1 and αVβ6 integrin function-blocking antibodies. Values are means ± SEM of 9 observations where **p<0.01 *** p<0.001 compared with control as determined by Student's unpaired t-test.***

Effect of IGFBP-5 on ILK in MCF-7 cells

Consistent with the integrin-mediated action of IGFBP-5, Integrin-Linked Kinase (ILK) has been associated with many cellular functions including cellular adhesion. Of relevance to aforementioned studies, I have examined the expression of ILK in IGFBP-5 treated and transduced MCF-7 cells. To achieve this purpose, I have employed both exogenous addition of recombinant IGFBP-5 and Ad-IGFBP-5.

Briefly, 300,000 MCF-7 cells/ml were seeded into 24 well plates at serum-free conditions and treated with 10 µg/ml of recombinant IGFBP-5 at 24 h time intervals. On the other side, 1,500,000 adenovirally-transduced MCF-7 cells/ml were transduced with empty and IGFBP-5 adenovirus at 200 m.o.i and seeded into 6 well plates at serum-free, 0.1% and 1% serum concentrations. Cells were lysed at 24 h time intervals using RIPA buffer. Total protein concentration was estimated and immunoblotted with α-ILK Ab. Results showed increase in ILK expression in IGFBP-5 treated MCF-7 cells both with recombinant IGFBP-5 at 24 & 48 h in serum free conditions and with Ad-IGFBP-5 at 48 & 72 h in 0.1% and 1% low-serum concentrations respectively (Refer to figures 3.3.5 and 3.3.6).

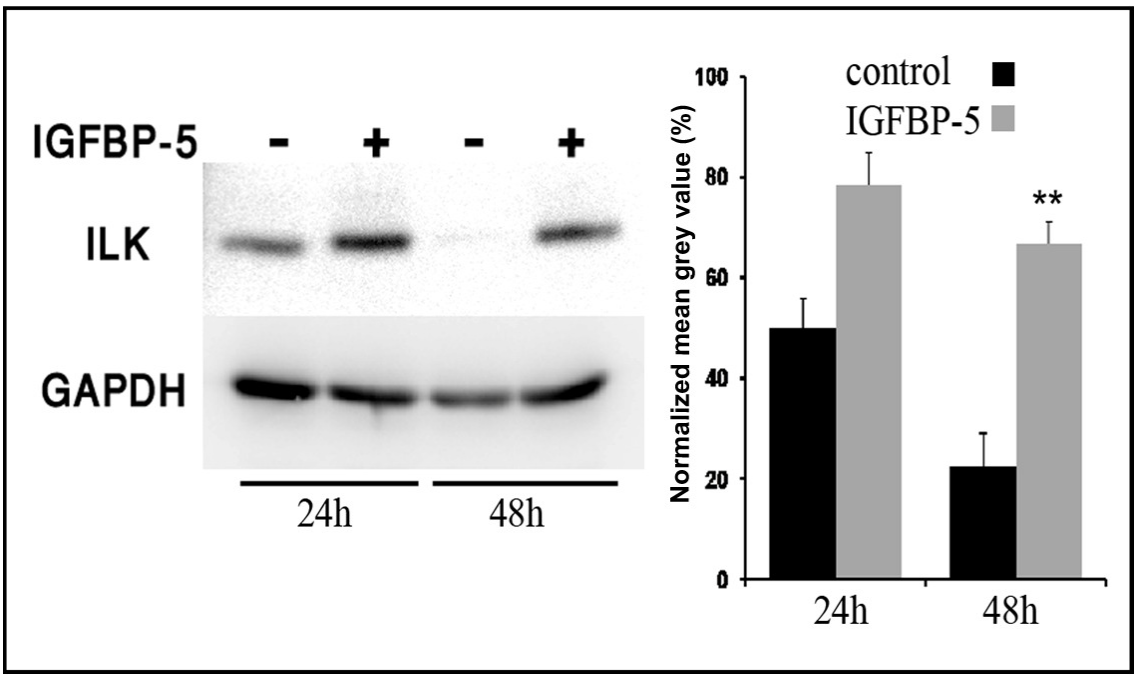


Fig. 3.3.5: ***ILK expression in IGFBP-5 treated MCF-7 cells. (A) Western blotting analysis of MCF-7 cells seeded in serum-free medium and treated with exogenous IGFBP-5 for 24 and 48 h time intervals. Cell lysates were immunoblotted with α-ILK (top) and re-probed with α-GAPDH (bottom). (B) Corresponding graph showing the percentage of mean gray values of analysed blot normalised with GAPDH blot. Values are mean ± SEM of three independent blots where * indicates p<0.01 as compared with control (Student's unpaired t-test).***

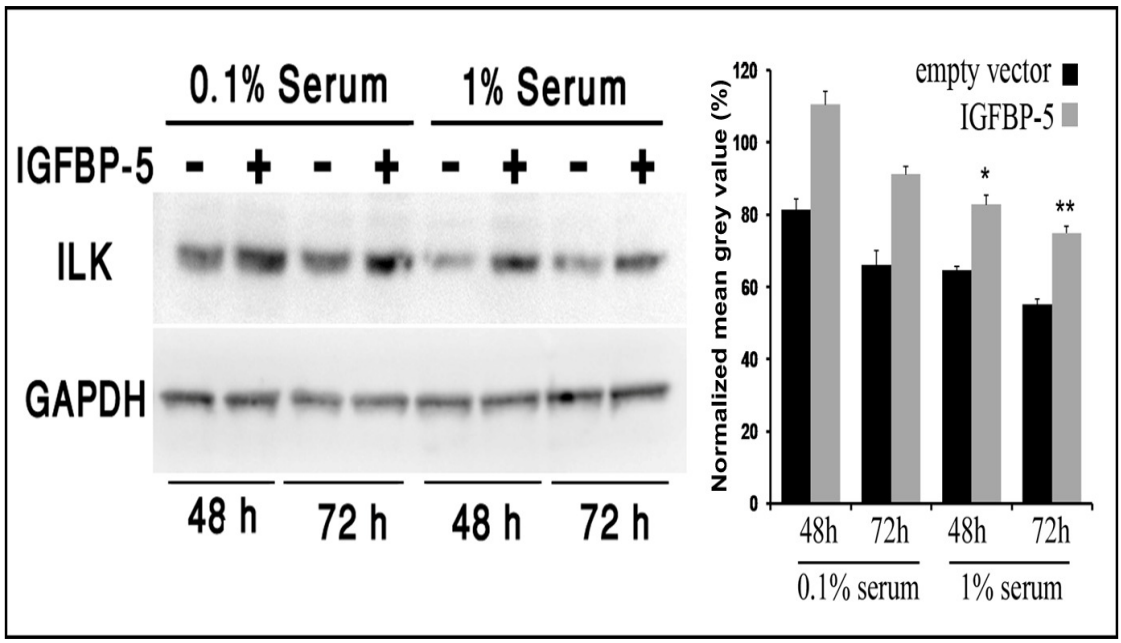


Fig. 3.3.6: ***ILK expression in IGFBP-5-transduced MCF-7 cells. (A) Western blotting analysis of adenovirus-transduced IGFBP-5 MCF-7 cells seeded at low serum concentrations and performed mock transduction with 200 MOI for 48 and 72 h time intervals. Cells were harvested after indicated time intervals and treatments. Cell lysates were immunoblotted with α-ILK (top) and re-probed with α-GAPDH (bottom). (B) Corresponding quantified graph showing the percentage of mean gray values of analysed blot normalised with GAPDH blot. Values are mean ± SEM of three independent blots where * indicates p<0.05, ** p<0.01 compared with control (Student's unpaired t-test).***

Integrin-Mediated Cell Adhesion Assay Studies

α, β - Integrin Binding Assay with MCF-7 cells:

In order to study the effect of IGFBP-5 on cell adhesion, integrin binding to IGFBP-5-transduced MCF-7 cells was examined since they mediate cell adhesion either to the extracellular matrix or to cell-surface receptors during cell-cell interactions. IGFBP-5-transduced MCF-7 cells and primary HDFs were added to α and β integrin plates 4d post-transfection. The results showed that Ad-IGFBP-5 considerably decreased the binding of α 5, α V receptors in MCF-7 cells. The results showed that IGFBP-5 considerably decreased the binding of β1 receptors and αVβ5, α5β1 integrin receptors in MCF-7 cells (Figures 3.3.7 and 3.3.8). The αVβ5, α5β1 integrins are receptors for the ligands fibronectin and vitronectin respectively.

α, β - Integrin Binding Assay with HDF cells:

IGFBP-5-transduced HDFs were added to α, β integrins binding plates 4d post-transfection. The results showed in contrast to IGFBP-5-transduced MCF-7 cells, IGFBP-5 significantly increased the binding to panel α and β integrins in HDFs (fig. 3.3.9 & 3.3.10).

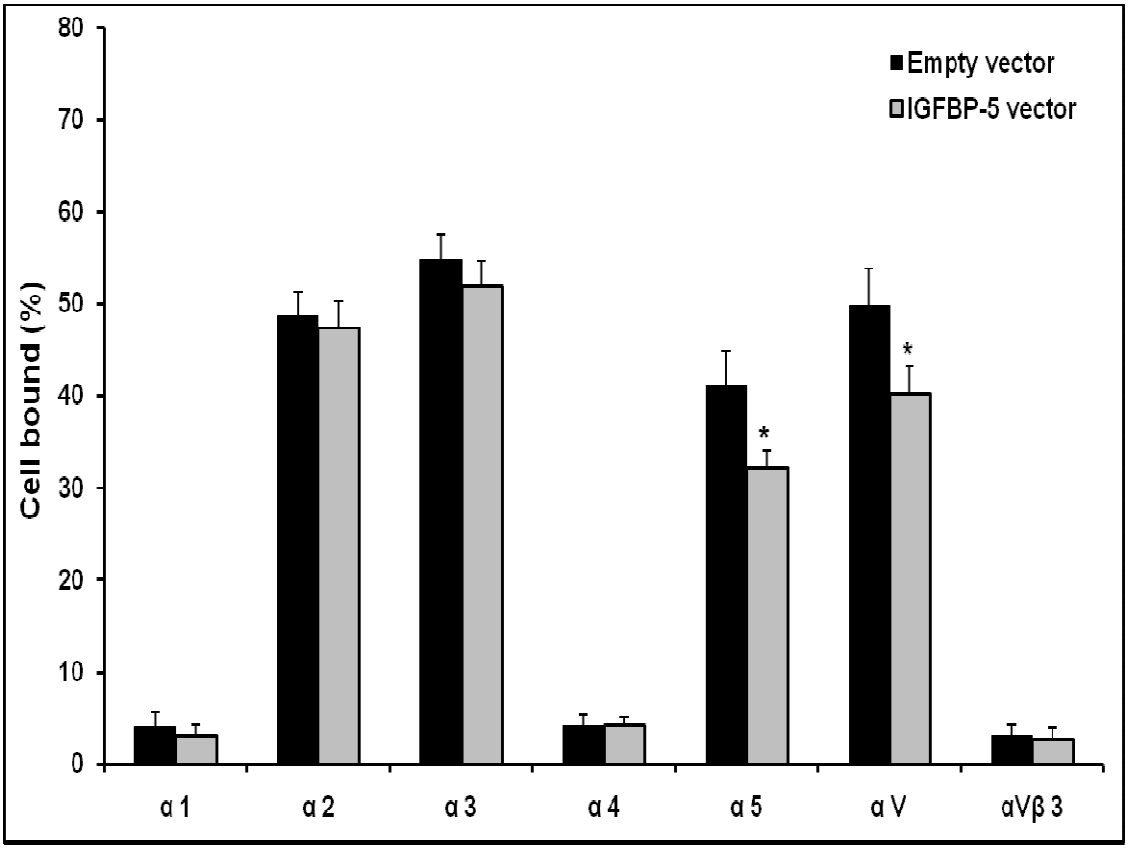


Fig 3.3.7: ***α - Integrin Binding Assay of IGFBP-5-transduced MCF-7 cells. Values are mean ± SEM of 9 observations where * indicated as p < 0.05 as compared with control and determined by Students paired t-test. n=3***

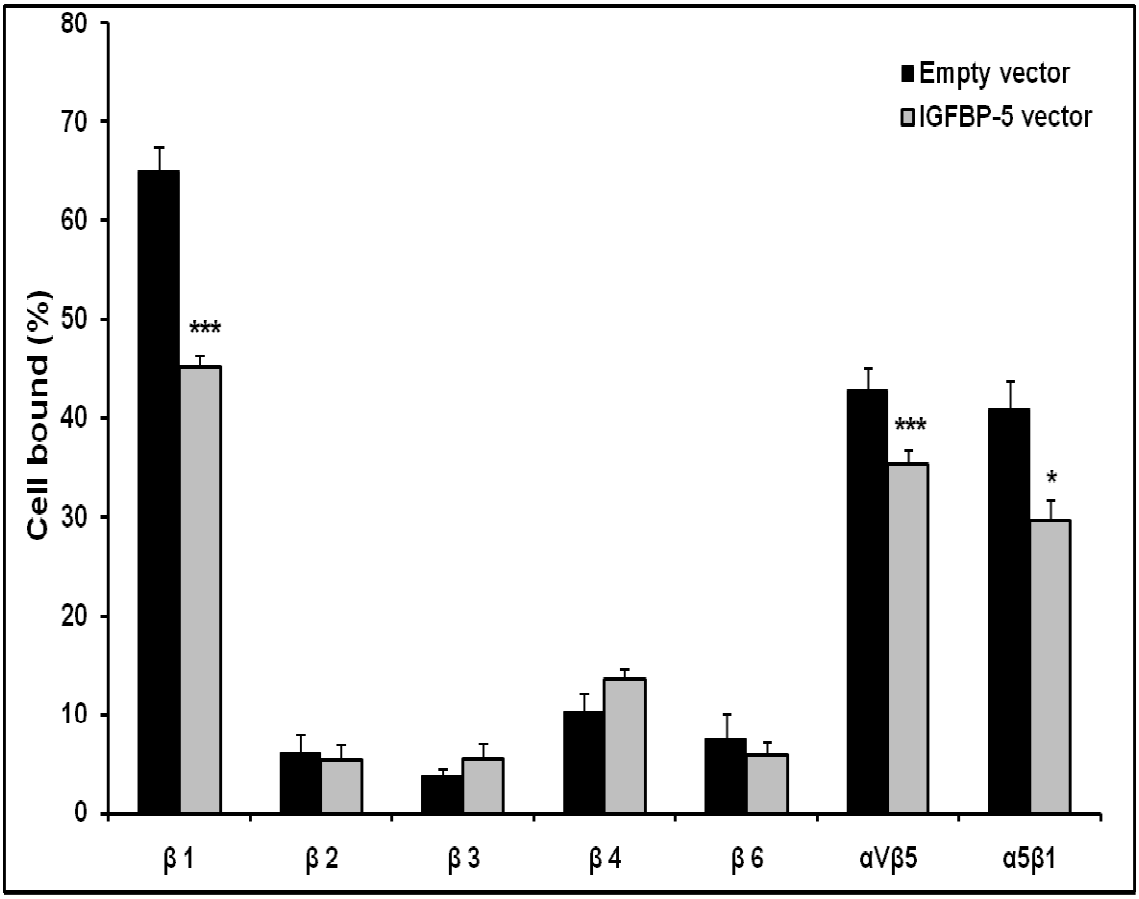


Fig 3.3.8: β - *Integrin Binding Assay of IGFBP-5-transduced MCF-7 cells. Values are mean ± SEM of 9 observations where * indicated as p < 0.05 ** p < 0.01 *** p < 0.001 as compared with control and determined by Students paired t-test. n=3*

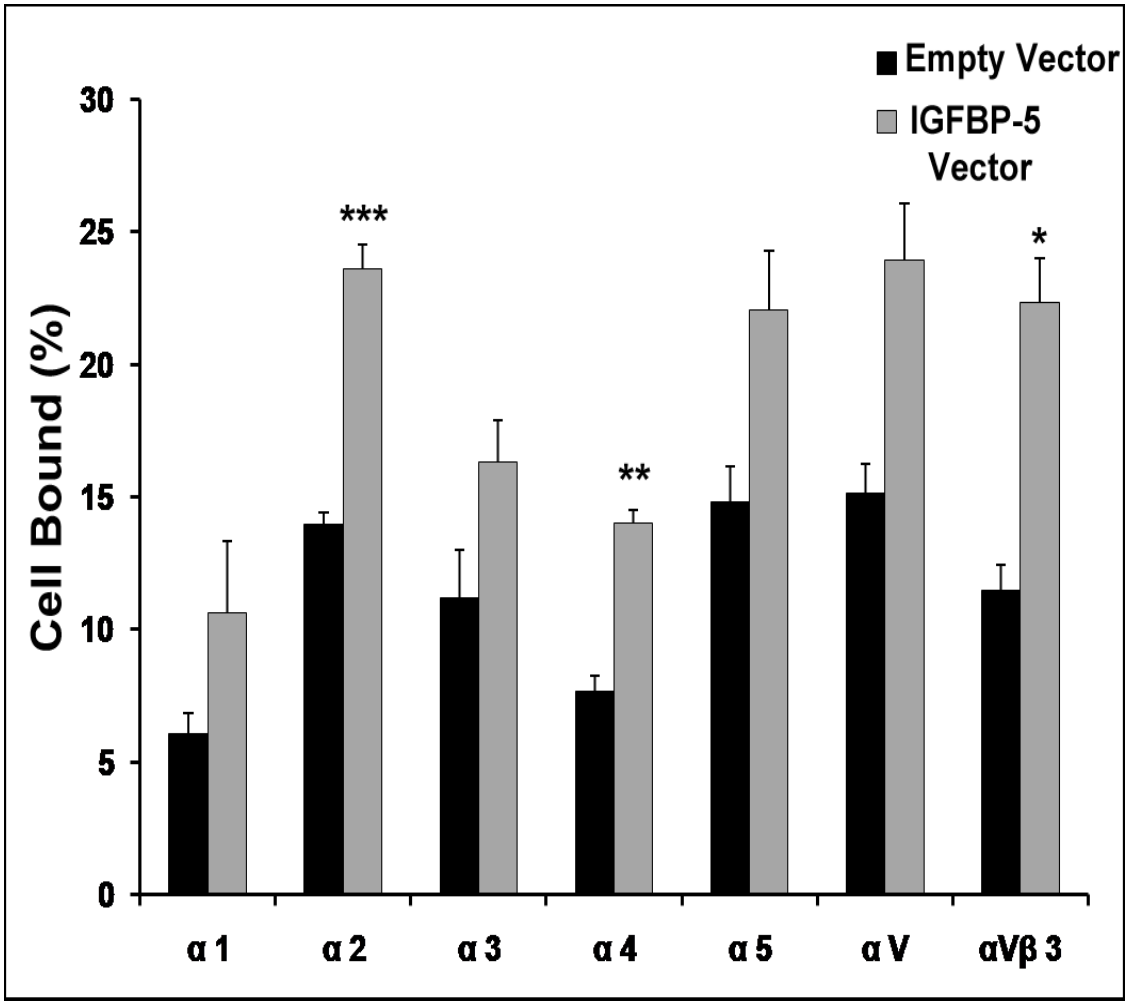


Fig 3.3.9: ***α - Integrin Binding Assay of IGFBP-5-transduced HDFs. Values are mean ± SEM of 9 observations where * indicated as p < 0.05 ** p < 0.01 *** p < 0.001 as compared with control and determined by Students paired t-test. n=3***

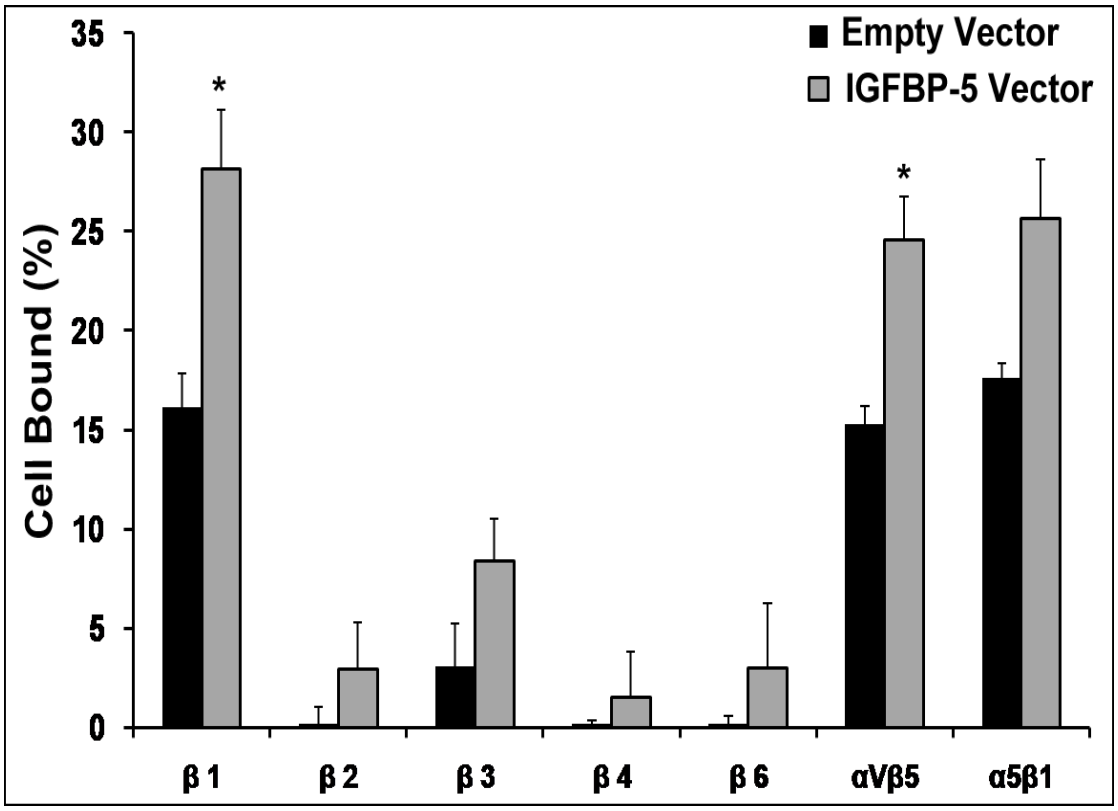


Fig 3.3.10: ***Beta-Integrin Binding Assay of IGFBP-5-transduced HDFs. Values are mean ± SEM of 9 observations where * indicated as p< 0.05 as compared with control and determined by Students paired t-test. n=3***

Effect of Function Blocking Integrin Antibodies on IGFBP-5 Cell Migration

To understand the mechanism of inhibition of IGFBP-5 migration, function blocking integrins antibodies α2 and β1 were employed. Briefly, 50,000 MCF-7 cells/chamber were seeded into an insert at 10% FCS medium pre-incubated with and without 10µg/ml IGFBP-5. The concentration of integrins antibodies used were 50 µg/ml. Results showed that function blocking integrins antibodies α2- and β1- failed to inhibit the IGFBP-5 inhibition of migration and hence IGFBP-5 inhibition of migration is α2- and β1-integrins independent in MCF-7 cells (Fig. 3.3.11).

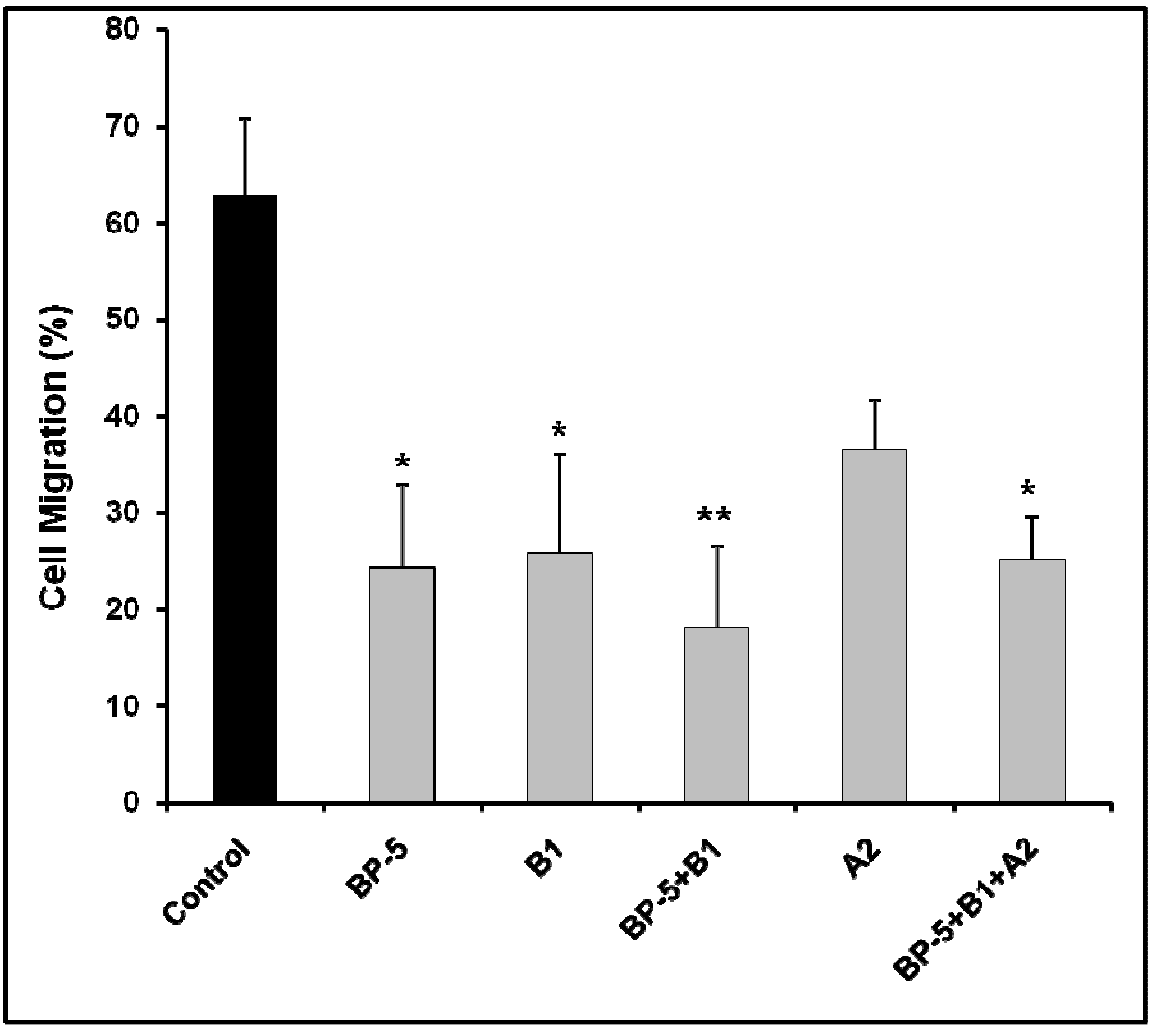


Fig. 3.3.11: ***Graph showing the IGFBP-5 inhibition of migration effect is independent of α2- and β1- integrins in MCF-7 cells. Function blocking integrins antibodies α2- and β1- failed to inhibit the IGFBP-5 inhibition action of migration. Values are mean ± SEM of 3-4 observations where * indicates p<0.05 and ** p<0.01 compared with control as determined by Student's paired t-test. n=3***

Effect of Kinase Inhibitors on IGFBP-5-induced Cell Adhesion

The intracellular mechanisms involved in the increased cell adhesion and survival induced by IGFBP-5 were examined using various kinase inhibitors. In order to study mechanism of action of IGFBP-5, effect of various kinase inhibitors for signalling pathways leading to cell proliferation, stress activation and smad signalling were used. For this purpose, MCF-7 cells were treated with various kinase inhibitors either alone or in the presence of IGFBP-5. The inhibitors were prepared in 0.1% DMSO and this was used as a control for cells not receiving any drug. After overnight culture, the cells in the wells were fixed, stained, solubilised and read spectrophotometrically at 540 nm. Interestingly, results showed that sphingosine kinase type I inhibitor prevented the effect of the cell: substratum adhesion property of IGFBP-5 in MCF-7 cells (Figures 3.3.12; 3.3.13 & 3.3.14).

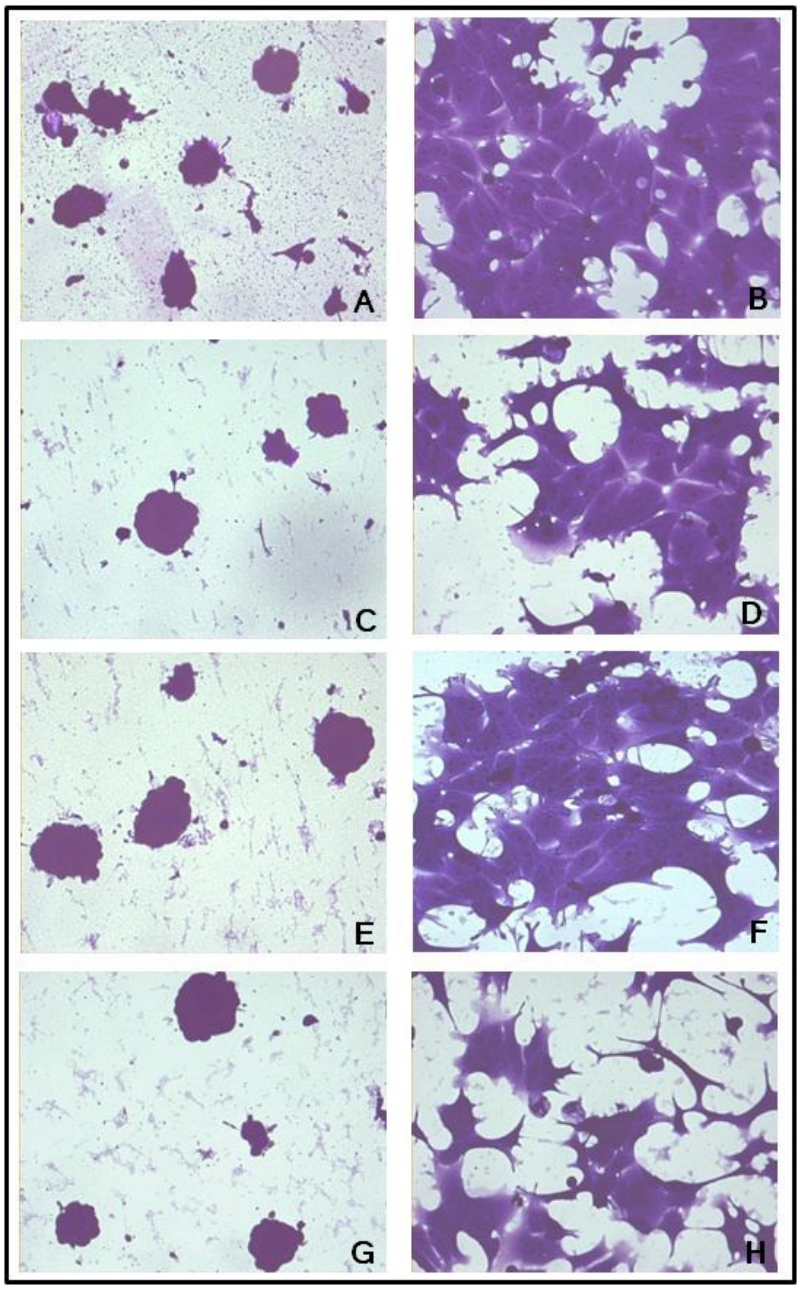


Fig 3.3.12a: ***Crystal violet stained images showing the effect of various kinase inhibitors on IGFBP-5 cell adhesion in MCF-7 cells. In 0.1% DMSO serum-free conditions, (A) Control MCF-7 cells without the addition of IGFBP-5 and/or drug. (B) MCF-7 cells in the presence of 10 ug/ml IGFBP-5 showing cell: substratum adhesion. (C) MCF-7 cells in the presence of LY294 002 (D) MCF-7 cells in the presence of LY 294 002 and IGFBP-5 (E) MCF-7 cells in the presence of PD 98059 (F) MCF-7 cells in the presence of PD 98059 and IGFBP-5 (G) MCF-7 cells in the presence of U 0126 (H) MCF-7 cells in the presence of U 0126 and IGFBP-5.***

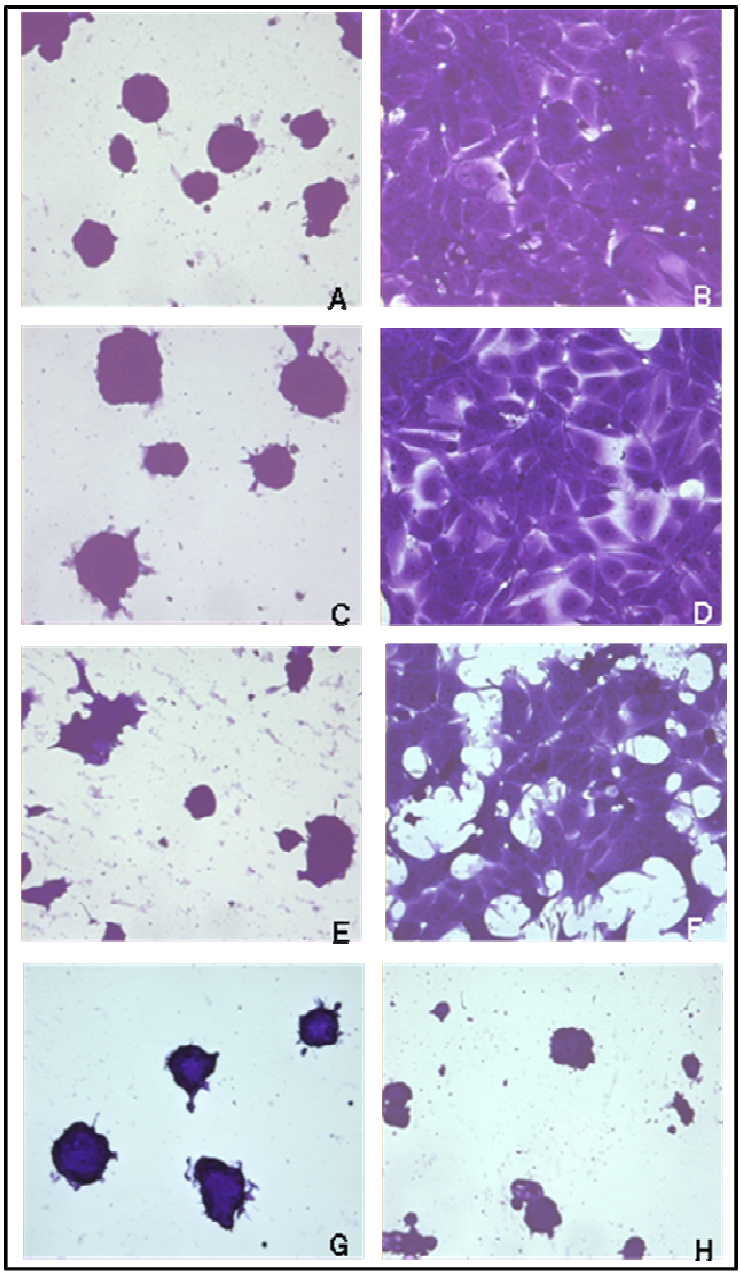


Fig 3.3.12b: ***Effect of various kinase inhibitors on IGFBP-5 cell adhesion in MCF-7 cells. In 0.1% DMSO serum-free conditions, (A) MCF-7 cells in the presence of SB 203580. (B) MCF-7 cells in the presence of 10 ug/ml IGFBP-5 and SB 203580. (C) MCF-7 cells in the presence of A 83.01. (D) MCF-7 cells in the presence of A 83.01 and IGFBP-5. (E) MCF-7 cells in the presence of SB 4131542 (F) MCF-7 cells in the presence of SB 4131542 and IGFBP-5 (G) MCF-7 cells in the presence of SK1 inhibitor (H) MCF-7 cells in the presence of SK1 inhibitor and IGFBP-5 showing the cell: substratum adhesion has been reversed.***

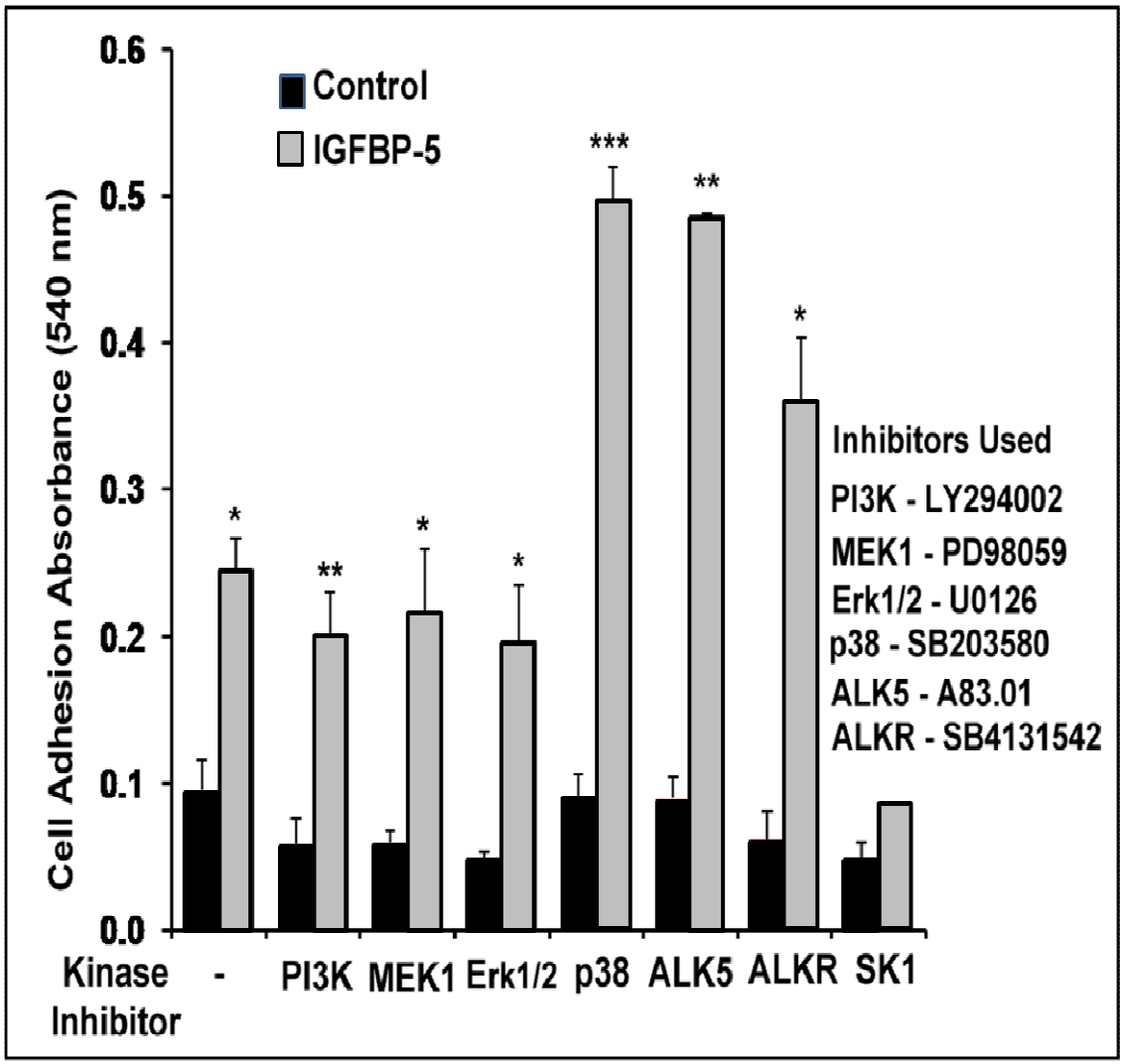


Fig 3.3.14: ***Quantified data showing the effect of various kinase inhibitors on IGFBP-5 cell adhesion in MCF-7 cells. SK1 inhibitor significantly decreased the effect of IGFBP-5 on cell adhesion. Values are means ± SEM of 9 observations. * p<0.05; ** p<0.01; *** p<0.001 as compared with control and determined by Student's unpaired t-test. n=3***

Gene Expression Profiling Studies of IGFBP-5-Adenoviral mediated Transduction

In order to further characterize the nature of the cell: substratum adhesion process, an array of 84 genes involved in cell: cell interactions and cell: extracellular matrix adhesion was used. To this end, super-array PCR system was employed to study the human extracellular matrix and cell adhesion gene expression profiling in IGFBP-5-transduced MCF-7 cells. The results suggested that IGFBP-5 inhibited expression of matrix metallopeptidase genes namely MMP1 and MMP2 by 3 and 1.9 fold respectively and collagen, type VII, alpha 1 (Col 7A1) by 2.4 fold and stimulated neural cell adhesion molecule 1 (NCAM 1) 7 fold.

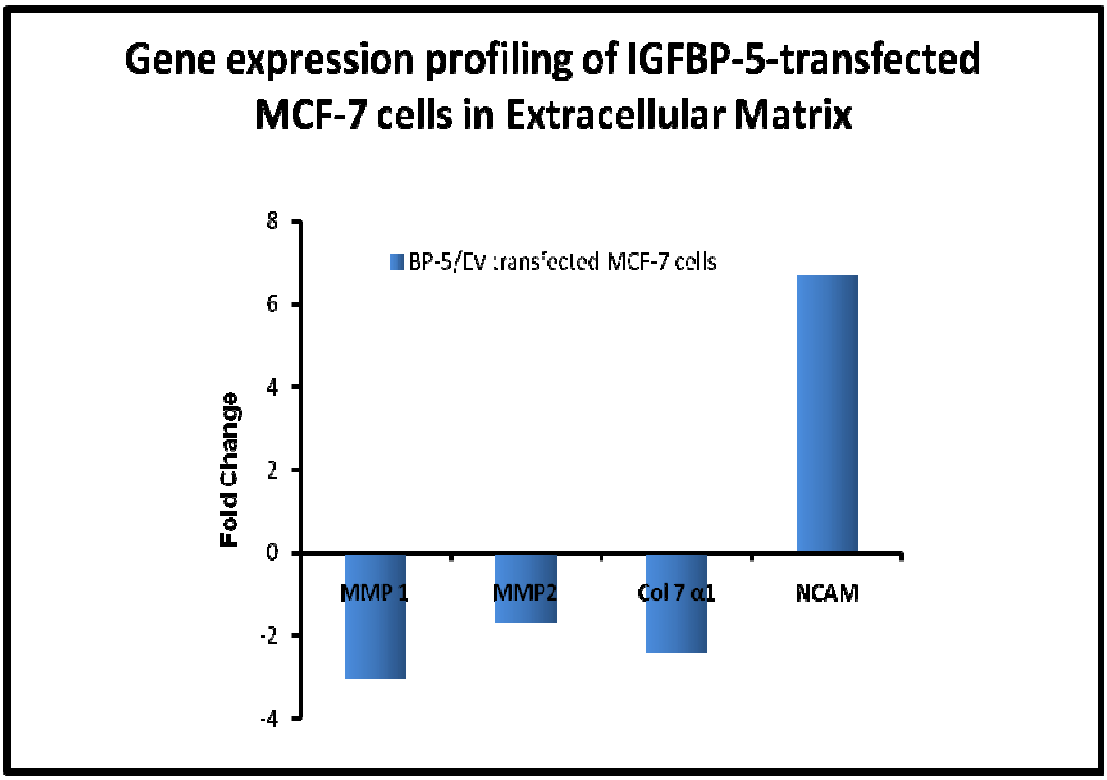


Fig 3.3.15: ***Fold Change of extracellular matrix and cell adhesion genes in IGFBP-5-transduced MCF-7 cells***.

Human Phospho Kinase Array Studies

To further characterize the intracellular signalling responses to IGFBP-5 and also to determine the relative levels of IGFBP-5 mediated protein phosphorylation profiles, we have employed a Human Phospho-Kinase Array Kit which contains 35 phospho kinases and 42 phosphorylation sites. Exogenous IGFBP-5 treated or adenovirus-transduced-IGFBP-5 MCF-7 cells were seeded at 1,000,000 cells/ml in triplicates. Cells were lysed at 6h and 48h after the treatment. Results showed several significant changes in response to IGFBP-5 treatment. After 6h there was a decrease in p38MAPK, which was consistent with the enhanced effects of IGFBP-5 which were evident when p38MAPK was inhibited. IGFBP-5 also induced a 4-fold increase in phosphorylation of p53 on S392 and a 3-fold increase on S46, although S15 was unaffected (Fig. 3.3.16).

After 48h, changes in p53 (S392) were still evident but decreased β-catenin phosphorylation. In addition there was a 2-fold increase in p70S6k (T389) and a dramatic 6-fold increase in phosphorylation of Akt on S473 but not on S308 (Figure 3.3.17). This phosphorylation pattern for Akt is consistent with activation by ILK or mTOR, rather than PI3-kinase. However, we found no evidence of activation of mTOR and thus our results suggest an integrin-mediated activation of this survival/ proliferation pathway. Similarly, despite the activation of Akt there was no evidence of activation of focal adhesion kinase (Fig. 3.3.17.). The differences in phosphorylation at early (6h) and later (48h) time intervals of phopho kinase array profiles would indicate the time dependent effects of IGFBP-5.

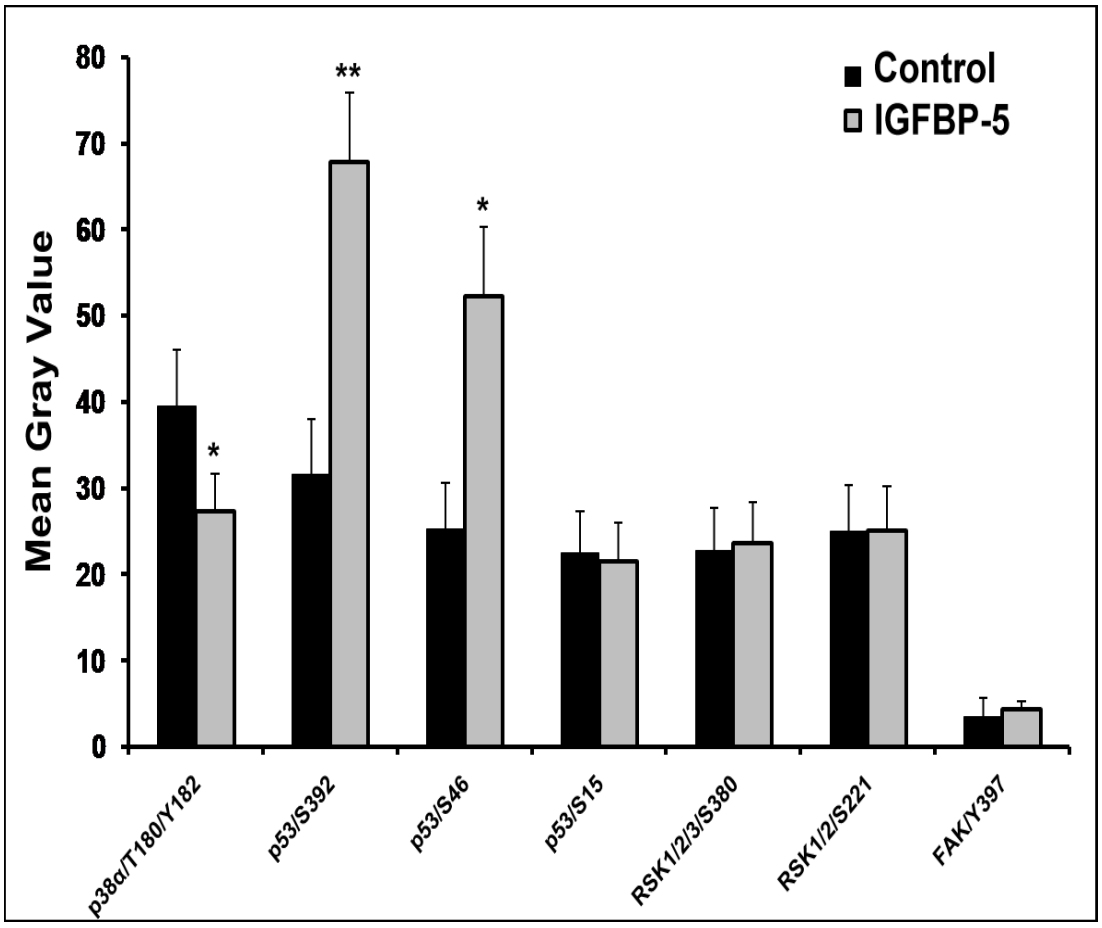


Fig. 3.3.16: ***Selected profile of* exogenous *IGFBP-5 treated phospho kinase array of MCF-7 cells after 6 h. Values are mean ± SEM of mean gray values of 4 observations where * indicated as p < 0.05; ** indicates p < 0.01 and *** p < 0.001 as determined by Students paired t-test. n=2***

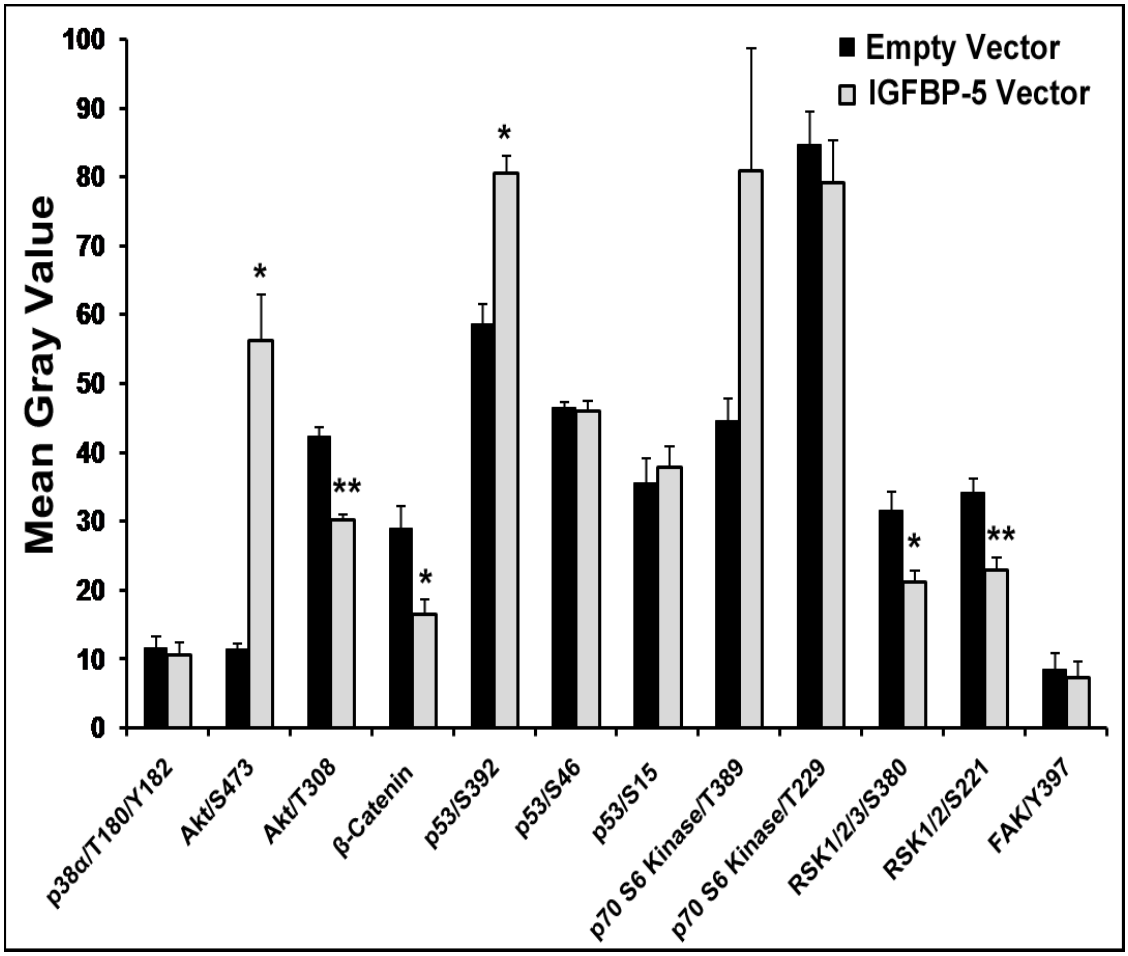


Fig. 3.3.17: ***Selected profile of Adenovirus transduced IGFBP-5 treated phospho kinase array of MCF-7 cells after 48 h. Values are mean ± SEM of 4 observations where * indicates p < 0.05; ** indicates p < 0.01 as determined by Students paired t-test. n=2***

Immunocytochemistry Studies

To examine whether or not IGFBP-5-treated MCF-7 cells have undergone epithelial-mesenchymal transition, expression of E-cadherin and N-Cadherin using immunocytochemistry was determined. Results showed that considerable expression of E-cadherin was evident even after IGFBP-5-induced cell: ECM adhesion (see Figures. 3.3.18 & 3.3.19).

To identify the molecular changes in IGFBP-5-treated MCF-7 cells, expression of proteins employing immunofluorescence staining was carried out in migration experiments. IGFBP-5 treated MCF-7 cells were stained with markers namely E-cadherin, Paxillin and Vinculin. Results indicate that IGFBP-5 maintains E-cadherin expression and reduces stress fibre formation at the wound edges (See figure 3.3.20). F-actin formation in IGFBP-5 treated MCF-7 cells appears to be realigned alongside the plasma membrane with smaller focal complexes. Whereas, control cells were found to be possessing large actin stress-fibre formation with abundant stress fibres crisscross in all the directions.

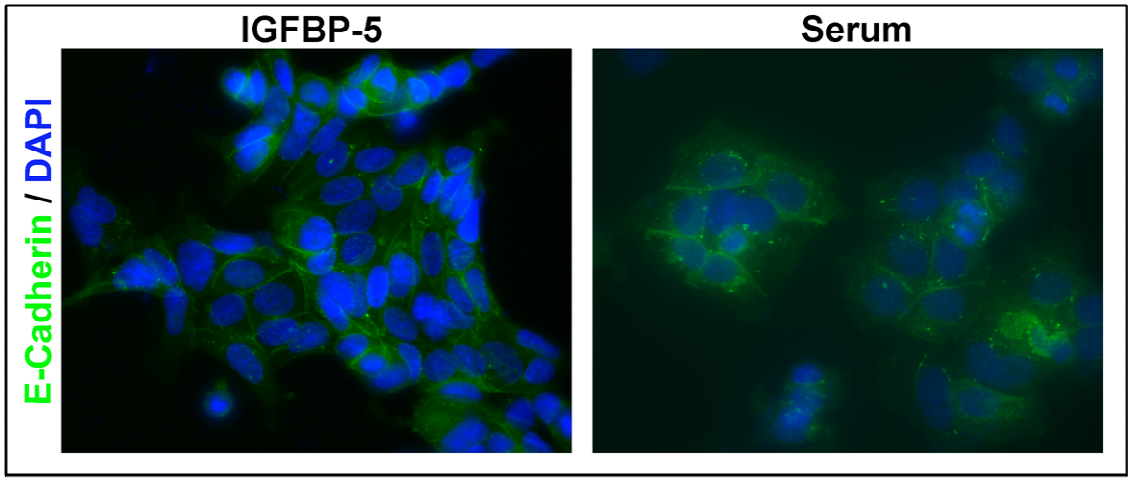


Fig 3.3.18: ***Immunofluorescence images showing the expression of E-Cadherin in IGFBP-5 treated MCF-7 cells. E-Cadherin is stained in green and nuclei were stained with blue. In serum treated MCF-7 cells, the expression of E-Cadherin was clearly evident. Images were photographed at 400x magnification.***

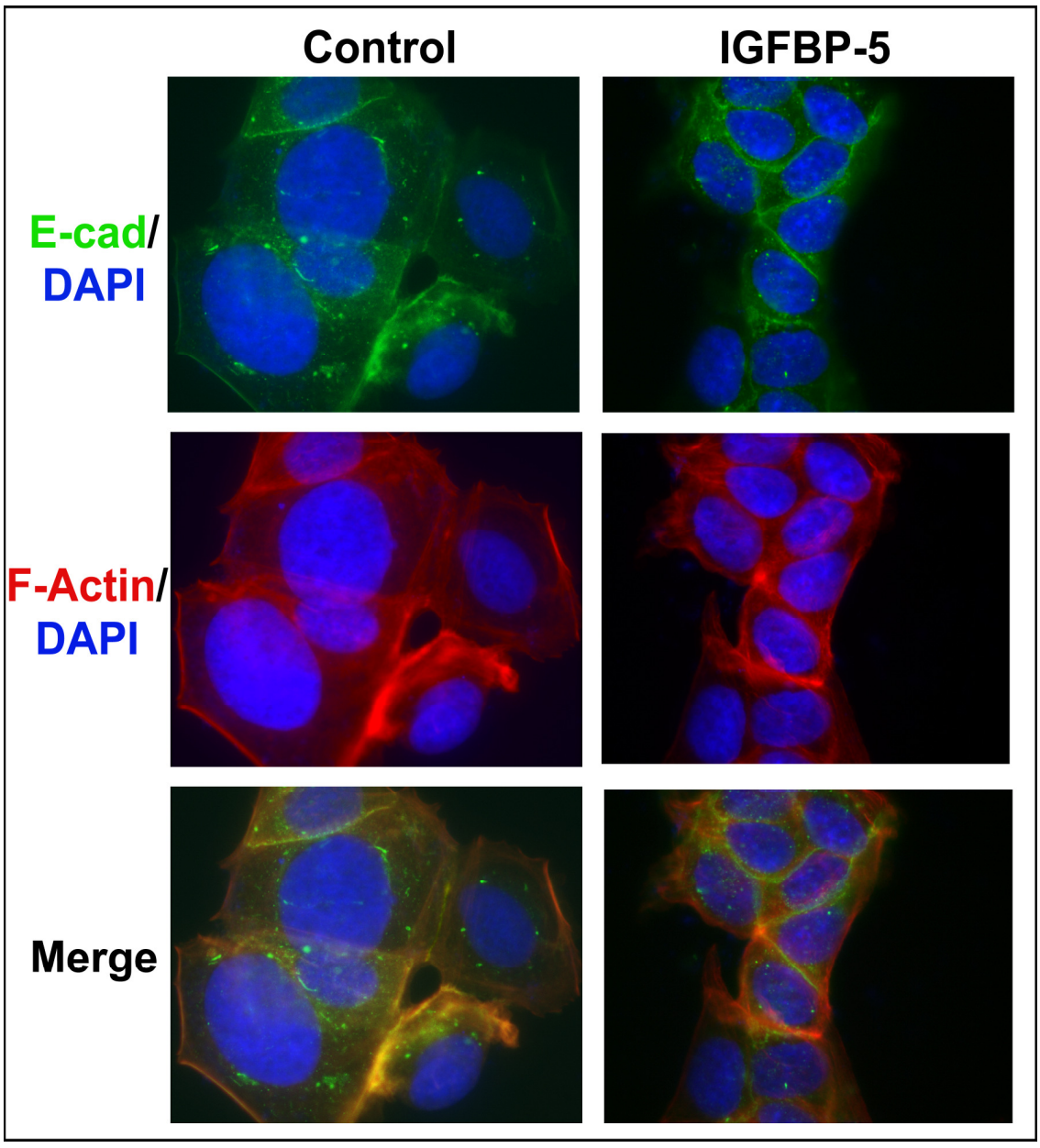


Fig: 3.3.19: ***Immunofluorescence images showing IGFBP-5-treated MCF-7 cells expressing E-Cadherin as compared to 10% FCS treated MCF-7 cells. Images were photographed at 1000x magnification.***

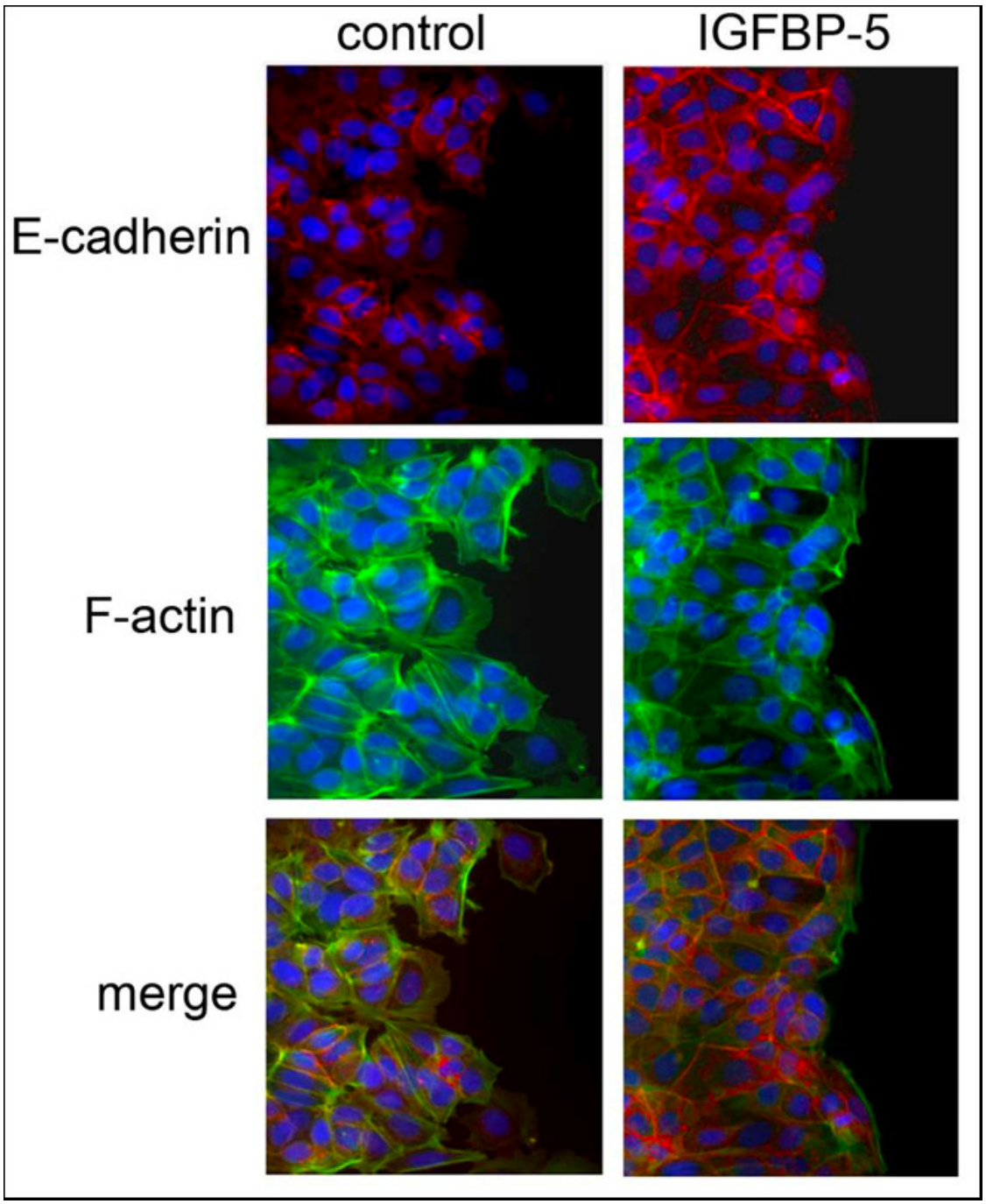


Fig 3.3.20: ***Immunofluorescence images showing IGFBP-5 maintaining expression of E-cadherin (Red) and reorganisation of F-actin (Green) at the ends of wound sites. Control cells were found to be having abundant stress-fibre formation at the wound regions. The merge image showing E-Cadherin expression and F-actin reorganization in IGFBP-5-treated MCF-7 cells and abundant stress fibre formation in control cells. Images were photographed at 200x magnification.***

In order to further examine the effect of IGFBP-5 on focal adhesions, expression of vinculin and paxillin was studied in IGFBP-5-treated MCF-7 cells. Results indicated that vinculin expression is evident in serum treated but not in IGFBP-5 treated MCF-7 cells. Whereas, paxillin expression is evident in both serum treated and in IGFBP-5 treated MCF-7 cells. And to further examine the expression of paxillin at wound sites, migration experiments were undertaken with and without the presence of IGFBP-5 in 10% serum. Results showed that IGFBP-5 treated MCF-7 cells exhibited paxillin expression alongside the wound sites with actin alignment as compared to migrating control cells with paxillin expression at the point of focal adhesions and with reorganisation of actin cytoskeleton.

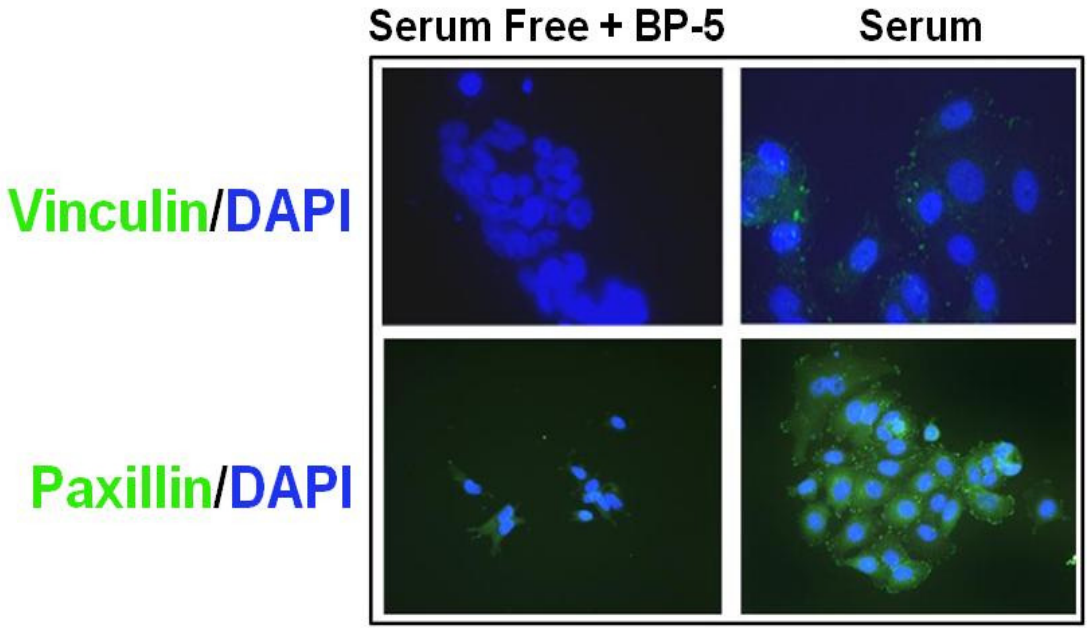


Fig 3.3.21: ***Immunofluorescence images showing the effect of IGFBP-5 on the expression of focal adhesions namely vinculin and paxillin in IGFBP-5 treated mCF-7 cells. Vinculin expression (green) is evident in serum-treated but not in IGFBP-5 treated MCF-7 cells. Whereas, paxillin expression (green) is evident in both IGFBP-5-treated and serum-treated MCF-7 cells. Images were photographed at 400x magnification.***

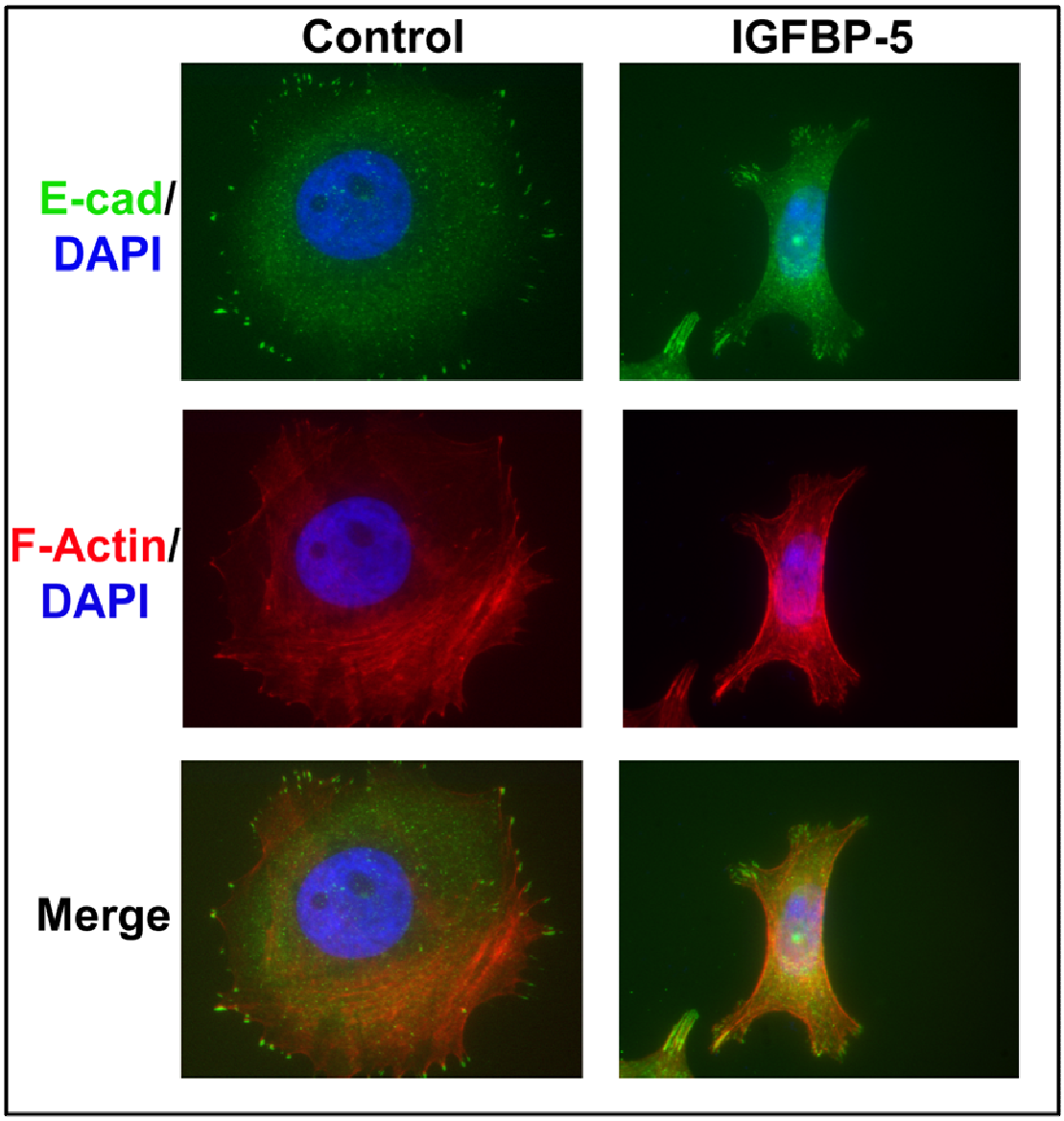


Fig 3.3.22: ***Immunofluorescence images showing the expression of paxillin and alignment of F-actin in IGFBP-5 treated MCF-7 cells. IGFBP-5 showed expression of paxillin at the 4- point focal adhesion and alignment of F-actin alongside of the plasma membrane. Serum treated MCF-7 cell showed abundant reorganization of actin cytoskeleton and paxillin expression at the sites of focal adhesion. Images were photographed at 1000x magnification.***

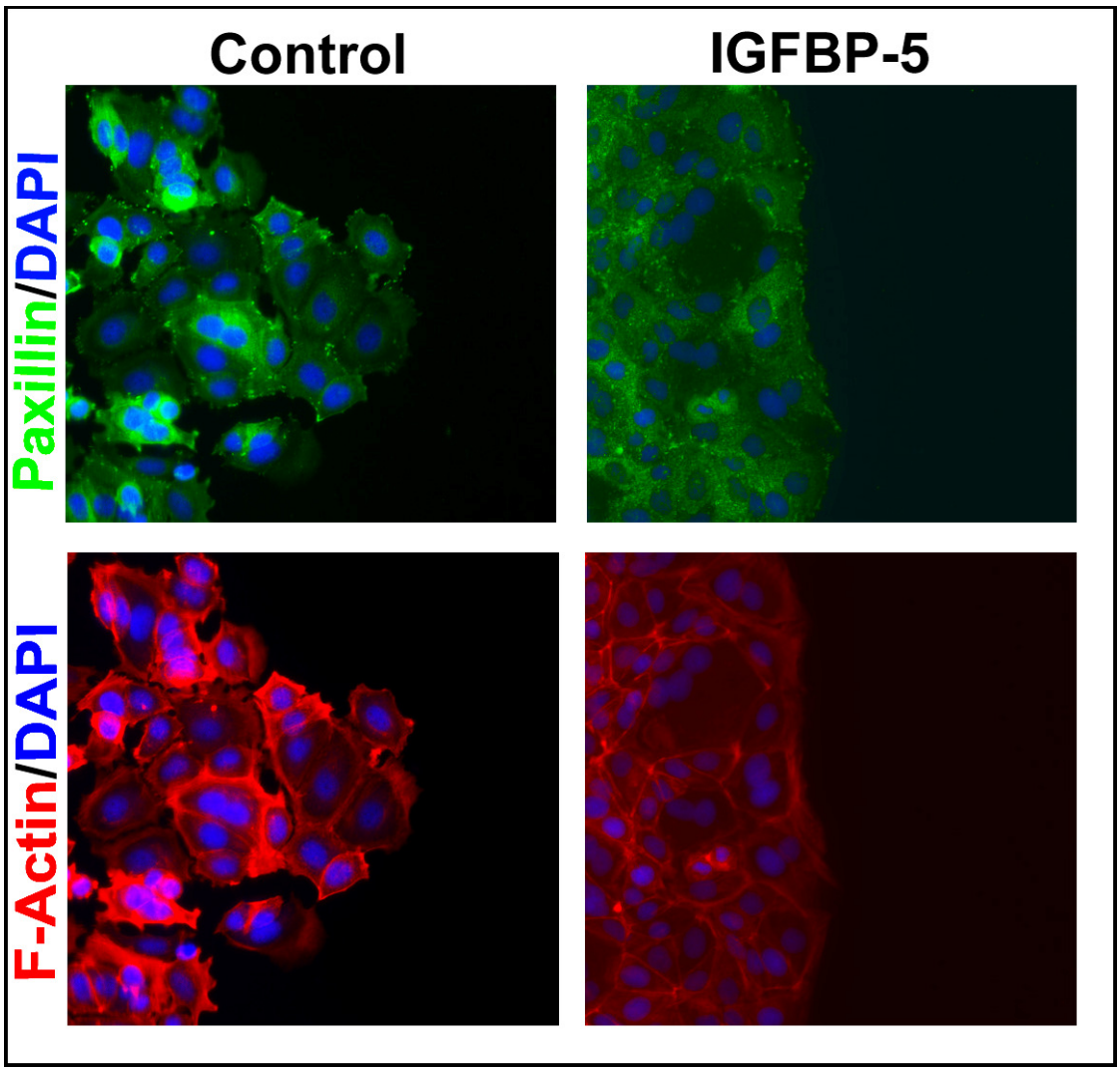


Fig 3.3.23*: Immunofluorescence images showing the expression of paxillin in IGFBP-5-treated MCF-7 cells. In both serum-treated and IGFBP-5 + serum treated MCF-7 cells, paxillin expression is evident. Stress fibres are evident and running criss cross in all directions in controls whereas, F-actin is organised along the plasma membrane in case of IGFBP-5 treated cells. Images were photographed at 200x magnification.*

Discussion

To study mechanistically the action of IGFBP-5 in human breast cancer cells, various basal environments were employed to assess the effects of IGFBP-5 on cell to substratum adhesion. Results showed that IGFBP-5-induced cell adhesion is promoted only in the presence of collagen and fibronectin but not in the presence of laminin in MCF-7 cells. This suggested that IGFBP-5-induced cell adhesion is only effective when epithelial cells are exposed to a mesenchymal environment. This supports the concept that this action of IGFBP-5 relates to either injury or activation of proteases, perhaps from tumour cells, resulting in break-down of the basement membrane, thereby exposing cells to a collagen- and fibronectin-rich environment. IGFBP-5-induced cell adhesion to extracellular matrix was observed only in the presence of laminin but not in the presence of collagen and fibronectin. In contrast, IGFBP-5 demonstrated increased cell adhesion in laminin but not in collagen and fibronectin in case of HS 578T cells. However, IGFBP-5 showed increased cell adhesion in all the basal substrata employed with highly metastatic breast cancer MDA-MB-231 cells. These observations suggest that IGFBP-5-induced cell adhesion is cell-type specific and possibly receptor specific is worth further study of interest.

Following the demonstration of increased cell: substratum adhesion and the involvement of IGFBP-5 in fibronectin production, the effect of IGFBP-5 on various α and β integrins expression was examined. The interaction of integrins, heterodimeric transmembrane glycoproteins with extracellular matrix is considered to be an important

factor in cell: cell and cell: ECM adhesion in tumour invasion and metastases (Jaskiewicz et al., 1993; Juhasz et al., 1993) For example, an increase in β1 integrin has been implicated in many cancer metastases (Schäfer and Werner, 2008).

The studies with function-blocking antibodies to integrins demonstrated that the cell: substratum adhesion action of IGFBP-5 was inhibited by antibodies to α2- and β1-integrins as well as (to a lesser extent) by αvβ6 integrin.

Results of α- and β- integrin-mediated cell adhesion assay in IGFBP-5-transduced MCF-7 cells rather surprisingly showed that IGFBP-5 decreased the levels of α-5, α-V, β1 integrin and αVβ5 (vitronectin receptor), α5β1 (fibronectin receptor) integrins. It was surprising to see a decrease in integrin expression when cell adhesion actually increased but this presumably indicates that MCF-7 cells are increasing adhesion through other integrins or other cell: ECM contact (Arias-Salgado et al., 2005; Levy et al., 2000). IGFBP-5 decreasing β1 integrin and fibronectin and vitronectin receptors indicates that it may be involved in limiting the metastatic potential and also it appears that is associated in the repair of epithelial cell injury and influence cell migration (Caswell et al., 2007; Cordes and Park, 2007; Yao et al., 2007).

In contrast, the α and β integrin-mediated cell adhesion assay in IGFBP-5-transduced HDFs demonstrated that IGFBP-5 significantly increased all of the integrins measured consistent with the suggestion that IGFBP-5 activates fibroblasts (Yasuoka et al., 2009a; Yasuoka et al., 2009b). In addition, IGFBP-5-transduced HDFs showed a difference in morphological appearance with elongated cytoplasmic tails and aligned appearance (Fig. 3.2.14) indicating activation of fibroblasts.

Activation of fibroblasts is one of the hallmark events of fibrosis. Other evidence that IGFBP-5 has been implicated in fibrosis comes from reports that IGFBP-5 interacts with matricellular proteins and monocyte activation (Yasuoka et al., 2009b), which are expressed during wound healing and metastasis and which play a role in cellular de-adhesion and migration.

In order to address the intracellular signalling pathways affected by IGFBP-5 was studied using a variety of inhibitors to various pathways including PI-3K, MAPK, MEK SK1 and Smads were used. Results showed that sphingosine kinase type I inhibitor significantly decreased the IGFBP-5-induced cell adhesion whereas TGF β 1 inhibitors namely A 83. 01 (ALK R) and SB 4131542 (ALK R) and p38 MAPK inhibitor SB 203580 significantly increased IGFBP-5-induced cell adhesion. These findings implicate a stress-activated pathway (p38MAPK) and also intriguingly suggest that TGF β may suppress the actions of IGFBP-5 in epithelial cells, may suppress TGF β1 actions, rather than acting in similar fashion as a pro-fibrotic agent in fibroblasts. Thus my findings have clearly demonstrated the potential of IGFBP-5. These differences were also confirmed in studies in which TGF β1 failed to mimic the induction of cell adhesion which occurred in response to IGFBP-5. Studies involving addition of both IGFBP-5 and TGF β1 may be useful to determine whether or not these molecules actually have antagonistic actions on epithelial cells.

Employing a Super-array PCR system, extracellular matrix and cell adhesion gene-expression profiling of IGFBP-5-transduced MCF-7 cells was assessed. Preliminary results showed that adenoviral expression of IGFBP-5 inhibited matrix metallopeptidases, namely MMP1 and MMP2 as well as collagen, type VII, alpha 1 (Col

7A1) and stimulated neural cell adhesion molecule 1 (NCAM 1). These changes are consistent with IGFBP-5 acting as an anti-migratory molecule (inhibiting MMPs) and pro-adhesion via NCAM-1, as well as restricting collagen secretion in the epithelial compartment. The effect of IGFBP-5 on these four genes will be studied in greater detail employing quantitative real time PCR using specifically designed primers.

After 6h, phosphorylation events of IGFBP-5-induced cell adhesion revealed a 4-fold increase in phosphorylation of p53 on S392 and a 3-fold increase on S46, although S15 was unaffected. After 48 h, 6 fold increase in Akt phosphorylation (S473) in addition to phosphorylation of p53 (S392) is evident. In both early and later time intervals, FAK phosphorylation is not observed. Further to this, there was a 2-fold increase in p70S6k (T389) phosphorylation and decreased β-catenin phosphorylation. This phosphorylation pattern for Akt is consistent with ILK or mTOR activation, rather than PI3-kinase. I showed increase in ILK expression and hence suggest an integrins-mediated activation of survival/proliferation pathway.

Following the IGFBP-5-induced cell adhesion and spreading, expression of certain important proteins in the process of cell adhesion were examined with the use of immunocytochemistry. E-Cadherin expression was found to be evident in IGFBP-5-induced cell adhesion of MCF-7 cells. Further, there was no indication of any N-Cadherin expression in IGFBP-5-treated MCF-7 cells (results not shown). This demonstrates that IGFBP-5 maintains adherens junctions by expressing E-Cadherin in MCF-7 cells making it unlikely for the cells to metastasize.

Chapter IV

General Discussion

The primary objective of the present study was to demonstrate the molecular mechanisms of action underlying novel cellular effects of IGFBP-5 involved in breast cancer metastasis, cell senescence and additional cellular responses in mesenchymal cells of relevance to debilitating diseases which include fibrosis as a component.

General Discussion:

In this study we investigated the actions of IGFBP-5 on signal transduction pathways in human mammary epithelial cells (MCF-7) which are weakly metastatic and represent an early stage of tumour development and progression. Here I have identified a novel role for IGFBP-5 in the induction of cell adhesion and spreading which could play an important part in the repair of epithelial tissues after injury. I demonstrated that IGFBP-5 induces cell to extracellular matrix adhesion, typically by a 3- or 4- point angular cell attachment using focal complexes and an effect which is dependent upon α2β1 integrins, and which invokes a survival signal at least in these cells. This cell to substratum adhesion was driven by the presence of collagen or fibronectin, components of the mesenchymal matrix but was markedly inhibited in the presence of laminin, the major component of the epithelial basement membrane. Whereas in HS 578T cells, the adhesion was found to be evident only in the presence of epithelial environment but not in the presence of mesenchymal environment. However, in case of MDA-MB-231 cells, the adhesion was found to be increased in the presence of both epithelial and mesenchymal environments consistent with its high metastatic characteristics. I also demonstrated that IGFBP-5 induces cell to extracellular matrix de-adhesion which eventually leads to cell death termed 'anoikis' in normal mammary epithelial cells

(Schafer et al., 2009). Further, Tonner and her co-workers demonstrated that IGFBP-5 induces programmed cell death in normal mammary epithelial cells of transgenic mice (Tonner et al., 2002). Although there is increased cell adhesion and spreading in MCF-7 cells, the expression of integrins namely α5- and β1- on the cell surface were found to be decreased but integrin α2- has showed no effect. In contrast, integrins α2-, β1- and to some extent αVβ6 were found to be essential for the action of IGFBP-5-induced cell adhesion in MCF-7 cells. In addition to this, using biosensor technology, we showed a novel, direct and high affinity interaction of IGFBP-5 with α2 and β1 integrins (results not shown).

In relation to this, IGFBP-5 showed a six fold increase in activation of Akt on S473 via increased integrin-linked kinase (ILK) expression rather than PI3-kinase which phosphorylates S308. As well as serving as an important survival signal, activation of Akt often leads both to proliferation signals and activation of FAK which, in turn, increases migratory potential. For example, both EGF and HGF have been shown to stimulate migration through a α2β1 integrin-mediated process (Maldonado and Furcht, 1995; Matsumoto et al., 1994). On the other hand, I demonstrated that IGFBP-5 significantly decreased cell migration in response to growth factors namely IGF, EGF and also serum. In addition to this, IGFBP-5 showed decrease in cell migration in response to serum in case of MDA-MB-231 cells. However, in agreement with studies in HUVECs, I have showed that IGFBP-5 induced a sustained phosphorylation of p53 in MCF-7 cells. p53 is known to inhibit cell cycle progression and such an effect of IGFBP-5 on HUVECs, with increased numbers of cells in G1 phase of the cell cycle, has been demonstrated (Kim et al., 2007). Of relevance to this study, p53 also inhibits

phosphorylation of FAK (Golubovskaya et al., 2008). We thus propose that activation of p53 by IGFBP-5 prevents both proliferation and migration of MCF-7 cells. Consistent with this, IGFBP-5 induced smaller focal complexes with considerable expression of paxillin and very little or no expression of vinculin in serum-free conditions when compared with the induction of large focal adhesions, with high levels of paxillin and vinculin expression produced by serum. However, expression of paxillin found to be evident in IGFBP-5 treated MCF-7 cells in both serum free and serum concentrations. This is followed by the association of integrin signaling associated proteins called adhesomes including ILK, paxillin, α- Parvin and other adaptor proteins (yet unidentified) at the cytoplasmic domains of α2- and/or β1- interest is an area of worthy of further investigation (Knoll et al., 2007; Legate et al., 2009; Streuli and Akhtar, 2009). Thus, IGFBP-5 forms smaller focal complexes and appear to be filopodia and such adhesions normally involve the small GTPase cdc42 rather than Rho or Rac (Krugmann et al., 2001; Nobes and Hall, 1995). A similar, cdc42-dependent stimulation of adhesion by IGFBP-5, has been described in neuronal cells (Abrass and Hansen, 2010; Berfield et al., 2000).

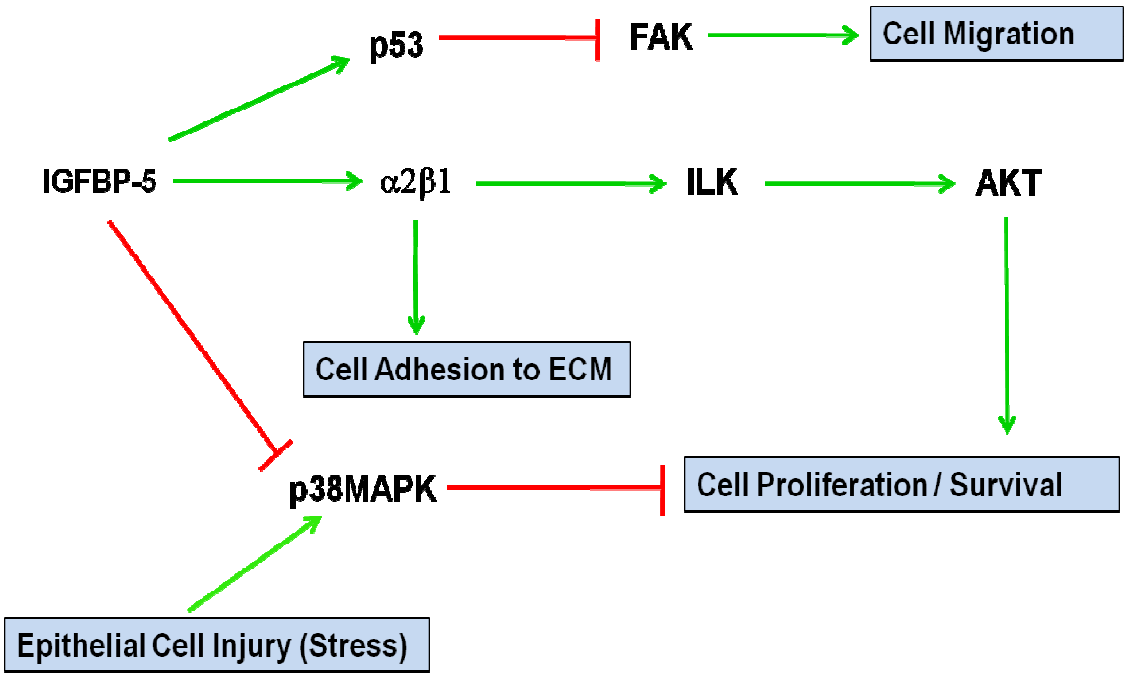
Serum starvation induces a stress response in cells which includes phosphorylation of p38MAPK (Lu et al., 2008). Interestingly, IGFBP-5 rapidly suppressed this response and inhibition of p38MAPK with a drug further increased cell to substratum adhesion. Whether this represents the inhibition, or limitation, of an apoptotic pathway is unclear but combined with the integrin-dependent activation of Akt, inhibition of p38MAPK would provide a powerful survival stimulus. The strength of this pro-survival signal was evident from the impressive ability of cells to remain adherent in nutrient-depleted

conditions for at least 14 days, whereas control cells underwent cell detachment through anoikis from as early as 8-10 days. Taking these findings, along with those in vascular endothelial cells HUVECs, there is mounting evidence for a role of IGFBP-5 in the cellular response to stress, one in which IGFBP-5 attenuates the stress response and enhances adhesion-mediated cell survival. By inhibiting proliferation and cell migration these cells are retained at the site of the insult or in this case epithelial injury and are potentially converted to senescent cells and able to spread and thereby potential to eliminate apoptotic cells and reduce stress in the surroundings in a hostile wound environment. These secreted molecules could thus act upon neighbouring epithelial cells, or indeed more distal fibroblasts, as has been demonstrated in fibrosis. Evidence is now accumulating which suggests that the senescence messaging secretome (SMS) plays an important tumour suppressor role in this manner (Adams, 2009).

In relation to cell surface integrin expression, IGFBP-5 did not increase the amount of $\alpha 2\beta 1$ integrins on the cell surface so the manner by which it influences integrin adhesion/activation is of obvious importance in understanding its mechanism of action. ILK has been shown to phosphorylate $\beta 1$ integrin and phosphorylation of $\beta 1$integrin has, in turn, been shown to promote adhesion but inhibit directed migration in fibroblasts and teratocarcinoma cells (Mulrooney et al., 2001). This aspect of possible integrin activation and or recycling is worth of further study in relation to the actions of IGFBP-5 particularly inhibition of cell migration in MCF-7 cells.

These results are consistent with the hypothesis that IGFBP-5 can induce epithelial cell adhesion to exposed mesenchymal environments and that this may be important to aid

re-epithelialisation. In circumstances where this cannot occur, IGFBP-5 secretion from epithelial cells undergoing apoptosis may activate fibroblasts either directly (stimulating integrins expression and fibronectin and collagen secretion) or indirectly by secreting fibronectin from epithelial cells, which aids/directs fibroblast migration and to allow scarring (fibrosis) to occur. Thus IGFBP-5 may act as a central mediator in the initial response of epithelial cell injury. These wide-ranging responses could be anticipated to enhance re-epithelialisation during injury by increasing the surface area of epithelial cells when epithelia are exposed to a mesenchymal environment, whilst concurrently inhibiting their migration into the mesenchymal compartment. Nonetheless, there is still much to learn about the interactions between IGFBP-5 and integrins and their downstream signalling pathways in the epithelial repair regulation and its contribution as a pro-fibrotic agent in idiopathic pulmonary fibrosis.



In conclusion, these findings suggest that IGFBP-5 induces cell to extracellular matrix adhesion involving α2β1 integrins by an IGF-I independent mechanism and increased expression of ILK and also Akt and p53 phosphorylation and decreased p38MAPK phosphorylation which results in increased cell survival and inhibition of cell migration. Taken together, I have described an integrin mediated cell survival pathway induced by IGFBP-5 which involves p53 activation. To date there is no evidence as to how IGFBP-5 activates p53 and understanding this process has obvious implications for driving cellular senescence or autophagy. Whilst I have demonstrated that IGFBP-5 activates p53 and is a potent inhibitor of cell migration, I have also shown that the response to IGFBP-5 included the maintenance of E-cadherin expression in cells at the extreme edges of the wound site. E-cadherin is a well-known inhibitor of cell migration and is consistently down-regulated during metastasis (Makrilia et al., 2009). I believe that this action of IGFBP-5 leads to an adhesive, anti-migratory phenotype, and, as such, IGFBP-5 may on its own provide important insights into limiting or control of the metastatic process. Thus IGFBP-5 may be a more relevant target than TGFβ1 in therapies of wound repair, since it enhances fibrosis, whilst simultaneously limiting scar formation to the mesenchymal compartment, thereby preventing excessive disruption of the epithelium. In summary, IGFBP-5 serves to enhance epithelial cell adhesion and spreading over mesenchymal matrix whilst inhibiting epithelial invasion into the mesenchyme at wound sites in an integrin-dependent manner.

Future Directions:

- IGFBP-5 delaying the increase in β-galactosidase activity at pH 4 and its mechanism could be further investigated and the downstream mediators of IGFBP-5 on lysosomal activity in IGFBP-5-transduced MCF-7 cells is worthwhile study of interest.

- If time permitted, expression and purification of recombinant IGFBP-5 mutants would have been undertaken in order to examine the mechanism of IGFBP-5 inhibition of cell migration which might be involved in other domains in human breast cancer cells.

- If time permitted, interactions of IGFBP-5 with matricellular proteins, namely osteopontin, thrombospondin, tenascin -X and -C would have undertaken.

Chapter V

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Abbreviations

Ad-IGFBP-5	Adenovirus expressing IGFBP-5
ACS	Accelerated Cellular Senescence
ALS	Acid-labile subunit
ATCC	American Type Culture Collection
CTGF	Connective Tissue Growth Factor
CYR61	Cysteine-rich heparin-binding protein 61
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FCS	Fetal Calf Serum
bFGF	Basic Fibroblast Growth Factor
GH	Growth Hormone
GJ	Gene Jammer Transfection Reagent
GSK	Glycogen synthase kinase
HUVECs	Human Umblical Vein Endothelial Cells.
HDFs	Human Dermal Fibroblasts
IGF	Insulin-like Growth Factor
IGFBPs	Insulin-like Growth Factor Binding Proteins
IGFBP-rps	Insulin-like Growth Factor Binding Protein Related Proteins
IGFR	Insulin-like Growth Factor Receptor
IGF-IIR	Type II – Insulin-Like Growth Factor Receptor
IGF-IR	Type I – Insulin-Like Growth Factor Receptor
ILK	Integrin-Linked Kinase
IPF	Idiopathic Pulmonary Fibrosis
LB	Lysogeny Broth or Luria Broth medium
M.O.I.	Multiplicity of Infection
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated Protein Kinase
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NLS	Nuclear Localization Sequence
OIS	Oncogene-induced Senescence
OPN	Osteopontin
PDGF	Platelet derived growth factor
PI3K	Phosphotidyl inositide 3-kinase
pRB	Retinoblastoma protein
PTEN	Phosphotase and Tensin homologue deleted from Chr. 10
RIPA	Radioimmunoprecipitation assay buffer
RS	Replicative Senescence
RT-RT-PCR	Reverse Transcription Real-Time PCR
STAT 3	Signal transducer and activator of transcription 3
TDLU	Terminal Duct Lobular Unit
TGF-β	Transforming Growth Factor - Beta
TNF	Tumour Necrosis Factor
TSP	Thrombospondin
VEGF	Vascular Endothelial Growth Factor
005	C-terminal domain of IGFBP-5
500	N-terminal domain of IGFBP-5
555 or wt IGFBP-5	Full length IGFBP-5

List of Publications

Publications

1. **Sureshbabu A.,** Okajima H., Yamanaka D., Tonner E., Shastri S., Maycock J., Szymanowska M., Shand J., Takahashi S-I., Beattie J., Allan G., Flint D (2010). IGFBP-5 induces cell adhesion, increases cell survival and inhibits cell migration in human breast cancer cells. ***Cell Res.*** Under Review

2. **Sureshbabu A.,** Allan G., Flint D (2011). Relative roles of IGFBP-5 and TGF-β in Idiopathic Pulmonary Fibrosis. ***BMC Pulm Med.***

3. **Sureshbabu A.,** Okajima H., Yamanaka D., Shastri S., Tonner E., Rae C., Szymanowska M., Shand J., Takahashi S., Beattie J., Allan G., Flint D. (2009). IGFBP-5 induces epithelial and fibroblast responses consistent with the fibrotic response. ***Biochemical Society Transactions***, 37(4): 882 - 5.

4. **Sureshbabu V.S. Angara,** Tapan Kumar Barik, I. Namita, I. Prem Kumar (2008). Radioprotective properties of *Hippophae rhamnoides* (sea buckthorn) extract *in vitro*. ***International Journal of Health Sciences***, Qassim University, 2(2): 27 - 34.

Invited Talks

- ***The Physiology Society Conference:*** Presented a scientific talk and poster **"IGFBP-5 promotes cell survival by inducing cell: ECM adhesion and by inhibiting cell migration through α2β1 integrin-dependent manner in human breast cancer cells"** on Young Physiologists Symposium "Physiology and Disease: Advances and Perspectives" at University of Manchester from 28th - 29th June, 2010.

- ***Biochemical Society Conference*:** Presented a scientific talk and poster titled **"IGFBP-5 induces epithelial and fibroblast responses consistent with a fibrotic response"** at Biochemical Society Conference on **"Biochemical Basis on Respiratory Diseases"** at Astrazeneca, Loughborough, United Kingdom from 5th - 6th March, 2009.
  http://www.biochemistry.org/Portals/0/Conferences/abstracts/SA098/SA098P013.pdf

Selected Poster Presentations

- ***International Beatson Cancer Centre Symposium on Cancer Research:*** Presented a poster **"IGFBP-5 promotes cell survival by inducing cell: ECM adhesion and by inhibiting cell migration through α2β1 integrin-dependent manner in human breast cancer cells"** at Beatson Institute for Cancer Research, Glasgow, June 18th, 2010.

- ***Gordon Research Conference:*** Presented a scientific poster **"IGFBP-5 induces cell adhesion and spreading, increases cell survival and inhibits cell migration in human breast cancer cells"** on **Mammary Gland Biology** at Lucca (Barga), Italy, June 6 - 11, 2010.

- ***University of Strathclyde Research Day:*** Presented a scientific poster **"Fibrosis: Premature Ageing of Multiple Organs"** at Barony Hall, University of Strathclyde, Glasgow, 17th June, 2009.

- ***The Physiological Society Conference:*** Presented a scientific poster **"IGFBP-5: A Key Molecule with Novel Effects in Fibrosis and Cancer Metastasis"** at Physiological Society Conference focusing on **"Young Physiologists Symposium on Physiological Signalling: Genes to Function"** at Firth Hall, University of Sheffield, United Kingdom from 6th - 7th April, 2009.
  http://www.physoc.org/site/cms/contentviewarticle.asp?article=886