MOLECULAR REGULATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-5 BY SIGNALING MOLECULES DOWNSTREAM OF THE IGF-I RECEPTOR IN MAMMARY EPITHELIAL CELLS

by

JEFFREY ALAN BRANDIMARTO

A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Microbiology & Molecular Genetics

written under the direction of

Dr. Wendie S. Cohick

and approved by

_____

_____

_____

New Brunswick, New Jersey

[January, 2009]

ABSTRACT OF THE THESIS

Molecular regulation of insulin-like growth factor binding protein-5 by signaling molecules downstream of the IGF-I receptor in mammary epithelial cells

By JEFFREY ALAN BRANDIMARTO

Thesis Director:
Dr. Wendie S. Cohick

The insulin-like growth factor binding proteins (IGFBP) are important regulators of mammary epithelial cell (MEC) growth and can either enhance or inhibit IGF-I action. IGF-I activates the IGF-I receptor (IGF-IR) to initiate two well-characterized signaling pathways; the phosphoinositide 3-kinase pathway (PI3K) and the mitogen activated protein kinase (MAPK) pathway. In the bovine MEC line MAC-T, the PI3K pathway is required for both basal and IGF-I stimulated IGFBP-5 expression. In contrast, inhibition of the MAPK pathway with the chemical inhibitor PD98059 leads to an increase in IGFBP-5 expression in both basal and IGF-I treated conditions. In the present study, we identified molecules downstream of the IGF-IR that might play a role in the inhibitory regulation of IGFBP-5 expression via the MAPK pathway.

The MAPK pathway terminates with the activation of ERK1/2. Activated ERK1/2 enters the nucleus where it affects numerous transcriptional factors. ERK1/2 has been shown to inhibit activation of the peroxisome proliferator-activated receptor gamma (PPARγ). Inhibition of PPARγ with the chemical inhibitor GW9662 led to a decrease in IGFBP-5 message in PD98059-treated MAC-T. Activation of PPARγ and PPARβ/δ via

the agonists Rosiglitazone and GW0742, respectively, was found to increase basal IGFBP-5 mRNA expression in murine MEC but not in MAC-T cells.

While PPARγ contributed to the PD98059-stimulated increase in IGFBP-5 message, it was unable to account for the total increase. We therefore examined the promoter region of IGFBP-5 to identify factors that could be affected by mitogens. Both mouse and human IGFBP-5 promoters contain a consensus NFκB binding site. In the present study, phenethyl caffeiate, an inhibitor of NFκB, almost completely inhibited the increase in IGFBP-5 observed with PD98059 and IGF-I + PD98059-stimulated IGFBP-5 mRNA and protein expression in MAC-T cells. Interestingly, IGFBP-3 expression was inversely regulated by phenethyl caffeiate.

In conclusion the synergistic increase in IGFBP-5 expression observed with IGF-I and inhibition of the MAPK pathway may be due to the formation of a PPARγ – NFκB complex that binds to the promoter region of IGFBP-5. Rapid ERK dephosphorylation has been reported in involution, therefore this regulation may be important in inducing IGFBP-5 during involution in the bovine mammary gland.

Table of Contents

List of illustrations

Chapter One

Review of Literature

THE IGF SUPER-FAMILY

General Overview of the IGF Super-family

The IGF super-family consists of two peptide growth factors (IGF-I and IGF-II), two cell surface receptors (IGF-IR and IGF-IIR), and six soluble IGF-binding proteins (IGFBP-1–6). Interactions among members of the family modulate growth and development (1).

IGF-I and IGF-II are mitogenic peptides that bind the IGF-IR, ultimately causing stimulation of cell growth and inhibition of cell death (2, 3). Regulation of IGF-I has a substantial role in the differentiation and growth of many tissues such as bone and the mammary gland (4, 5). Moreover IGF-I has been shown to affect aging; overexpression of IGF-I in mouse heart cells alleviated aging-induced cardiac contractile abnormalities, reducing age-associated degeneration of heart tissue (6).

IGF-I is expressed ubiquitously in tissue, although in a highly regulated manner. Locally secreted IGF-I can either enter the blood stream or act in a paracrine factor to act on nearby tissue. The main source of IGF-I found in circulation is the liver; hepatic IGF-I CRE/lox knockout mice demonstrated a dramatic reduction in circulating IGF-I levels (7). The total concentration of IGF-I and -II in human blood is about 100 nmol per liter, which is 1000 times greater than the concentration of circulating insulin (8, 9). Circulating IGF-I levels are hormonally controlled by the growth hormone/IGF-I axis (10). Growth hormone (GH) is a peptide hormone synthesized and secreted by the anterior pituitary gland. GH binds the growth hormone receptor (GHR) which increases IGF-I production (11), conversely, decreases in both insulin and IGF-I production were

shown by the deletion of GHR (10, 12, 13). Moreover there is support that increased IGF-I can cause negative-feedback, leading the pituitary to inhibit GH secretion (14).

High concentrations of circulating IGF-I are made possible by an array of six IGFBPs. A major function of IGFBPs is to bind and regulate the actions of IGF-I (9, 15-18). IGF-I/IGFBP complexes function to maintain stable pools of bioactive IGF-I, increasing the half-life of IGF-I by sequestering IGF-I in circulation. IGFBPs prevent IGF-I from binding to the IGF-IR, thus inhibiting cell proliferation, differentiation, survival, and other IGF-stimulated signaling events (19). The IGFBPs bind IGF-I at high affinity (K_D = 0.1 nM); this high binding affinity prevents IGF-I from binding to the IGF-IR, which has a lower affinity for IGF-I (K_D= 1 nM). Like IGF-I, the IGFBPs are secreted by many different cell types with regulation of individual IGFBPs varying in a cell- and tissue type-dependent manner.

Greater than 95% of IGF-I in circulation is bound to IGFBPs. IGF-I is carried in ternary complexes of 150 kDa consisting of one molecule each of IGF, IGFBP-3 or IGFBP-5, and acid-labile subunit (ALS). Each component of the complex is necessary to maintain IGF-I levels in circulation. Transgenic mice produced by a single IGFBP knockout have been created, however they do not show phenotypic changes in general growth rate, most likely due to the redundancy of the other IGFBPs. A triple knockout for IGFBP-3, -4 and -5 in adult mice lead to significant growth reduction (78% of wild type), decreased fat pad accumulation with lower circulating levels of total IGF-I (45% of wild type), and decreased IGF-I bioactivity (37% of wild type) (20). ALS knockout mice have reduced circulating IGF-I and a 90% reduction in circulating IGFBP-3 (21).

The IGF-IR is a heterotetrameric trans-membrane protein complex that functions as a tyrosine kinase receptor (22). Upon binding IGF-I, the IGF-IR intrinsic tyrosine kinase activates intracellular signaling pathways that include the mitogen activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K) pathway, both of which enhance growth and survival (23). Moreover the IGF-IR can dimerize with other receptors including the insulin receptor (IR) and epidermal growth factor receptor (EGFR) (24, 25). Increased levels of IGF-I in the blood are associated with an increased risk of breast cancer, and overexpression of IGF-IR and its ligands has been associated with the development and maintenance of breast cancer (26, 27).

IGF-II can bind to the IGF-II receptor (IGF-IIR), a large 280 kDa single transmembrane protein with no homology to IGF-IR or IR (28). The IGF-IIR is also known as the mannose 6-phosphate receptor. The receptor is ubiquitously expressed, its major known function is to transport lysosomal enzymes (28). Its influence on other signaling pathways is not well defined.

Molecular Design of the IGF Super-family

IGF-I is composed of four domains (A, B, C and D) that form a single 71 amino acid complex held together through three intramolecular disulfide bonds (29). The A and B domains make up the bulk of the molecule and are connected by the C domain (29). Similarly IGF-II consists of a 67 amino acid single polypeptide chain with similar domains, 58% of which are identical to IGF-I (29). The amino acid sequences of insulin**,** IGF-I and IGF-II from all vertebrate groups currently known share a high degree of similarity (30).

The six IGFBPs vary in length from 201 to 289 residues, after post-translational modifications they all fall in the range of 24–44 kDa. IGFBP members have a conserved cysteine-rich N-terminal domain, share homology in the C-terminal region, but vary in the mid-region of the protein.

Origins of the IGF Super-family

The name insulin-like growth factor is derived from the fact that the growth factors and the IGF-IR are similar to insulin and the IR, respectively. An ancestral duplication of an insulin-like predecessor gave rise to insulin and the insulin-like factor, a subsequent duplication of the insulin-like gene developed into the two modern day factors IGF-I and IGF-II (29). The A and B domains of IGF-I are nearly fifty percent identical to the A and B chains of insulin (31). The primary structures of the IR and IGF-IR proteins share a high degree of similarity (32). The overall similarity between both receptors is 58%, the highest similarity (84%) between both molecules is found in the tyrosine kinase domain of the β subunit (32). Although the receptors are similar to one another, each ligand exhibits high-affinity binding to its cognate receptor (32).

The ancestral origins of the IGFBP genes appear to be linked to the homeobox (HOX) genes. Homebox is a 180 bp gene sequence that encodes DNA binding proteins that are associated with morphogenesis (33). IGFBP-1 and -3 are localized next to each other on chromosome 7 in humans, 20 million basepairs downstream is the HOXA cluster. Similarly, IGFBP-2 and IGFBP-5 genes are located in the same chromosomal region as the HOXD cluster. IGFBP-4, which is not paired with another IGFBP, is found near the HOXB genes. The final set of HOX genes, the HOXC cluster, is in the vicinity of IGFBP-6. The pattern of these gene locations in relation to one another indicates that

they were derived from the same chromosome. This suggests that this portion of the chromosome was linked prior to the initial duplication event, furthermore that additional duplications have taken place (34). The origins of IGFBP predate mammalian evolution, and are present even in the serum of reptiles and bony fish (30).

Cell Signaling in the IGF Super-family

IGF-I binding to IGF-IR induces phosphorylation of three cytoplasmic tyrosine residues, recruitment of adapter molecules and initiation of signaling cascades. The activated IGF-IR initiates two well characterized signaling pathways; the PI3K is initiated by the phosphorylation of the insulin receptor substrates (IRSs), and the MAPK via the adaptor molecules SHC and GRB2. In addition significant cross-talk can occur between these two pathways. Dysregulation of these pathways is often responsible for the aggressive phenotype of breast cancer (24, 35, 36).

MAPK Pathway

Activated SHC recruits the guanine nucleotide exchange factor SOS/GRB2 complex to the plasma membrane. The change in position brings the SOS/GRB2 into contact with Ras, where Ras is converted from the GDP-bound state to the GTP-bound state. The GTP-bound form of Ras is needed for proliferation in most cell types (37), however over-expression or failure to deactivate the Ras-GTP complex is common in tumors (37). Activated Ras leads in turn to the activation of a cascade of serine/threonine kinases (Raf and Raf-like kinases).

Raf and other kinases phosphorylate MAPK kinase one and two (MEK1/2). MEK-1 and -2 are dual-specificity protein kinases that function in the MAPK cascade controlling cell growth and differentiation (38-40). MEK1/2 can be activated by growth

factors, cytokines, and influxes of calcium (40). MEK1/2 in turn phosphorylates extracellular signal-regulated kinases one and two (ERK1/2) on two proximal tyrosine residues, Tyr185 and Tyr183 (37). MEK1/2 has no other known substrate then ERK1/2. The final step in the cascade is for activated ERK1/2 to enter the nucleus and phosphorylate any of a large number of proteins, including transcriptional factors and other kinases.

PD98059 is a highly selective inhibitor of MEK1 and to a lesser degree MEK2; it does not inhibit activation of other highly related dual-specificity protein kinases (such as MKK3 or MKK4) or the activity of other Ser/Thr protein kinases (38, 39). PD98059 binds MEK1/2 and prevents activation by upstream activators, the inhibitor binds to a region of MEK1/2 outside of the ATP-binding site that prevents the un-phosphorylated protein from undergoing the conformational change needed to activate ERK1/2 (38). Since the inhibitor does not bind to the phosphorylated region, previously activated MEK1/2 is free to activate ERK1/2 (38, 39).

Activation of ERK1/2 plays a key role in repressing the transcriptional effects of peroxisome proliferator-activated receptors (PPAR) that belong to the nuclear hormone receptor family. The PPARs function as transcription factors to regulate growth and differentiation in various organs (41, 42). PPARγ activation results in repression of cell cycle progression via increased expression of cyclin-dependent kinase inhibitors. Repression of the cell cycle decreases cell growth and in turn increases caspase activity (43, 44). It has been shown that PPARγ serves as a substrate for phosphorylation by the various MAPKs (45-47), including ERK1/2. ERK1/2 is known to attenuate PPARγ 's transactivation function either by an inhibitory phosphorylation or by modulating

PPARγ's nucleo-cytoplasmic compartmentalization (48). The latter is achieved by the mitogen-induced nuclear export of PPARγ through its direct interaction with the ERK cascade (48). Upon phosphorylation active ERK1/2 enters the nucleus; the molecule is then rapidly exported from the nucleus along with PPARγ (48).

ERK1/2 phophorylates PPARγ through a putative MAPK phosphorylation site of PPARγ at Ser82 and Ser112 located on the ligand-independent transactivation domain. Phosphorylation of this domain down-regulates PPARγ activity (45, 46). Modification of this domain alters the interactions between PPARγ and its many co-repressors or coactivators (47), furthermore phosphorylation of PPARγ may activate proteasomal degradation (49). MEK inhibitors such as PD98059 abolish ERK1/2-dependent PPARγ phosphorylation.

PI3K Pathway

The second major pathway activated by IGF-I is the PI3K pathway. Phosphorylation of the adaptor molecule IRS-1 recruits PI3K to the plasma membrane (50). There the PI3K kinase converts phosphoinositol lipids into phosphoinositol-3-phosphates, which attracts and phosphorylates proteins such as serine/threonine protein kinase (Akt) and pyruvate dehydrogenase kinase (PDK) to the plasma membrane (50). Akt is a serine-threonine kinase that phosphorylates and regulates the activity of a number of targets including kinases, transcription factors and other regulatory molecules.

Nuclear factor-kappa B (NFκB) is one of the many factors that can be activated by Akt (51). NFκB is a protein complex found in almost all animal cells that regulates cellular responses to external stimuli such as stress and cytokines. NFκB is known to play a key role in the immune response to infection. Inactive NFκB is bound to its

inhibitor, termed IκB, and localized to the cytoplasm (51-53). Akt associates and activates the IκB kinase (IκKs); activated IκKs phosphorylates IκB targeting it for degradation by the proteasome. This allows NFκB to translocate to the nucleus and activate transcription of a variety of substrates including anti-apoptotic genes (51-53).

IGFBP-5

Molecular Design of IGFBP-5

The IGFBP-5 gene in mouse, human, and rat consists of four exons separated by three introns, with the first intron accounting for roughly half the gene. The promoter region in the mouse, human, and rat is typical for eukaryotic genes; there is a TATA box and CAAT sequence that are conserved among the mentioned species all located a few hundred basepairs upstream of the transcription start site (see Figures 1 and 2) (34, 54, 55).

The IGFBP-5 genomic sequence is the most conserved of the IGFBPs among species (34). Gene maps of mouse, rat, porcine and human IGFBP-5 cDNAs indicate a high degree of sequence conservation (34, 54-56). In humans, IGFBP-5 spans 33 kb and is located on chromosome 2 (34). In the mouse, the IGFBP-5 gene has a length of 17 kb and is localized on chromosome 1 (54). Interestingly, the IGFBP-5 gene is located adjacent to the IGFBP-2 gene but orientated in a head-to-head fashion in mouse, bovine, and human (see Figure 3). A similar pattern can be seen in the relative positions of IGFBP-1 and IGFBP-3. The distance between both genes comprises only 20 kb in humans (34) and 5 kb in mice (54). The similarities in gene location and sequence conservation between the IGFBPs suggest that these proteins have developed from a common ancestral IGFBP.

The IGFBP-5 protein comprises 252 residues (28.5 kDa) and is expressed with a hydrophobic signal sequence (19 to 20 residues) that is characteristic of secreted proteins. The protein is slightly basic (pI =8.5), making it the most basic IGFBP. The primary sequence of IGFBP-5 contains a large number of cysteine residues that are highly conserved across other IGFBPs; there are twelve cysteine residues in the N-terminal and six cysteines in the C-terminal regions. Like all the IGFBPs these conserved residues indicate the position of disulfide bonds in the C- or N-terminus (57-62). The IGFBP-5 protein is comprised of three discrete regions; C- and N-terminal domains, and a less conserved 'hinge region.'

The C-terminal domain of IGFBP-5 is distinctive due to the presence of a consensus long heparin-binding sequence (BBBXXB; B=basic amino acid; X=any amino acid), which is surrounded by basic residues. This region is important in binding extra-cellular matrix (ECM) components as well as other proteins. The C-terminal domain has a structure that is analogous to a thyroglobulin type-1 fold with an initial α-helix connected by a loop to a three-stranded antiparallel β-sheet structure leading finally to a flexible disulfide-bonded second loop (63). The six cytosine residues in the C-terminus form intradomain disulfide bridges; there is evidence that these cysteines also interact with each other (18). Another feature is the basic region found in the C-terminal domain of IGFBP-3, -5 and -6 that inhibits degradation by IGFBP-4 (64). Also located in this region is the IGF-II ligand binding domain that is found on the first β-turn and β-bulge of the central β-sheet structure. While not home to the IGF-I binding site, the C-terminal region is essential for high affinity binding of IGF-I. Site-specific mutagenesis within the C-terminal domain at residues Gly203 or Gln209 reduces the affinity of IGFBP-5 for rat

IGF-I (65, 66). Moreover, truncation or complete deletion of the C-terminus weakens the affinity for IGF-I 10-200 fold (59, 67).

The cysteine rich N-terminus of IGFBP-5 contains twelve cysteines that form six intradomain disulfide bonds (18). This domain is a rigid globular structure that consists of three beta sheets held together by disulfide bonds (59). The N-terminus contains the primary binding site for IGF-I, located between residues Ala40 and Ile92. Within the IGF-I binding site are residues Val49, Tyr50, Pro62 and Lys68–Leu75, which comprise a hydrophobic patch that is on the exterior surface of the protein (68). Substitutions in this region markedly alter IGF-I binding as well as biological activity.

The 'hinge like' central domain of IGFBP-5 is unique due to its amino acid sequence that is non-homologous to any of the other IGFBPs. Originally this portion was viewed only as a structural hinge between the other two highly conserved domains. Further investigation revealed that the central domain has an important physiological role, for many post-translational modifications occur only in this region. Hence this region is mainly responsible for the different functions of each of the six IGFBPs.

Post-translational Modifications of IGFBP-5

Proteolysis

Plasmin, serine proteases cathepsin G, elastase, thrombin and pregnancy associated plasma protein A (PAPP-A) act to cleave IGFBP-5 (69-72). Plasmin degrades IGFBP-5 in osteoblasts, however proteolysis can be mitigated by the occupation of the heparin binding domain which is known to bind various extra-cellular proteins (69). The neutrophil proteases, cathepsin G and elastase, proteolysis various IGFBPs to regulate IGF and IGFBP functions during inflammation and wound healing (70). Thrombin, a

serine protease that converts fibrinogen to an active form that assembles into fibrin, is secreted by porcine smooth muscle cells and was found to cleave IGFBP-5 between the amino acids Lys138 and Lys139 (72, 73). Physiologically, thrombin is found on vessel walls, its ability to degrade IGFBP-5 may be a mechanism for controlling IGF-I bioactivity (72). PAPP-A is a zinc-dependent metalloprotease that cleaves IGFBP-5 between Ser143 and Lys144 (71). IGFBP-5 is protected from proteolysis when it binds either IGF-I, heparin, or other components of the ECM. The IGFBP-5 protein contains two heparin-binding domains, the large consensus site lies in the C-terminus, while a short heparin-binding consensus sequence is found between residues 131 and 140 (74). This amino acid sequence is prone to proteolysis, which decreases the ability of IGFBP-5 to bind IGF-I therefore, proteolysis may be an innate mechanism for regulating free IGF-I.

Glycosylation

IGFBP-5 is glycosylated at residue Thr152 located in the hinge region (75). Glycosylation of IGFBP-5 was first indicated by the presence of a triplet at 29 kDa, 32 kDa, and 34 kDa in conditioned media from the U2 human osteosarcoma cell line (76). Each of the bands has the ability to bind IGF-I, however their ability to bind to cell surfaces may be affected by their level of glycosylation as has been reported for IGFBP-3 and -6 (77, 78). Treatment with neuraminidase and O-glycanase reduces the three bands to the 29 kDa core protein, confirming that IGFBP-5 is O-glycosylated (76). The O-linked glycopeptide is made of a combination of hexose (Hex), *N*-acetylhexosamines, (HexNAc), and *N*-acetylneuraminic acid (NeuAc) (75).

Phosphorylation

The least well-understood modification of IGFBP-5 is phosphorylation. Phosphorylation of any site on a given protein can change the function or localization of that protein. The majority (56%) of IGFBP-5 secreted by human cells is phosphorylated, with phosphorylation sites at Ser96 and Ser248 (75). The phosphorylation site at Ser96 lies in the hinge domain, its location in regards to the N terminus indicates a possible role in regulating the folding (75). The second phosphorylation site at Ser248 is in the C-terminus, perhaps affecting its C-terminal IGF-I-independent functions in the ECM and nucleus (75). The effect of phosphorylation on the ability of IGFBPs to bind IGF-I varies based on the IGFBP. While phosphorylation of IGFBP-1 was shown to increase its affinity for IGF-I (79), phosphorylation did not appear to influence the IGF-I binding affinity of IGFBP-3 (80). Another role of phosphorylation in IGFBP biology is to decrease susceptibility to proteolysis (80). Other possible biological actions of phosphorylated IGFBPs remain to be established.

Cellular Functions of IGFBP-5

IGFBP-5 has been shown to be multifunctional by virtue of its ability to act through both IGF-dependent and –independent mechanisms. A major role of IGFBP-5 is to mitigate the cell survival actions of IGF-I, there are also various IGF-independent actions of IGFBP-5 including interactions with the ECM, nuclear localization, and interactions with transcriptional regulators. Multiple roles for IGFBP-5 have been reported in cell growth, differentiation, and apoptosis with many papers reporting conflicting actions (79, 81-84).

IGFBP-5 and the Mammary Gland

Milk derived from lactating rats contains very low levels of IGFBPs with a pattern characteristic of serum (85). Many studies have shown a strong positive relationship between IGFBP-5 expression in the mammary gland and the occurrence of cell death during the process of involution. IGFBP-5 mRNA and protein are significantly upregulated during weaning or forced involution in rats, mice and cows (86-88). IGFBP-5 was found to be synthesized within the secretory epithelial cells, which are known to undergo apoptosis or programmed cell death at this stage. Results from several previous studies have led to the proposal that IGFBP-5 is important in promoting cell death and remodeling during involution, through both IGF-dependent and IGF-independent actions (89). Moreover, transgenic overexpression of IGFBP-5 in mammary tissue resulted in increased apoptotic death of the epithelial cells (90).

Addition of exogenous IGFBP-5 to mammary epithelial cells suppressed IGF-I-mediated survival, resulting in three-fold greater apoptosis in cells compared to rates when IGF-I was added alone (91). Administration of exogenous IGFBP-5 to murine MEC suppressed IGF-I mediated survival and *in vivo* administration of recombinant IGFBP-5 during the periparturient period resulted in impaired development of mammary alveoli and reduced invasion of the mammary fat pad, indicative of increased rates of apoptosis (91, 92). Furthermore, addition of exogenous IGFBP-5 to human breast cancer cells inhibited growth *in vitro* (81). Transgenic mice expressing IGFBP-5 in the mammary gland, using a mammary-specific promoter, β-lactoglobulin (BLG), had significantly decreased cell number and milk production (90). Additionally, the transgenic mice had higher concentrations of the pro-apoptotic molecules caspase-3 and

plasmin, while the concentrations of two pro-survival molecules, Bcl-2 and Bcl-xL were significantly reduced (90).

IGFBP-5 and Bone Formation

Regulation of IGF-I can impact bone growth and overall bone mass. IGF-IR knockout mice have retarded skeletal development accompanied by delayed ossification, as well as many other severe systemic defects (93). The effect of IGFBP-5 has been extensively studied in the area of bone formation. IGFBP-5 mediates differentiation of osteoblasts via mechanisms that are both dependent on and independent of IGF-I. During fetal development IGFBP-5 was found to be expressed in differentiating chondrocytes and osteoblasts (94, 95), while in adult primary osteoblast cultures, IGFBP-5 was secreted by pre-osteoblasts but decreased in abundance during their differentiation and maturation (96).

In IGF-I knockout mice, addition of IGFBP-5 promoted cell growth, alkaline phosphatase activity and osteocalcin expression as strongly as in wild type mice (97). When IGFBP-5 was administered locally to the outer periosteum of the parietal bone of IGF-I knockout mice, serum levels of IGFBP-5 exhibited a positive correlation with bone formation markers and bone density independent of IGF-I levels (97). Thus, IGFBP-5 functions as a growth factor that mediates its actions, in part, via an IGF-independent mechanism.

In contrast, transgenic mice over-expressing IGFBP-5 have impaired osteoblast function and osteopenia (98). Increased IGFBP-5 in the bone microenvironment could inhibit osteoblast formation through the binding and sequestering of IGF-I and II or by impairing the function of other factors involved in bone development such as vitamin D.

Targeted knockout of IGFBP-5 in murine osteoblasts enhanced the 1,25-$(OH)_2D_3$-response (vitamin D response) (99). The role of vitamin D is to stimulate calcium absorption in the intestine and to maintain and regulate bone mineralization. The actions of vitamin D are mediated through the vitamin D receptor (VDR), which acts as a ligand-activated transcription factor to regulate the expression of target genes. The VDR heterodimerizes with retinoid X receptor (RXR), which in turn associates with a vitamin D response element site on the promoter region of target genes. IGFBP-5 can interact with VDR to prevent RXR:VDR heterodimerization, which suggests that IGFBP-5 may attenuate vitamin D-induced expression of bone differentiation markers (99).

IGFBP-5 and the Extracellular Matrix

IGFBP-5 can directly bind to components of the ECM (Type III and IV collagen, laminin and fibronectin) and extracellular components such as dermatan sulphate, dextran sulphate, and other anionic polysaccharides (100, 101). The IGFBP-5–ECM association is responsible for many of the biological functions of IGFBP-5 that are dependent on the C-terminus of the molecule. Intact IGFBP-5 and fragments containing the C-terminus region (the heparin-binding domain) can stimulate cell motility in mesangial cells independent of IGF-I (102).

IGFBP-5 also binds plasminogen activator inhibitor-1 (PAI-I) a protein that is heavily involved with organ remodeling and wound healing (103). PAI-1 is a part of the ECM, it has been found in fibroblast and smooth-muscle cell cultures. Binding of PAI-1, as well as TSP-1, and vitronectin have no impact on the affinity of IGFBP-5 for IGF-I. Binding of IGFBP-5 may inhibit the action of PAI-1, activating plasminogen and the consequent breakdown of the ECM that takes place during tissue remodeling. Therefore,

IGFBP-5 may coordinate cell death and ECM remodeling that takes place during mammary gland development and involution by regulating both IGF-I and PAI-1 actions. Both IGFBP-5 and tissue-type plasminogen activator (tPA) interact with the milk protein αs2-casein (104). This suggests that IGFBP-5 may be an important regulator of this system, perhaps involving a multimolecular complex associated with the casein micellar fraction of milk. Thus, IGFBP-5 appears to be important for regulating both apoptosis and cell proliferation in the mammary gland during development, lactation and involution.

IGFBP-5 and the Nucleus

A growing area of interest in IGF research involves the nuclear actions of IGFBP-5. The C-terminal ends of IGFBP-3 and IGFBP-5 contain a bipartite nuclear localization signal (105, 106). The localization of these IGFBPs has been shown to occur via the nuclear transporter protein, importin, in breast cancer cells (107). Recently it has been shown that IGFBP-3 and -5 each bind retinoic X receptor (RXR), retinoic acid receptor (RAR) and VDR in human osteosarcoma cell lines U2-OS and MG-63 (99). Therefore it is possible that IGFBP-5 can modify the transcriptional activities of RXR, RAR and VDR in MEC as well. IGFBP-5 undergoes nuclear uptake in human osteoblasts, where the zinc finger protein four and a half LIM protein 2 (FHL2) acts as a binding partner for IGFBP-5 (108). IGFBP-5 and FHL2 co-localize in the nucleus as a complex, this complex functions as a transcriptional factor (108). FHL2 itself does not contain a nuclear localization sequence; it is possible that the IGFBP-5-FHL2 complex is shuttled to the nucleus.

Recently it was discovered in transgenic mice overexpressing IGFBP-5 that nuclear IGFBP-5 may impact prolactin production. Increased IGFBP-5 did not accelerate remodeling of the extracellular matrix during involution (109). Increased IGFBP-5 had no effect on IGF signaling during lactation, however it did affect prolactin cell signaling, as judged by decreased phosphorylation of STAT5 (109). This suggests that intracellular IGFBP-5 may interact directly with the prolactin signaling pathway. The presence of prolactin in the mammary gland serves as a survival factor and controls several MMPs needed for tissue remodeling.

Regulation of IGFBP-5

IGFBP-5 is regulated by hormones such as progesterone, androgens, estrogen, glucocorticoids (GC) and prolactin. Progesterone (PG) increased IGFBP-5 expression in normal human osteoblasts and increased IGFBP-5 transcription in U2 human osteosarcoma cells. The human IGFBP-5 promoter contains five putative GRE/PRE half-sites that have been shown to bind the receptors and effectively mediate ligand-dependent transactivation through synergistic interactions between the multiple half-sites (110). Testosterone treated mice have increased IGFBP-5 mRNA expression levels 10- to 12-fold above wild type controls (111). This increase is specific for IGFBP-5 as the levels of the other IGFBP mRNAs did not change following androgen withdrawal and replacement (111).

Estradiol suppressed expression of IGFBP-5 *in vivo* in the uterus to less than 15% of control values (112). Furthermore use of the antiestrogen ICI 182780 on MCF-7 cells increased IGFBP-5 mRNA and protein accumulation in the conditioned medium (113). Glucocorticoids modulate IGF-I action in bone. Treatment with GCs resulted in time-

and dose-dependent decreases in IGFBP-3, -4, and -5 levels in culture medium, with corresponding decreases in mRNA levels (114). Concurrent treatment of growth hormone and prolactin inhibits about 90% of IGFBP-5 mRNA and protein levels in rat mammary tissue (104).

Non-hormonal factors such as sonic hedgehog (SHH) and TGFβ can also affect IGFBP-5 expression. IGFBP-5 is regulated in chicken embryos by SHH. SHH functions in embryonic development as a morphogen responsible for the development of limbs, brain structure, and the spinal cord. IGFBP-5 and SHH co-localize to the same areas of the embryo during development, and IGFBP-5 is known to have a pivotal role in embryonic limb development. Addition of cyclopamine, a potent inhibitor of SHH signaling, leads to the down-regulation of IGFBP-5 expression (115). Addition of TGFβ (0.5-10 ng/mL) to human prostate cells increased IGFBP-5 protein and mRNA in a time- and dose-dependent manner. At high concentration (10 ng/mL) TGFβ increased IGFBP-5 protein levels 9- to 14-fold above controls (116).

Perhaps the most consistent regulator of IGFBP-5 is the IGF-I growth factor itself. IGF-I upregulates IGFBP-5 in porcine smooth muscle cells, human neural cells, and murine and bovine MEC lines (88, 117-119). Regulation of IGFBP-5 has been characterized in primary bovine mammary epithelial and fibroblast cells. The addition of IGF-I induced large dose-dependent increases of IGFBP-5 mRNA in mammary fibroblasts, however the same treatment had little to no effect on primary MEC (120).

Objective of the Thesis

IGF-I signaling classically consists of PI3K and MAPK pathways. Inhibition of the PI3K pathway completely inhibited the effect of IGF-I on IGFBP-5 mRNA levels in

mammary fibroblasts. Inhibition of the MAPK pathway with PD98059 alone did not affect basal IGFBP-5 expression in mammary fibroblasts; it did however increase IGFBP-5 mRNA levels in mammary epithelial cells (118). In addition, no other IGFBP was increased in either cell type when MAPK activity was inhibited (118). Furthermore addition of IGF-I with the MAPK inhibitor synergistically increased IGFBP-5 mRNA in bovine and murine MEC (118). The present study focuses on the regulation of IGFBP-5 in MEC thought IGF-IR signaling. The specific objectives are:

1. To determine if protein expression correlates to increases seen in IGFBP-5 mRNA.
2. To identify the downstream signaling molecules of MAPK that activate IGFBP-5 expression in MEC
3. To formulate a mechanism responsible for the synergistic increase in IGFBP-5 observed in response to IGF-I and PD98059

Chapter Two

Molecular regulation of insulin-like growth factor binding protein-5 expression by signaling molecules downstream of the IGF-I receptor in mammary epithelial cells

Introduction

The IGF system regulates the growth, development, and function of the mammary gland through complex interactions with other growth factors and hormones. The IGF system is composed of two polypeptide growth factors (I and II), two receptors, and six high affinity-binding proteins (IGFBP-1 through -6). The temporal expression of ligands, receptors, and each of the IGFBPs has been reported for the mammary gland. The IGFBPs function to transport, prolong the half-life of, and regulate the bioavailability of the IGFs. However, studies are continually emerging suggesting that both IGF-dependent as well as IGF-independent roles for the IGFBPs at the cellular level are important in mammary gland physiology.

Many studies have shown a strong positive relationship between IGFBP-5 expression in the mammary gland and the occurrence of cell death during the process of involution. IGFBP-5 mRNA and protein are significantly upregulated during weaning or forced involution in rats, mice and cows (86-88). Overexpression of IGFBP-5 specifically in the mammary gland in transgenic mice increases cell death in MEC (87). In contrast, there are several reports that suggest IGFBP-5 is not exclusively pro-apoptotic in mammary cells. During post-natal development IGFBP-5 is highly expressed in terminal end buds and is present during mid-pregnancy in ductal and alveolar cells (121). Also IGFBP-5 levels increase following lactogenic hormone-induced differentiation in murine MEC (86, 122).

To study the role of IGF/IGFBPs in MEC our laboratory uses the MAC-T bovine mammary epithelial cell line. This line was established by stable transfection of primary bovine mammary secretory alveolar cells with simian virus 40 (SV-40) (123). MAC-T

cells display morphological characteristics similar to MEC *in vivo*, and retain their ability to differentiate in culture (124). They possess IGF-IR and are responsive to IGF-I, in addition they synthesize IGFBP-2, -3, -4, -5, -6 (125).

IGF-I signaling classically consists of the PI3K and MAPK pathways. We have reported that inhibition of the PI3K pathway completely abrogated the effect of IGF-I on IGFBP-5 mRNA levels in both primary mammary fibroblasts and MAC-T cells. Similar effects were observed when the MAPK pathway, was blocked in primary mammary fibroblasts. In contrast, when the MAPK pathway was inhibited in MAC-T cells, both basal and IGF-I stimulated IGFBP-5 mRNA levels were dramatically increased (118). The goals of this study were to further examine the specificity of this response, as well as to determine the factors downstream of the IGF-IR that are involved in regulation of IGFBP-5 expression in MEC by IGF-I and the MAPK pathway.

Materials and Methods

Materials

Reagents used for cell culture were obtained from Invitrogen Co. (Carlsbad, CA), except for fetal bovine serum that was from Gemini Bio-Products (Woodland, CA) and phenol-red free Dulbecco's Modified Eagles Medium from Sigma Aldrich (St. Louis, MO). Bovine insulin and glucose were from Sigma Aldrich. MAPK inhibitor PD98059 was purchased from Calbiochem (La Jolla, CA), resuspended at 10 mM stock solution in dimethyl sulfoxide (DMSO) and stored at -20^{o}C. PPAR γ antagonist GW6992, IκB inhibitor phenethyl caffeiate, 1α 25-dihydroxy Vitamin D_3 (Vitamin D), PPAR agonists Rosiglitazone, Fenofibrate, Bezafibrate, and GW0742 were purchased from Cayman Chemical (Ann Arbor, MI) and resuspended in DMSO according to manufacturer's recommendations for maximal solubility. Tissue culture plasticware was from Becton-Dickinson (Franklin Lakes, NJ).

IGFBP-5 antibody H-100 that recognizes cow, mouse, and human IGFBP-5 was obtained from Santa Cruz (Santa Cruz, CA).

Cell Culture Experiments

Primary mammary epithelial cells (MEC) were isolated from lactating cows as previously described (Fleming et al. 2005) (120). The bovine mammary epithelial cell line MAC-T was established from primary bovine MEC of a lactating cow, by immortalization with the SV40 large-T antigen (12). The murine MEC line NMuMG (CRL-1636) and human MEC line MCF-10A (CRL-10317) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Stock cultures of primary bovine MEC, MAC-T, and NMuMG cells were maintained in phenol-red containing

DMEM supplemented with 4.5 g/L D-glucose (i.e. DMEM-H), 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin (Complete medium) with the addition of 15 μg/ml bovine insulin. Stock cultures of MCF-10A cells were maintained in DMEM medium supplemented with 20 ng/ml EGF, 10 μg/ml insulin, 50 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin. All cell lines were incubated at 37^{o}C in a humidified atmosphere with 5% CO2

For all experiments, cells were plated in DMEM-H lacking phenol-red or insulin with 10% FBS and antibiotics. Cells were grown to confluence, rinsed in serum-free medium, and incubated overnight in phenol-red free serum-free DMEM with 0.2% BSA and 30 nM Sodium selenite. The spent medium was aspirated and replaced with phenol-red free serum-free DMEM plus antibiotics ± treatments. For RNA analysis, cells were lysed in Trizol™ (Invitrogen Co.) and stored at -80^{o}C until analysis. For analysis of secreted proteins the conditioned medium was collected, cleared by centrifugation and stored at -80^{o}C.

Western Blotting

Total protein content of the conditioned medium was determined using The Thermo Scientific NanoDrop^{TM} 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Conditioned media containing equal amounts of protein were concentrated using Microcon Ultracel YM-10 centrifugal filter devices from Millipore Corporation (Bedford, MA). The concentrated protein was then separated by electrophoresis through 12.5% resolving SDS-polyacrylamide gels under reducing conditions. The proteins were transferred to a 0.2 μm PVDF membrane using the semi-

dry method as previously described in Fleming et al. 2005 (120). Membranes were blocked in tris buffered saline (TBS) + 0.5% tween (TBS-T) with 5% non-fat dried milk for one hour at room temperature, and then incubated with primary antisera overnight at 4^{o}C. The membranes were then washed with TBS-T then incubated with an appropriate secondary antibody conjugated to horseradish peroxidase. The membranes were washed with TBS-T, and then enhanced chemiluminescence substrate (ECL plus, Amersham) was added to detect peroxidase activity.

Ligand Blotting

Conditioned media containing equal amounts of protein were separated by electrophoresis through 12.5% resolving SDS-polyacrylamide gels under non-reducing conditions. The proteins were transferred to a 0.2 μm PVDF membrane using the semi-dry method. Briefly, ligand blotting was performed as previously described (118), membranes were washed, blocked with 1% BSA, washed then incubated overnight with [^{125}I] IGF-I from GE Healthcare (Waukesha, WI) and autoradiographed for 1 to 2 weeks with intensifying screens.

Quantitative Real Time PCR

Unless otherwise stated all reagents were purchased from Applied Biosystems (Foster City, CA). RNA was isolated using Qiagen RNeasy columns (Bothell, WA) and eluted with 30-50 μl of RNAse free dH_2O. Total RNA integrity was verified by inspection of the 18S and 28S rRNA bands after agarose gel electrophoresis. Filtered RNA (2 μg) was reverse transcribed using random primers and SuperScript II Reverse Transcriptase.

Individual standard curves were established for each primer set using serial dilutions (1:2, 1:20, 1:200, and 1:2,000) and a common pool of cDNA. Primer sets for bovine cyclophilin F 5'-GTT CCA AAG ACA GCA GA-3'; R-5'-CCT AAA TAG ACG GCT CC-3', bovine IGFBP-5 F 5'-AGT CGT GCG GCG TCT ACA CTG AG-3'; R 5'-AAG ATC TTG GGC GAG TAG GTC TCC-3', bovine IGFBP-4 F 5'-GAC AAG GCG TGT GCA TGG-3'; R 5' AGG GGC TGA AGC TGT TGT T-3', bovine IGFBP-6 F 5'-ACT CGG GGA CCT CTA CCA CT-3'; R 5'- AGC ACG GAG TCC AGA TGT TT-3', murine IGFBP-5 F 5'-AGA AAG GAC TCC ACA ACC AAG GCT-3'; R 5'-GGG TGT CAC TGA ATG CCA CAC TTT-3', murine cyclophilin F 5'-TGC TGG ACC AAA CAC AAA CGG TTC-3'; R 5'-CAA AGA CCA CAT GCT TGC CAT CCA-3', human IGFBP-5 F 5'-GGA GAC CTA CTC CCC CAA GA-3'; R 5'-CTG GGT CAG CTT CTT TCT GC-3', and human RPL13A F 5'-CCT GGA GGA GAA GAG GAA AGA GA-3'; R5'-TTG AGG ACC TCT GTG TAT TTG TCA A-3' were developed using PrimerQuest (IDT, Coalville, IA).

To determine gene expression profiles, individual samples were diluted 1:4; 9.5 µl diluted sample was amplified in a 27.5 µl reaction mix containing 10.5 µl Power SYBR Green MasterMix, 6.5 µl dH_2O, and 0.5 µl (~200uM) of each forward and reverse primer set. Fluorescent PCR products were detected in single wells of 96-well plates (TKR Biotech, Huntingdon Valley, PA) using an Applied Biosystems 7300 Real-Time PCR system. Fluorescence was measured following each cycle and displayed graphically (Applied Biosystems 7300/7500 SDS software, version 1.3.1, ABI, Foster City, CA), and mean expression levels relative to untreated samples were determined. Data values were normalized to cyclophilin for all genes except human IGFBP-5 which was normalized to

RPL13A. The software determined a cycle threshold value, which identified the first cycle at which the fluorescence was detected above the baseline for that sample or standard. Products were verified by agarose gel electrophoresis. Melting curves were generated for each sample.

Statistical Analysis

Data were analyzed by ANOVA or by Bonferroni *t*-test with differences considered significant where $P < 0.05$ or $T < 0.05$ respectively. Analyses were performed in SigmaStat (2.03) program for Windows (SPSS Inc., Chicago, IL, USA).

Results

The Inhibition of MAPK/ERK pathway increases basal and IGF-I-stimulated IGFBP-5 mRNA levels in MAC-T cells in a time-dependent manner

Previous work using Northern blotting techniques had shown that IGF-I and PD98059 each increased IGFBP-5 mRNA levels at 4 h, with a combination of the treatments inducing increases of up to 12-fold above serum-free controls. Since expression of IGFBP-5 mRNA is quite low in untreated control cells, our first goal was to use the more sensitive qRT-PCR to quantitate the fold-change with each treatment across time. As shown in Figure 4 the increase in IGFBP-5 mRNA expression in response to MAPK inhibition was rapid; increases were detected at 2 h with the peak of expression occurring between 2 and 4 h. Addition of IGF-I alone was shown to generate a small but consistent increase in IGFBP-5 mRNA, slightly less then 2-fold between 2- and 8 h. At all time points IGFBP-5 mRNA expression was increased to a greater degree in response to PD98059 compared to IGF-I. The treatment combination of IGF-I plus PD98059 led to a greater increase in IGFBP-5 mRNA expression relative to either treatment alone at all time points, with the greatest increase observed at 8 h.

Identification of IGFBP-5 by immunoblot analysis

We have previously shown that treatment with IGF-I plus PD98059 resulted in an increase in a specific IGFBP that binds IGF-I, as shown by ligand blotting with [^{125}I] IGF-I. This band migrates at the molecular weight of IGFBP-5. However, we have not previously had an antibody that specifically recognized bovine IGFBP-5. After trying several commercial antibodies, we determined that a polyclonal IGFBP-5 antibody from

Santa Cruz specifically recognized bovine IGFBP-5. As shown in Figure 5, a dramatic increase in IGFBP-5 protein was seen in conditioned media of cells treated with IGF-I and PD98059. The antibody also detected a non-specific band that migrated slightly above IGFBP-5 in all lanes. There was no regulation of this band and it was present in conditioned media of untreated cells that express very low levels of IGFBP-5 mRNA. Since Vitamin D has been reported to increase IGFBP-5 expression in other cell types, we treated cells with this agent in order to find a treatment that produced large increases in IGFBP-5 as a positive control for the antibody. Vitamin D did increase IGFBP-5 expression (data not shown). An even larger increase in IGFBP-5 protein was observed when cells were treated with a combination of vitamin D, PD98059 and IGF-I. However addition of PD98059 alone, a treatment that increased IGFBP-5 mRNA 4- to 5-fold over serum-free controls, had no effect on IGFBP-5 protein levels in conditioned media. We have inconsistently observed a 19-20 kDa fragment by immunoblot that is recognized by the IGFBP-5 antibody and may in fact be a proteolyzed fragment of IGFBP-5 (data not shown), this could explain the failure to detect an increase in IGFBP-5 protein in conditioned media under these conditions.

The stimulatory effect of IGF-I and PD98059 on IGFBP-5 is specific for this individual form of IGFBP

The activated IGF-IR classically signals through two pathways, the PI3K pathway and the MAPK pathway. The PI3K pathway is required for the activation of both IGFBP-3 and -5 in our bovine MEC model (126). However, the MAPK pathway has opposing effects on these two IGFBP; inhibition of the MAPK pathway decreases

IGFBP-3 mRNA levels, while it increases IGFBP-5 expression. To determine if this regulation was novel we investigated the other IGFBPs that are produced by MAC-T cells using qRT-PCR and Northern blotting. Figure 6 shows that mRNA levels of IGFBP-2, -4, and -6 were not increased by inhibition of the MAPK pathway. In fact, PD98059 decreased the basal levels of IGFBP-2 and -4. Additionally IGF-I or IGF-I + PD908059 had no effect on IGFBP-2, -4, or -6 mRNA levels. Therefore, these effects are specific for IGFBP-5.

The MAPK/ERK Pathway does not affect basal or IGF-I stimulated IGFBP-5 mRNA levels in human mammary epithelial cells

Previous experiments have shown that the novel regulation of IGFBP-5 expression by the combination of IGF-I and PD98059 that was found in MAC-T cells was also observed in murine MEC and primary bovine MEC, though to a lesser degree (118). Therefore we sought to determine if IGFBP-5 was regulated by similar factors in a human MEC model, the MCF-10A cell line. Similar to MEC from other species, addition of IGF-I increased IGFBP-5 mRNA expression approximately 2-fold. However, inhibition of the MAPK pathway had no effect on either basal or IGF-I-stimulated IGFBP-5 mRNA levels (Figure 7).

PPARγ antagonists partially blocks PD98059-induced IGFBP-5 mRNA in MAC-T cells

Given that inhibition of ERK1/2 activation in MAC-T cells increased IGFBP-5 mRNA expression, we hypothesized that ERK1/2 directly affected an inhibitor or activator of IGFBP-5. Promoter analysis elucidated two PPAR consensus sites in the

mouse and human sequence (Figures 1 and 2), suggesting that PPARs might be involved. All three members of the PPAR nuclear receptor family (PPARγ, PPARα, and PPARβ/δ) could potentially bind to this consensus sequence. We first concentrated on PPARγ since previous research has shown ERK1/2 regulation can affect PPARγ activation and location within the cell (48). In order to determine if PPARγ played a role in the ability of MAPK inhibition to regulate IGFBP-5 expression, cells were treated with GW9662, a highly selective PPARγ antagonist. As shown in Figure 8, addition of GW9662 inhibited the ability of PD98059 to stimulate IGFBP-5 mRNA expression approximately 50%, also GW9662 alone slightly repressed basal IGFBP-5 expression. To further confirm that PPARγ was affecting IGFBP-5 expression, a second selective PPARγ antagonist T0070907 was tested. This antagonist inhibits PPARγ via a different mechanism compared to GW9662. Results with this inhibitor were similar to those observed with GW9662 (data not shown). A next step was to determine if a PPARγ agonist would have the opposite effect. Treatment of MAC-T cells with the agonists Rosiglitzone (PPARγ), Fenifibrate (PPARα) or GW0742 (PPARβ/δ) failed to activate IGFBP-5. Since basal ERK activation may sequester PPAR to the cytoplasm, we also treated cells with PD98059 plus each agonist. However, this combination also failed to activate IGFBP-5 (data not shown).

PPAR agonists increase IGFBP-5 mRNA expression in murine MECs

In the murine MEC model NMuMG, cells have low basal phosphorylated ERK1/2 and PD98059 alone does not significantly increase IGFBP-5 expression. Therefore, we tested the PPAR agonists to determine if they would activate IGFBP-5 in these cells. As

shown in Figure 9, both the PPARγ agonist Rosiglitazone and the PPARβ/δ agonist GW0742 increased IGFBP-5 mRNA 2.3-fold above control at 8 h. The PPARα agonist Fenofibrate and pan-PPAR agonist Bezafibrate both repressed basal IGFBP-5 expression.

Inhibition of NFκB represses IGFBP-5 mRNA and protein expression in MAC-T cells

Human and mouse promoter sequence analysis elucidated a NFκB binding domain that is located in the proximal promoter region near the TATA box (Figure 2). Akt and members of the MAPK pathway, such as Raf, phosphorylate IκB which in turn allows the two NFκB subunits (P65 and P50) to be released from the complex and enter the nucleus. Phenethyl caffeiate, a selective inhibitor of NFκB nuclear translocation, almost completely silenced IGFBP-5 mRNA and protein levels in MAC-T cells treated with PD98059 ± IGF-I. As shown in Figure 10, phenethyl caffeiate added in conjunction with PD98059 reduced IGFBP-5 mRNA expression from a 10-fold increase to only a 2-fold increase. Addition of phenethyl caffeiate to PD98059 + IGF-I treated cells reduced expression from eighteen-fold to two-fold. Phenethyl caffeiate also highly repressed the basal mRNA expression level of IGFBP-5. Western ligand and immunoblots (Figure 11 A and B) indicated that the induction of IGFBP-5 protein by PD98059 + IGF-I was also significantly reduced when IκB was inhibited. Interestingly, in Figure 11 B ligand blot results indicated that IGFBP-3 was dramatically upregulated by the inhibition of NFκB alone and when treated with IGF-I and PD98059. This indicates that IGFBP-3 and IGFBP-5 are reciprocally regulated by NFκB.

Discussion

Insulin-like growth factors (IGFs) play a pivotal role in tissue homeostasis, regulating cell proliferation, differentiation and migration during development (1). In addition, IGFs are critical cellular survival factors. The IGFs (IGF-I and -II) signal through the IGF-IR, which is found on the surface of most cell types and is coupled to the PI-3 kinase and MAP kinase pathways. The actions of IGF-I are altered by a family of six IGFBPs. The mechanisms of IGFBP action are complex as they can either inhibit or augment the actions of IGF-I, as well as have IGF-independent effects, particularly in relation to apoptosis.

In the present study, we have shown that the MAPK pathway represses both basal and IGF-I stimulated IGFBP-5 production in bovine MEC. Inhibiting basal MAPK activation resulted in a four- to five-fold increase in IGFBP-5 mRNA levels, however this increase did not correspond to an increase in IGFBP-5 protein. IGFBP-5 is prone to degradation by various proteolytic enzymes, especially in the absence of IGF-I. PAPP-A a proteolytic enzyme naturally produced by the MAC-T cell line, is far more likely to degrade IGFBP-5 alone then when it is bound to IGF-I (127). Evidence of IGFBP-5's degradation was occasionally observed via western immunoblots in the form of a 19 kDA band that was only found in samples treated with PD98059. Inhibition of the MAPK pathway combined with the addition of IGF-I synergistically increased both IGFBP-5 mRNA and protein levels in MAC-T cells. This regulation was cell-type specific in the bovine mammary gland, since inhibition of the MAPK pathway decreased levels of IGFBP-5 mRNA and protein in basal and IGF-I stimulated bovine mammary fibroblasts.

Also, the ability of the MAPK pathway to repress expression of other IGFBPs was not regulated in this manner.

The role of the PI3K and MAPK pathways in the regulation of IGFBP-5 has been studied in tissues other than the mammary gland. IGFBP-5 production has been shown to be dependent on the PI3K pathway but not the MAPK pathway in rat smooth muscle cells, primary Schwann cells, and chondrocytes (94, 128, 129). Conversely, the MAPK pathway exclusively regulates IGFBP-5 transcription in rat intestine (130). Both the MAPK and the PI3K pathways control IGFBP-5 in human intestinal smooth muscle cells (131). While this is the first report that the MAPK pathway represses increases in IGFBP-5 mRNA and protein in response to a growth factor, it has been shown that the MAPK pathway represses epidermal growth factor (EGF)-stimulated increases in IGFBP-3 mRNA and protein in human esophageal cells (132).

A primary objective of this work was to find the molecules downstream of ERK that are responsible for the inhibition of IGFBP-5 expression. ERK is known to upregulate many transcription factors, but in this situation we were looking for a molecule that was inhibited by ERK. ERK has been reported to inhibit the actions of PPARγ. ERK directly phosphorylates PPARγ causing a conformational change that prevents ligands from binding PPARγ in murine fibroblasts (NIH-3T3) and preadipocytes (3T3-L1) (45, 46). Moreover, mitogenic activation by epidermal growth factor (EGF) or tetradecanoyl phorbol acetate (TPA) causes PPARγ to translocate from the nucleus to the cytoplasm in human embryonic kidney cells HEK-293 and the gastric epithelial cell line MKN45 (48, 133). Therefore inactivation of ERK1/2 could lead to increased action of PPARγ by keeping it in the nucleus. A role for PPARγ was supported by the finding that

addition of two highly selective PPARγ antagonists caused a decrease in PD98059-stimulated IGFBP-5 mRNA levels. To further confirm that PPARγ regulated IGFBP-5 expression we tested Rosiglitazone, a PPARγ agonist used to treat type 2 diabetes in humans, with the hypothesis that it would increase IGFBP-5 mRNA expression. Surprisingly, addition of Rosiglitazone alone did not result in increased IGFBP-5 message in MAC-T cells. This cell line is known to exhibit high levels of phosphorylated ERK1/2 under basal conditions. It is possible that this overabundance of phosphorylated ERK1/2 may sequester PPARγ to the cytoplasm where the agonist has no effect. To further investigate whether PPARγ positively regulates IGFBP-5 message we used another normal MEC line, the murine cell line NMuMG, which has low basal levels of phosphorylated ERK1/2. Basal levels of IGFBP-5 mRNA were higher in NMuMG cells then in MAC-T cells. Furthermore, while IGFBP-5 mRNA expression increased with both IGF-I and PD98059, the increases seen with MAPK inhibition tended to be less dramatic compared to MAC-T cells (118). Addition of agonists for PPARγ and PPARβ/δ agonists to NMuMG cells each increased IGFBP-5 message to a similar extent. This suggests that both of these PPAR receptors can bind to the PPRE binding sites in the IGFBP-5 gene. Furthermore, these two PPAR nuclear receptors could be utilizing the same coactivators to increase transcription, possibly a coactivator that PPARα does not recruit, since this form of PPAR had no effect on IGFBP-5 expression. There are many potential coactivators including CBP, PBP, PGC1, PRIP, p300 and SRC1 that bind to PPARs and to other nuclear receptors in an agonist-dependent manner.

While the PPARγ antagonist GW9662 reduced IGFBP-5 expression in PD98059 treated samples, it had no significant effect on IGFBP-5 mRNA levels in cells treated

with IGF-I + PD98059. Therefore, to identify other potential factors involved in the upregulation, we examined the known promoter regions of IGFBP-5. Both the mouse and human promoter sequences were found to have a consensus sequence for NFκB binding (see Figure 1 and 2). NFκB is activated by IGF-I through the PI3K pathway (134-136). NFκB is a protein complex found in almost all animal cells that regulates cellular responses to external stimuli such as stress and cytokines. Under basal conditions, inactive NFκB is bound to its inhibitor, termed IκB, and localized to the cytoplasm (51-53). Akt associates with and activates IκK; activated IκK phosphorylates IκB targeting it for degradation by the proteasome. This allows NFκB to translocate to the nucleus and activate transcription of a variety of substrates including anti-apoptotic genes (51-53). The chemical inhibitor phenethyl caffeiate prevents phosphorylation of IκB, thus NFκB is forced to stay in the cytoplasm. Addition of phenethyl caffeiate alone significantly reduced basal IGFBP-5 mRNA expression in the MAC-T cell line. Furthermore, addition of phenethyl caffeiate with IGF-I + PD98059 induced a dramatic decrease in both IGFBP-5 mRNA and protein. Surprisingly, addition of phenethyl caffeiate along with PD98059 also caused a major decrease in IGFBP-5 mRNA. This would suggest that either phosphorylated ERK1/2 inhibits NFκB activation or that upstream molecules, such as Raf, are inducing NFκB, as has been reported in NIH3T3 cells (137). The next step in this project would be to establish activation of NFκB via IGF-I and PD98059. In order to accomplish this we would need a reporter plasmid with an NFκB consensus binding domain. Another possibility would be to use the IGFBP-5 murine promoter with a mutated NFκB binding domain in a reporter assay.

It would seem that both regulatory elements NFκB and PPARγ are necessary for expression of IGFBP-5. Treatment with IGF-I activates NFκB, however we do not see a large increase in IGFBP-5 until PPARγ is unrepressed. The observed synergistic increase suggests that not only are both factors needed for full IGFBP-5 expression but also that these two factors must work together in a complex. NFκB has been reported to physically interact with PPARγ, which in some circumstances binds to DNA cooperatively with NFκB, further enhancing NFκB-DNA binding (138, 139). In addition PPARγ agonists were able to enhance the binding of NFκB to upstream regulatory elements (140). We propose that cooperative binding leads to the synergistic increase in IGFBP-5 that is seen in IGF-I + PD98059 treated cells (see Figure 12).

Studies with IκK2 knockout mice have supported a role for NFκB in mammary involution, IκK2 knockout mice have impaired mammary in concert with decreased rates of apoptosis (141, 142), however a role for PPARγ in involution would be novel. Rapid de-phosphorylation of ERK occurs during early stages of murine mammary involution *in vivo* (143). This decrease could in fact be a biological switch that allows PPARγ to enter the nucleus to promote pro-apoptotic genes that are responsible for mammary gland involution.

Figure 1

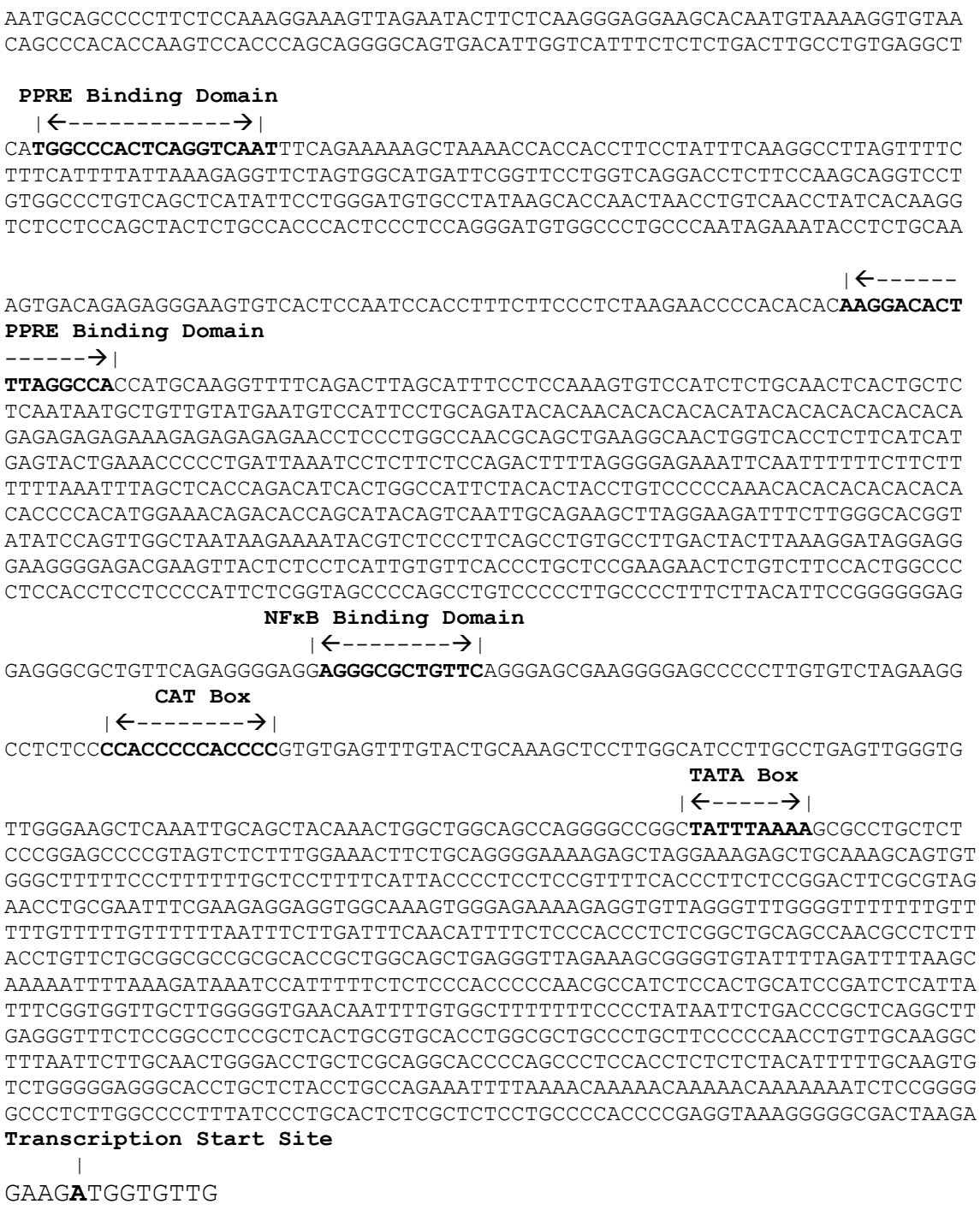


Figure 1. The proximal promoter region of IGFBP-5 in *Homo sapiens*

Figure 2

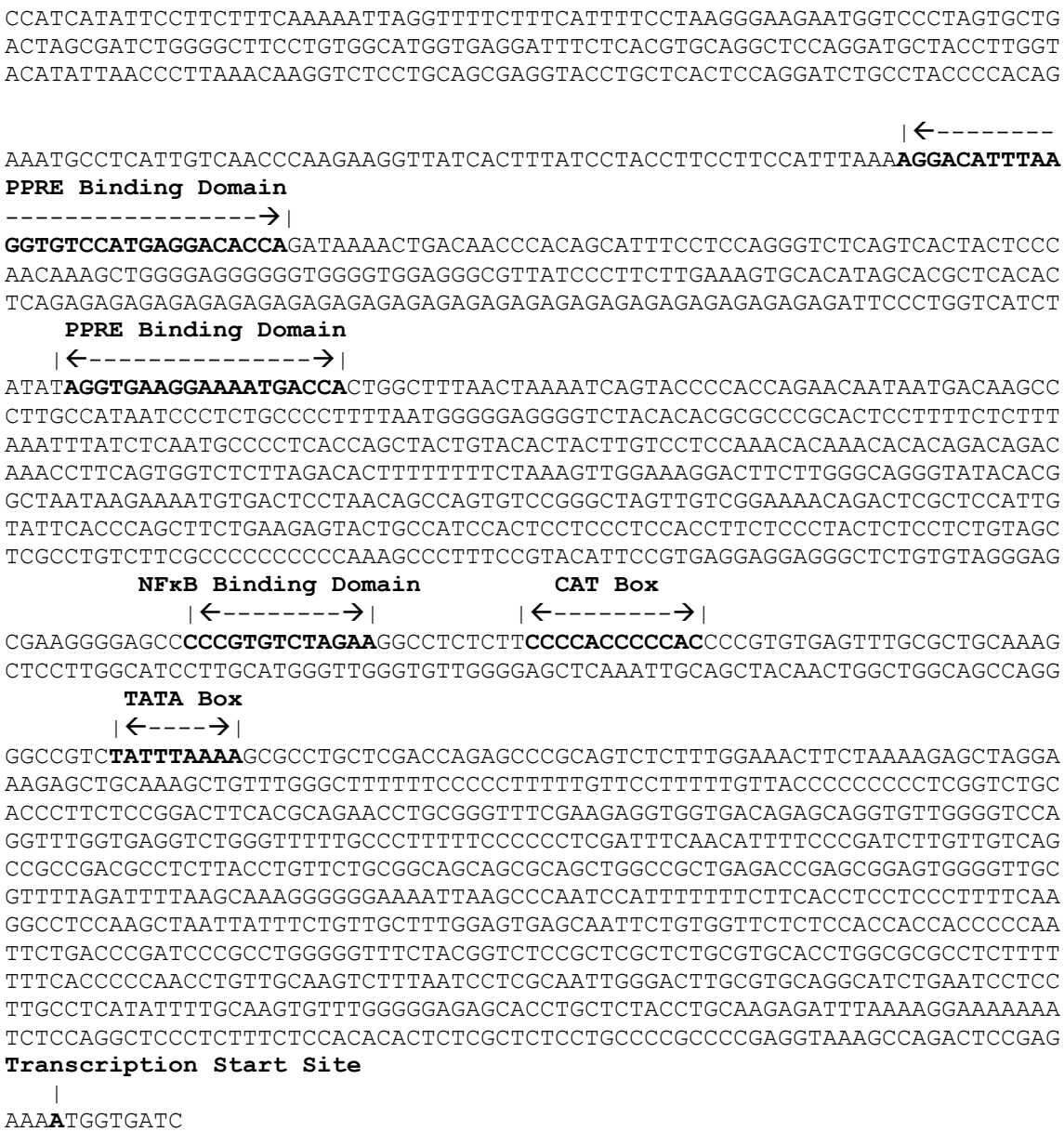
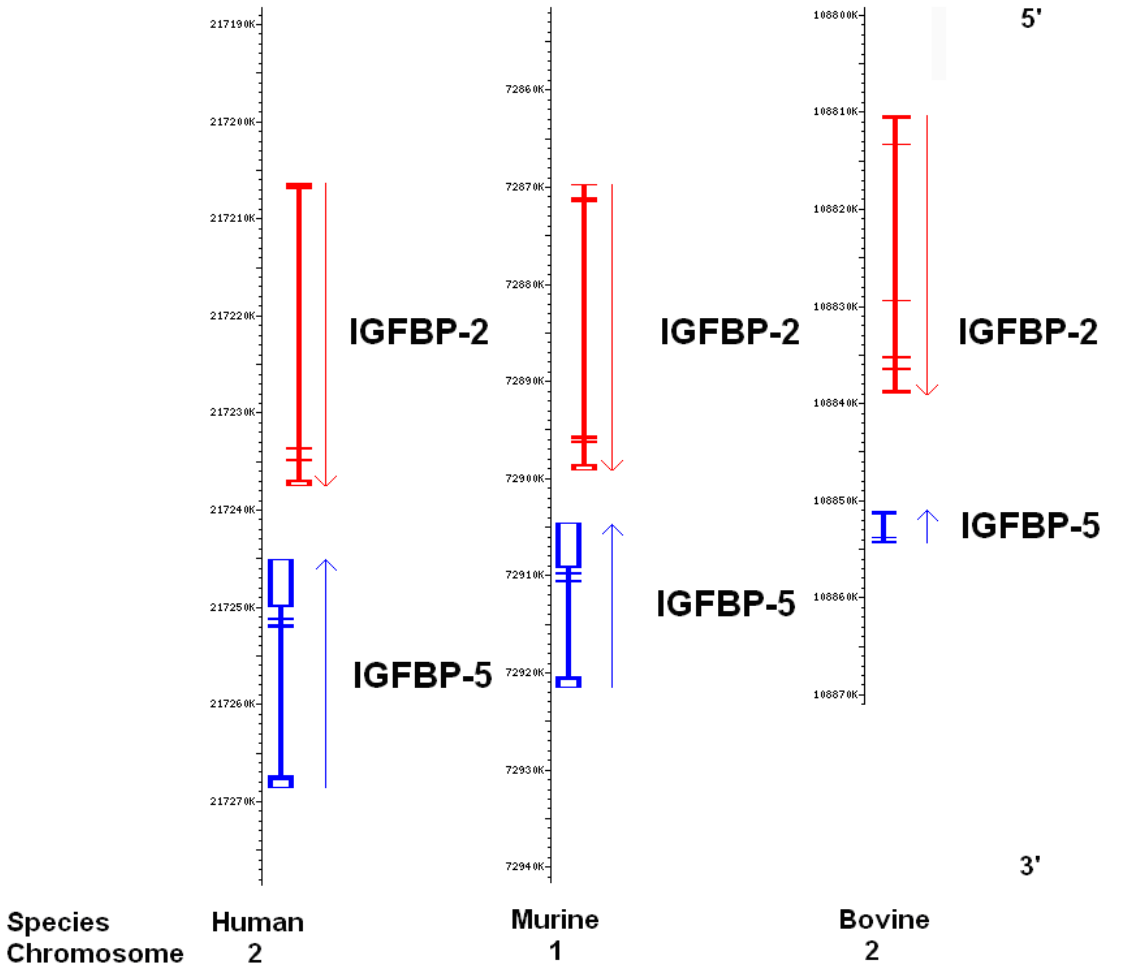


Figure 2. The proximal promoter region of IGFBP-5 in *Mus musculus*

Figure 3



[1]

Figure 3. Chromosomal location and orientation of IGFBP-5 and IGFBP-2

[1] Gene maps are from http://www.ncbi.nlm.nih.gov/

Figure 4

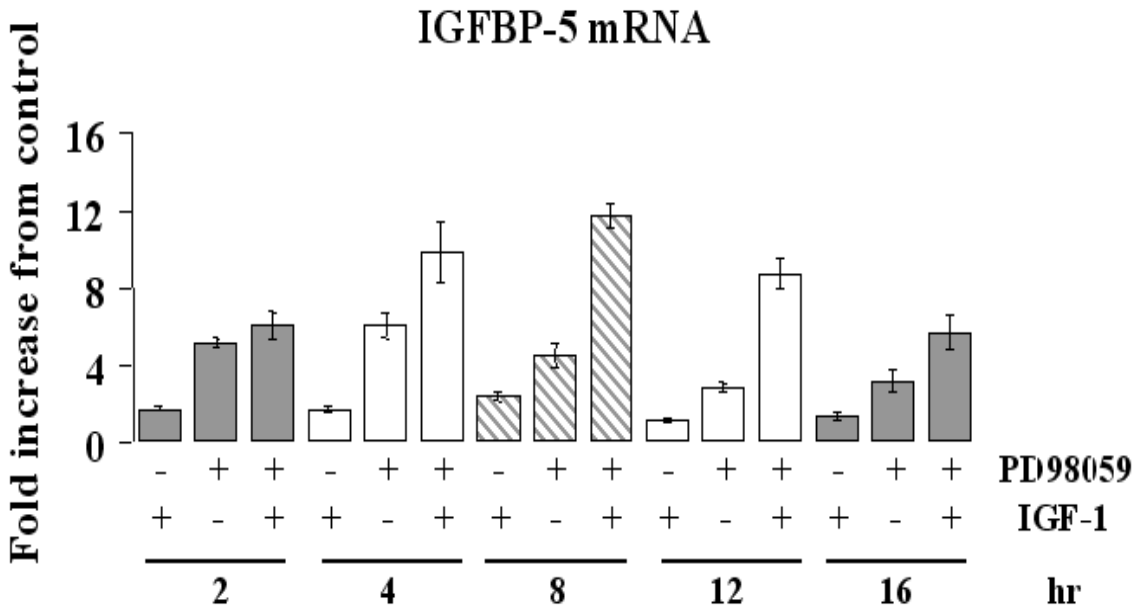


Figure 4. PD98059 ± IGF-I upregulates IGFBP-5 mRNA expression in MAC-T cells with peak expression occurring between 4 and 8 h. Confluent MAC-T cells were serum starved for 16 h, and then treated ± PD98059 (10 μM) ± IGF-I (100 ng/ml) for the indicated times. Total RNA was isolated and analyzed using quantitative RTPCR and normalized to cyclophilin. Graph represents mean ± S.E.M. of four separate experiments.

Figure 5

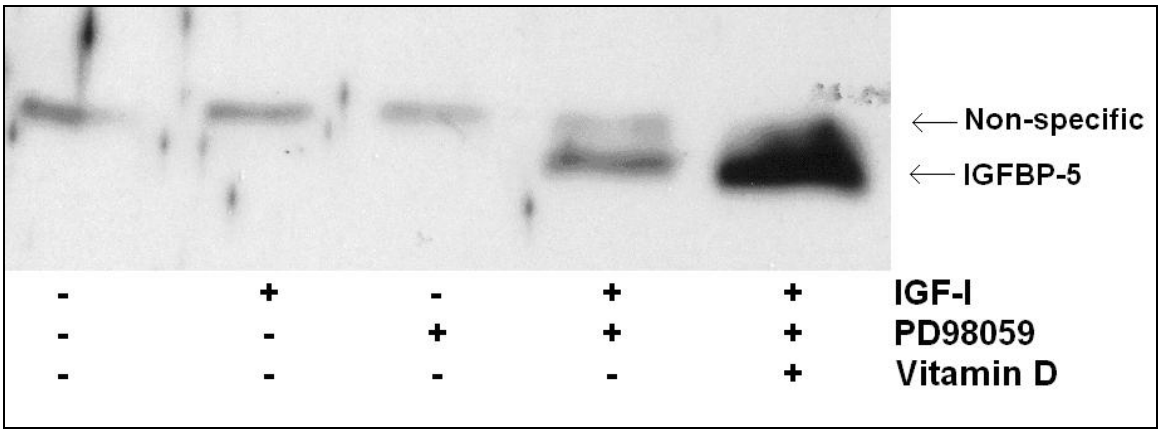


Figure 5. IGFBP-5 protein is increased in conditioned media of MAC-T cells by IGF-I, PD98059, and vitamin D. Confluent MAC-T cells were serum starved for 24 h before pretreatment with PD98059 (20 μM) for 30 min followed by simultaneous addition of IGF-I (100 ng/ml) and vitamin D (1 μM). After 24 h conditioned media were collected. Total proteins were separated by SDS-PAGE and Western immunoblotted for IGFBP-5.

Figure 6

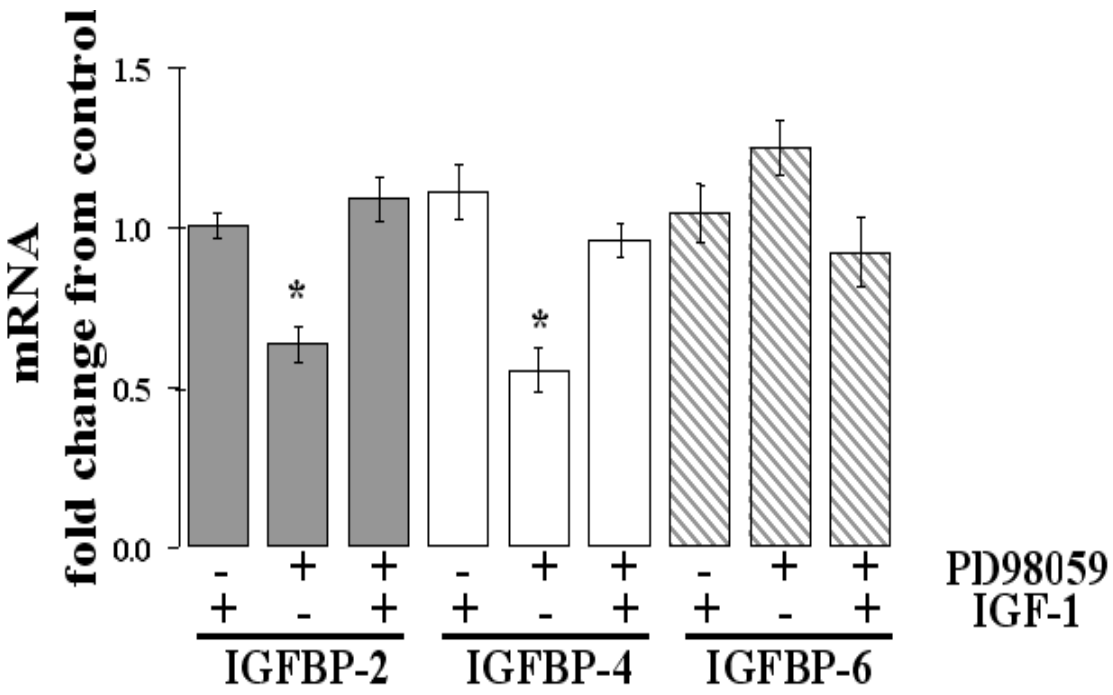


Figure 6. IGFBP-2, -4, and -6 mRNA levels in MAC-T cells are not increased by the MAPK inhibitor PD98059, and IGF-I. Confluent MAC-T cells were serum starved for 16 h, and then treated ± PD98059 (10 μM) and/or IGF-I (100 ng/ml) for 8 h. IGFBP-2 levels were determined by northern blotting and corrected for 18S. For IGFBP-4 and -6 total RNA was isolated and analyzed using quantitative RTPCR and normalized to cyclophilin. Graph represents mean S.E.M. of three separate experiments. * Indicates significant change from serum-free control ($P<0.05$)

Figure 7

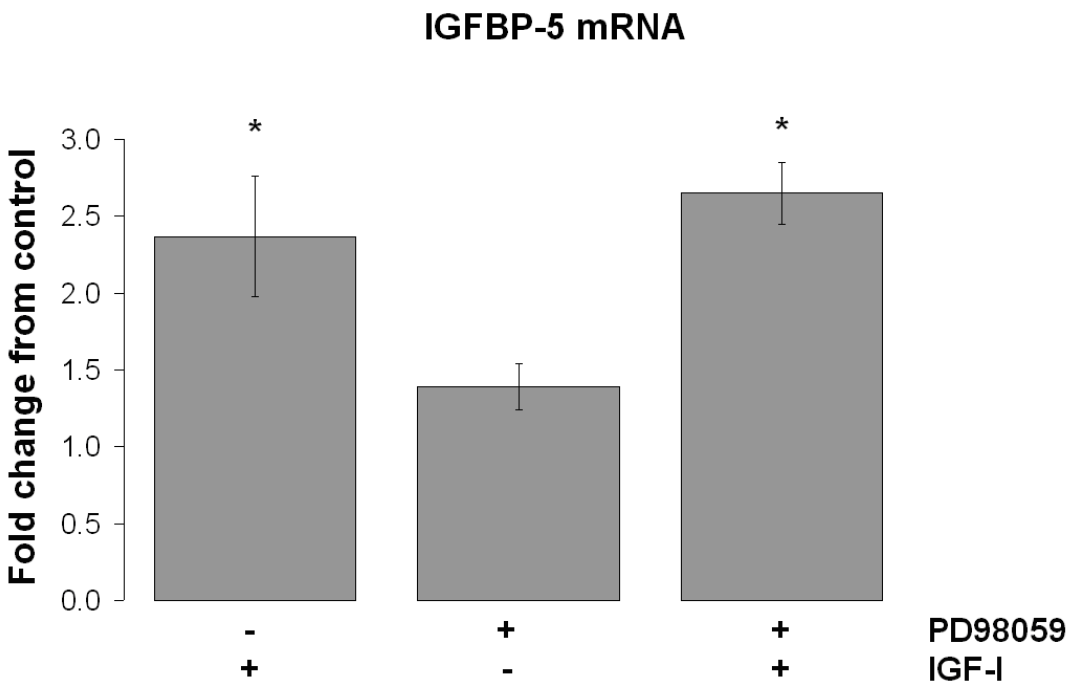


Figure 7. IGFBP-5 mRNA levels in human mammary epithelial cells are increased by IGF-I but not MAPK inhibitor PD98059. Confluent MCF-10A cells were serum starved for 16 h, and then treated ± PD98059 (20 μM) ± IGF-I (100 ng/ml) for 8 h. Total RNA was isolated and analyzed using quantitative RTPCR and normalized to cyclophilin. Graph represents mean ± S.E.M. of three experiments. * Indicates significant change from serum-free control ($P<0.05$).

Figure 8

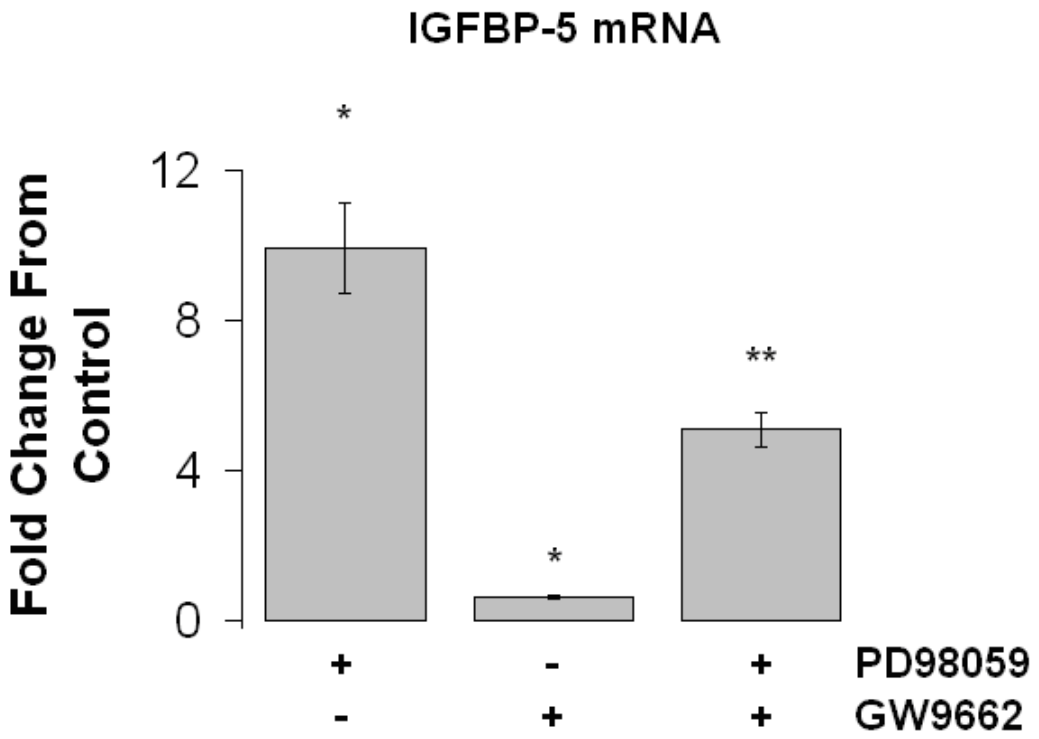


Figure 8. PPARγ antagonists partially blocks PD98059-induced IGFBP-5 mRNA in MAC-T cells. Confluent MAC-T cells were serum starved for 16 h, and then pretreated with PD98059 (20 μM) for thirty minutes, then treated ± GW9662 (25 μM) for 8 h. Total RNA was isolated and analyzed using quantitative RTPCR and normalized to cyclophilin. Graph represents mean ± S.E.M of three experiments. * Indicates significant change from serum-free control ($P<0.05$) ** Indicates significant change from PD98059 treated ($P<0.05$)

Figure 9

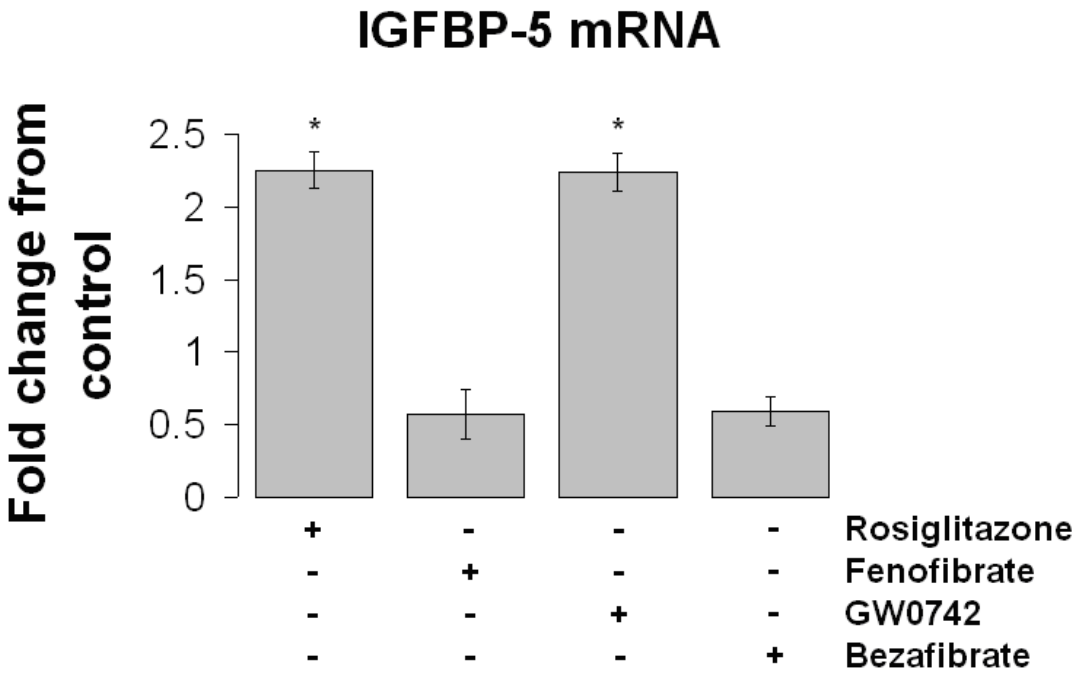


Figure 9. PPAR agonists increase IGFBP-5 mRNA expression in murine MECs Confluent NMuMG cells were serum starved for 16 h, and then treated with Rosiglitazone (20 μM), Fenofibrate (20 μM), GW0742 (2 μM), or Bezafibrate (200 μM) for 8 h. Total RNA was isolated and analyzed using quantitative RTPCR and normalized to cyclophilin. Graph represents mean ± S.E.M of three experiments. * Indicates significant change from serum-free control ($P<0.05$)

Figure 10

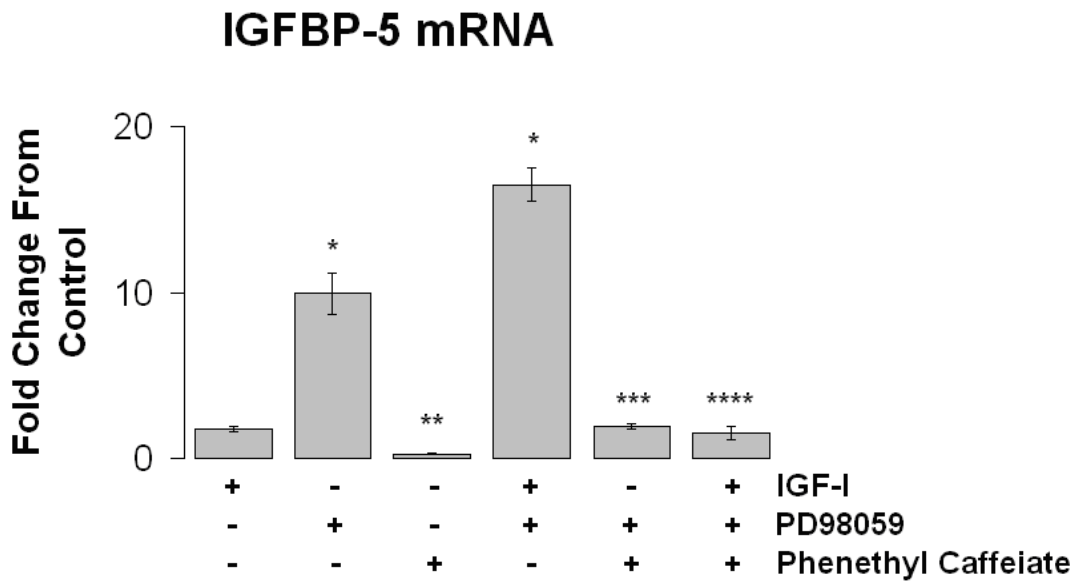


Figure 10. Inhibition of NFκB represses IGFBP-5 mRNA and protein expression in MAC-T cells. Confluent MAC-T cells were serum starved for 16 h, pretreated with the MAPK inhibitor PD98059 (20 μM) for thirty minutes, then treated ± IGF-I (100 ng/ml) ± phenethyl caffeiate (20 μM) for 8 h. Total RNA was isolated and analyzed using quantitative RTPCR and normalized to cyclophilin. Graph represents mean ± S.E.M. of three experiments. * Indicates significant change from serum-free control ($P<0.05$) ** Indicates a significant change from serum-free control ($T<0.05$) *** Indicates significant change from PD98059 treated sample ($P<0.05$) **** Indicates significant change from IGF-I + PD98059 treated sample ($P<0.05$)

Figure 11

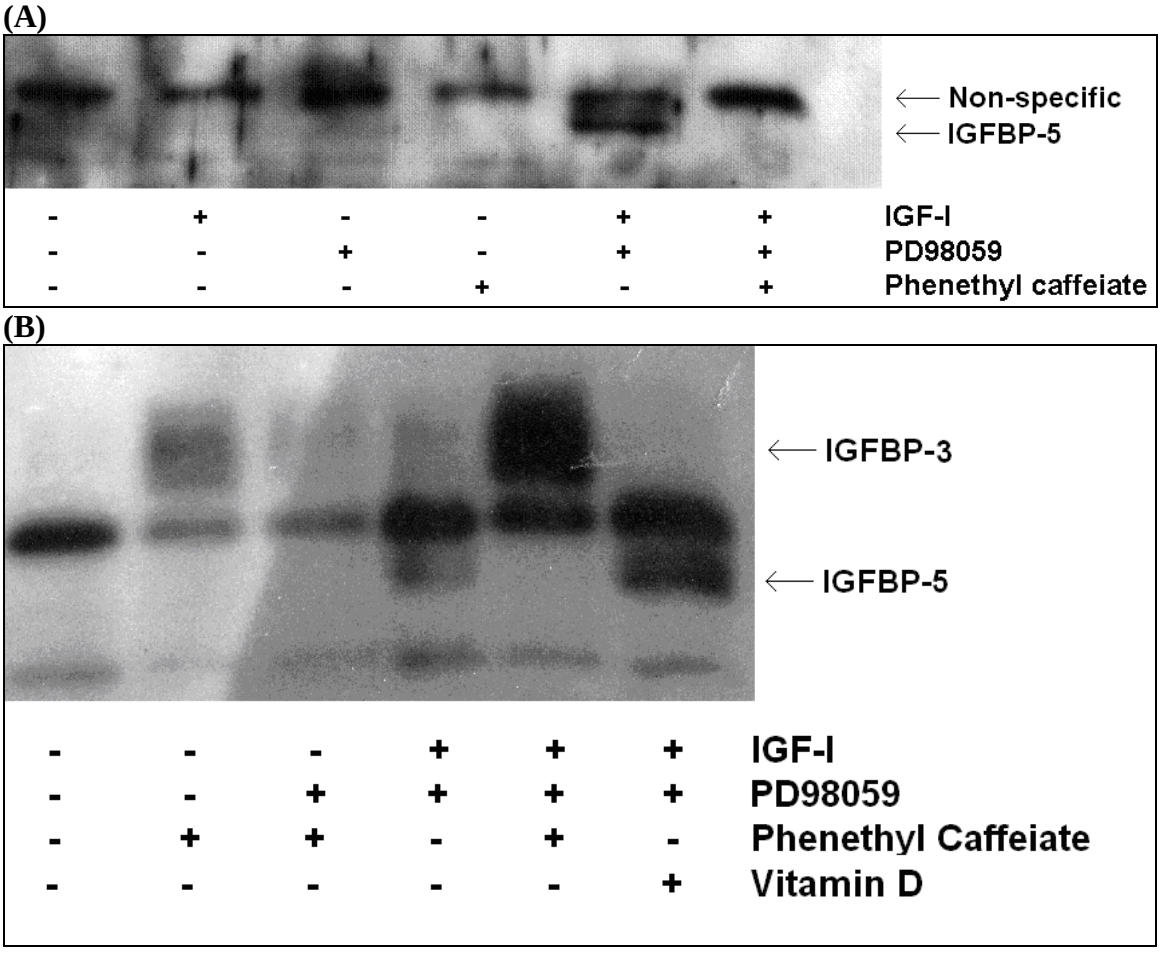


Figure 11. Inhibition of NFκB represses IGFBP-5 protein induced by PD98059 + IGF-I. Confluent MAC-T cells were serum starved for 24 h before pretreatment with PD98059 for 30 min followed by simultaneous addition of IGF-I ± phenethyl caffeiate. Conditioned media were collected after 24 h. Total protein (60 μg) were separated by SDS-PAGE. Membranes were immunoblotted with IGFBP-5 antibody (A) or ligand blotted with [^{125}I]-IGF-I (B).

Figure 12

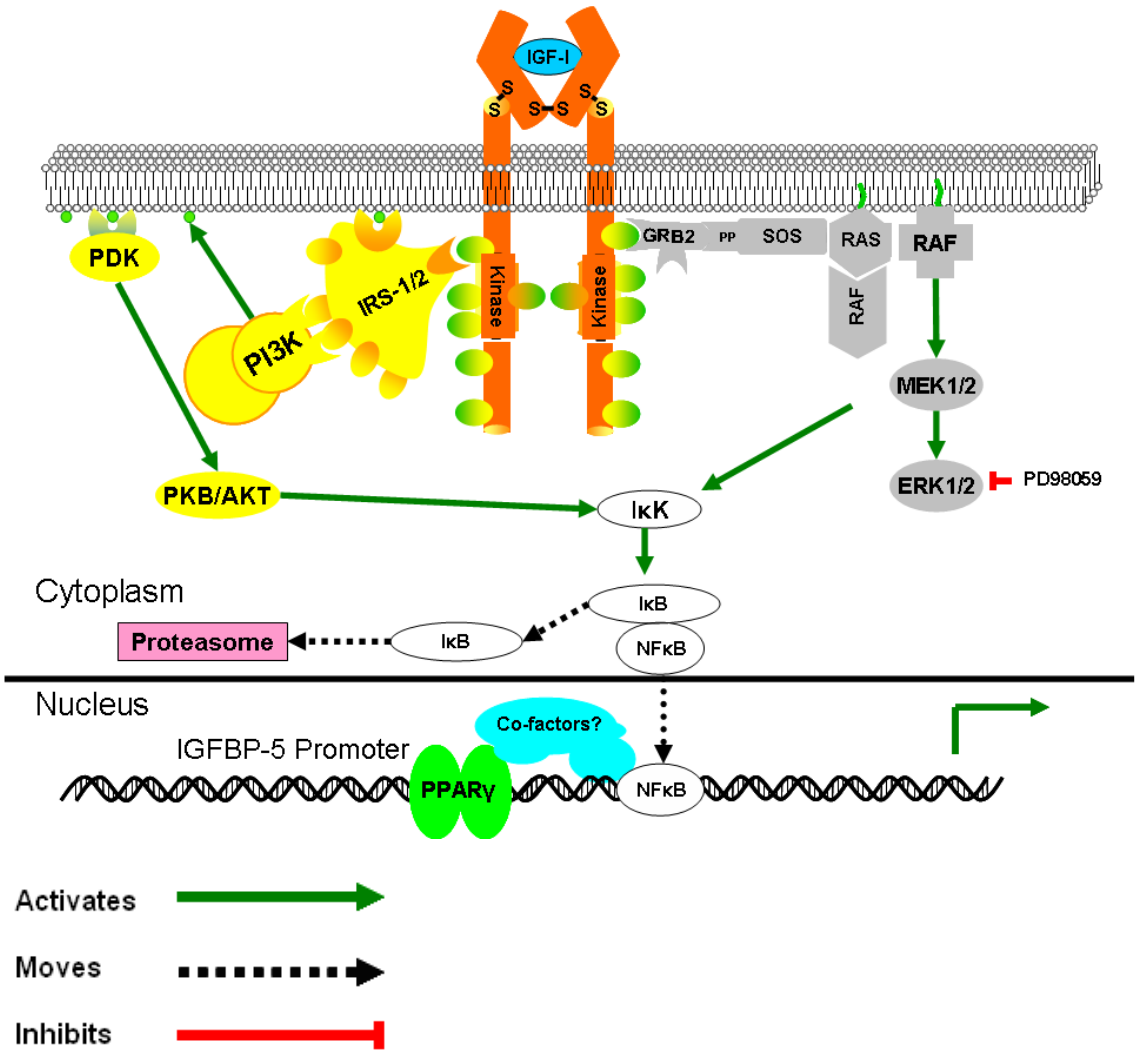


Figure 12. IGF-I targets the transcription factor NFκB to the IGFBP-5 promoter via the PI3K and MAPK Pathways. Blocking the phosphorylation of ERK1/2 allows PPARγ to remain in the nucleus to form a complex with NFκB.

References

1. **LeRoith D, Werner H, Beitner-Johnson D, Roberts CT, Jr.** 1995 Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 16:143-163
2. **Gluckman P, Klempt N, Guan J, Mallard C, Sirimanne E, Dragunow M, Klempt M, Singh K, Williams C, Nikolics K** 1992 A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. Biochem Biophys Res Commun 182:593-599
3. **Valentinis B, Baserga R** 2001 IGF-I receptor signalling in transformation and differentiation. Mol Pathol 54:133-137
4. **Hadsell DL, Bonnette SG, Lee AV** 2002 Genetic manipulation of the IGF-I axis to regulate mammary gland development and function. J Dairy Sci 85:365-377
5. **Yeh LC, Adamo ML, Duan C, Lee JC** 1998 Osteogenic protein-1 regulates insulin-like growth factor-I (IGF-I), IGF-II, and IGF-binding protein-5 (IGFBP-5) gene expression in fetal rat calvaria cells by different mechanisms. J Cell Physiol 175:78-88
6. **Li Q, Wu S, Li SY, Lopez FL, Du M, Kajstura J, Anversa P, Ren J** 2007 Cardiac-specific overexpression of insulin-like growth factor 1 attenuates aging-associated cardiac diastolic contractile dysfunction and protein damage. Am J Physiol Heart Circ Physiol 292:H1398-1403
7. **Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B, LeRoith D** 1999 Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A 96:7324-7329
8. **Mohan S, Baylink DJ** 1996 Insulin-like growth factor system components and the coupling of bone formation to resorption. Horm Res 45 Suppl 1:59-62
9. **Rajaram S, Baylink DJ, Mohan S** 1997 Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr Rev 18:801-831
10. **Coschigano KT, Holland AN, Riders ME, List EO, Flyvbjerg A, Kopchick JJ** 2003 Deletion, but not antagonism, of the mouse growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased life span. Endocrinology 144:3799-3810
11. **Scott CD, Martin JL, Baxter RC** 1985 Rat hepatocyte insulin-like growth factor I and binding protein: effect of growth hormone in vitro and in vivo. Endocrinology 116:1102-1107
12. **Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P, Le Bouc Y** 2003 IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 421:182-187
13. **Shimokawa I, Higami Y, Utsuyama M, Tuchiya T, Komatsu T, Chiba T, Yamaza H** 2002 Life span extension by reduction in growth hormone-insulin-like growth factor-1 axis in a transgenic rat model. Am J Pathol 160:2259-2265
14. **Melmed S, Yamashita S, Yamasaki H, Fagin J, Namba H, Yamamoto H, Weber M, Morita S, Webster J, Prager D** 1996 IGF-I receptor signalling: lessons from the somatotroph. Recent Prog Horm Res 51:189-215; discussion 215-186

15. **Baxter RC** 2000 Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am J Physiol Endocrinol Metab 278:E967-976
16. **Collett-Solberg PF, Cohen P** 2000 Genetics, chemistry, and function of the IGF/IGFBP system. Endocrine 12:121-136
17. **Ferry RJ, Jr., Cerri RW, Cohen P** 1999 Insulin-like growth factor binding proteins: new proteins, new functions. Horm Res 51:53-67
18. **Hwa V, Oh Y, Rosenfeld RG** 1999 The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev 20:761-787
19. **Firth SM, Baxter RC** 2002 Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23:824-854
20. **Ning Y, Schuller AG, Bradshaw S, Rotwein P, Ludwig T, Frystyk J, Pintar JE** 2006 Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. Mol Endocrinol 20:2173-2186
21. **Boisclair YR, Rhoads RP, Ueki I, Wang J, Ooi GT** 2001 The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. J Endocrinol 170:63-70
22. **De Meyts P** 1994 The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signalling. Diabetologia 37 Suppl 2:S135-148
23. **Nguyen KT, Wang WJ, Chan JL, Wang LH** 2000 Differential requirements of the MAP kinase and PI3 kinase signaling pathways in Src- versus insulin and IGF-1 receptors-induced growth and transformation of rat intestinal epithelial cells. Oncogene 19:5385-5397
24. **Esparis-Ogando A, Ocana A, Rodriguez-Barrueco R, Ferreira L, Borges J, Pandiella A** 2008 Synergic antitumoral effect of an IGF-IR inhibitor and trastuzumab on HER2-overexpressing breast cancer cells. Ann Oncol
25. **Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, Vigneri R** 2008 The role of insulin receptors and IGF-I receptors in cancer and other diseases. Arch Physiol Biochem 114:23-37
26. **Carboni JM, Lee AV, Hadsell DL, Rowley BR, Lee FY, Bol DK, Camuso AE, Gottardis M, Greer AF, Ho CP, Hurlburt W, Li A, Saulnier M, Velaparthi U, Wang C, Wen ML, Westhouse RA, Wittman M, Zimmermann K, Rupnow BA, Wong TW** 2005 Tumor development by transgenic expression of a constitutively active insulin-like growth factor I receptor. Cancer Res 65:3781-3787
27. **Jerome L, Shiry L, Leyland-Jones B** 2003 Deregulation of the IGF axis in cancer: epidemiological evidence and potential therapeutic interventions. Endocr Relat Cancer 10:561-578
28. **Braulke T** 1999 Type-2 IGF receptor: a multi-ligand binding protein. Horm Metab Res 31:242-246
29. **Pimentel E** 1987 Hormones, growth factors, and oncogenes. Boca Raton, Fla.: CRC Press
30. **Reinecke M, Collet C** 1998 The phylogeny of the insulin-like growth factors. Int Rev Cytol 183:1-94

31. **Daughaday WH, Rotwein P** 1989 Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr Rev 10:68-91
32. **Werner H, Weinstein D, Bentov I** 2008 Similarities and differences between insulin and IGF-I: structures, receptors, and signalling pathways. Arch Physiol Biochem 114:17-22
33. **Saha MS, Michel RB, Gulding KM, Grainger RM** 1993 A Xenopus homebox gene defines dorsal-ventral domains in the developing brain. Development 118:193-202
34. **Allander SV, Larsson C, Ehrenborg E, Suwanichkul A, Weber G, Morris SL, Bajalica S, Kiefer MC, Luthman H, Powell DR** 1994 Characterization of the chromosomal gene and promoter for human insulin-like growth factor binding protein-5. J Biol Chem 269:10891-10898
35. **Jones RA, Campbell CI, Gunther EJ, Chodosh LA, Petrik JJ, Khokha R, Moorehead RA** 2007 Transgenic overexpression of IGF-IR disrupts mammary ductal morphogenesis and induces tumor formation. Oncogene 26:1636-1644
36. **Sivaraman VS, Wang H, Nuovo GJ, Malbon CC** 1997 Hyperexpression of mitogen-activated protein kinase in human breast cancer. J Clin Invest 99:1478-1483
37. **Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, Cobb MH** 2001 MAP kinases. Chem Rev 101:2449-2476
38. **Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR** 1995 PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489-27494
39. **Davies SP, Reddy H, Caivano M, Cohen P** 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95-105
40. **Rosen LB, Ginty DD, Weber MJ, Greenberg ME** 1994 Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 12:1207-1221
41. **Grommes C, Landreth GE, Heneka MT** 2004 Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists. Lancet Oncol 5:419-429
42. **Nahle Z** 2004 PPAR trilogy from metabolism to cancer. Curr Opin Clin Nutr Metab Care 7:397-402
43. **Kato M, Kusumi T, Tsuchida S, Tanaka M, Sasaki M, Kudo H** 2004 Induction of differentiation and peroxisome proliferator-activated receptor gamma expression in colon cancer cell lines by troglitazone. J Cancer Res Clin Oncol 130:73-79
44. **Shimada T, Kojima K, Yoshiura K, Hiraishi H, Terano A** 2002 Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. Gut 50:658-664
45. **Adams M, Reginato MJ, Shao D, Lazar MA, Chatterjee VK** 1997 Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. J Biol Chem 272:5128-5132

46. **Camp HS, Tafuri SR** 1997 Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase. J Biol Chem 272:10811-10816
47. **Hu E, Kim JB, Sarraf P, Spiegelman BM** 1996 Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. Science 274:2100-2103
48. **Burgermeister E, Seger R** 2007 MAPK kinases as nucleo-cytoplasmic shuttles for PPARgamma. Cell Cycle 6:1539-1548
49. **Floyd ZE, Stephens JM** 2002 Interferon-gamma-mediated activation and ubiquitin-proteasome-dependent degradation of PPARgamma in adipocytes. J Biol Chem 277:4062-4068
50. **Paez J, Sellers WR** 2003 PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. Cancer Treat Res 115:145-167
51. **Kane LP, Shapiro VS, Stokoe D, Weiss A** 1999 Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol 9:601-604
52. **Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB** 1999 NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401:82-85
53. **Romashkova JA, Makarov SS** 1999 NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 401:86-90
54. **Kou K, Jenkins NA, Gilbert DJ, Copeland NG, Rotwein P** 1994 Organization, expression, and chromosomal location of the mouse insulin-like growth factor binding protein 5 gene. Genomics 20:412-418
55. **Zhu X, Ling N, Shimasaki S** 1993 Cloning of the rat insulin- like growth factor binding protein-5 gene and DNA sequence analysis of its promoter region. Biochem Biophys Res Commun 190:1045-1052
56. **White ME, Diao R, Hathaway MR, Mickelson J, Dayton WR** 1996 Molecular cloning and sequence analysis of the porcine insulin-like growth factor binding protein-5 complementary deoxyribonucleic acid. Biochem Biophys Res Commun 218:248-253
57. **Forbes BE, Turner D, Hodge SJ, McNeil KA, Forsberg G, Wallace JC** 1998 Localization of an insulin-like growth factor (IGF) binding site of bovine IGF binding protein-2 using disulfide mapping and deletion mutation analysis of the C-terminal domain. J Biol Chem 273:4647-4652
58. **Hashimoto R, Ono M, Fujiwara H, Higashihashi N, Yoshida M, Enjoh-Kimura T, Sakano K** 1997 Binding sites and binding properties of binary and ternary complexes of insulin-like growth factor-II (IGF-II), IGF-binding protein-3, and acid-labile subunit. J Biol Chem 272:27936-27942
59. **Kalus W, Zweckstetter M, Renner C, Sanchez Y, Georgescu J, Grol M, Demuth D, Schumacher R, Dony C, Lang K, Holak TA** 1998 Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. Embo J 17:6558-6572
60. **Neumann GM, Bach LA** 1999 The N-terminal disulfide linkages of human insulin-like growth factor-binding protein-6 (hIGFBP-6) and hIGFBP-1 are different as determined by mass spectrometry. J Biol Chem 274:14587-14594

61. **Siwanowicz I, Popowicz GM, Wisniewska M, Huber R, Kuenkele KP, Lang K, Engh RA, Holak TA** 2005 Structural basis for the regulation of insulin-like growth factors by IGF binding proteins. Structure 13:155-167
62. **Standker L, Braulke T, Mark S, Mostafavi H, Meyer M, Honing S, Gimenez-Gallego G, Forssmann WG** 2000 Partial IGF affinity of circulating N- and C-terminal fragments of human insulin-like growth factor binding protein-4 (IGFBP-4) and the disulfide bonding pattern of the C-terminal IGFBP-4 domain. Biochemistry 39:5082-5088
63. **Headey SJ, Leeding KS, Norton RS, Bach LA** 2004 Contributions of the N- and C-terminal domains of IGF binding protein-6 to IGF binding. J Mol Endocrinol 33:377-386
64. **Fowlkes JL, Thrailkill KM, George-Nascimento C, Rosenberg CK, Serra DM** 1997 Heparin-binding, highly basic regions within the thyroglobulin type-1 repeat of insulin-like growth factor (IGF)-binding proteins (IGFBPs) -3, -5, and -6 inhibit IGFBP-4 degradation. Endocrinology 138:2280-2285
65. **Bramani S, Song H, Beattie J, Tonner E, Flint DJ, Allan GJ** 1999 Amino acids within the extracellular matrix (ECM) binding region (201-218) of rat insulin-like growth factor binding protein (IGFBP)-5 are important determinants in binding IGF-I. J Mol Endocrinol 23:117-123
66. **Song H, Beattie J, Campbell IW, Allan GJ** 2000 Overlap of IGF- and heparin-binding sites in rat IGF-binding protein-5. J Mol Endocrinol 24:43-51
67. **Andress DL, Birnbaum RS** 1992 Human osteoblast-derived insulin-like growth factor (IGF) binding protein-5 stimulates osteoblast mitogenesis and potentiates IGF action. J Biol Chem 267:22467-22472
68. **Imai Y, Moralez A, Andag U, Clarke JB, Busby WH, Jr., Clemmons DR** 2000 Substitutions for hydrophobic amino acids in the N-terminal domains of IGFBP-3 and -5 markedly reduce IGF-I binding and alter their biologic actions. J Biol Chem 275:18188-18194
69. **Campbell PG, Andress DL** 1997 Plasmin degradation of insulin-like growth factor-binding protein-5 (IGFBP-5): regulation by IGFBP-5-(201-218). Am J Physiol 273:E996-1004
70. **Gibson TL, Cohen P** 1999 Inflammation-related neutrophil proteases, cathepsin G and elastase, function as insulin-like growth factor binding protein proteases. Growth Horm IGF Res 9:241-253
71. **Soe R, Overgaard MT, Thomsen AR, Laursen LS, Olsen IM, Sottrup-Jensen L, Haaning J, Giudice LC, Conover CA, Oxvig C** 2002 Expression of recombinant murine pregnancy-associated plasma protein-A (PAPP-A) and a novel variant (PAPP-Ai) with differential proteolytic activity. Eur J Biochem 269:2247-2256
72. **Zheng B, Clarke JB, Busby WH, Duan C, Clemmons DR** 1998 Insulin-like growth factor-binding protein-5 is cleaved by physiological concentrations of thrombin. Endocrinology 139:1708-1714
73. **Imai Y, Busby WH, Jr., Smith CE, Clarke JB, Garmong AJ, Horwitz GD, Rees C, Clemmons DR** 1997 Protease-resistant form of insulin-like growth factor-binding protein 5 is an inhibitor of insulin-like growth factor-I actions on porcine smooth muscle cells in culture. J Clin Invest 100:2596-2605

74. **Twigg SM, Kiefer MC, Zapf J, Baxter RC** 2000 A central domain binding site in insulin-like growth factor binding protein-5 for the acid-labile subunit. Endocrinology 141:454-457
75. **Graham ME, Kilby DM, Firth SM, Robinson PJ, Baxter RC** 2007 The in vivo phosphorylation and glycosylation of human insulin-like growth factor-binding protein-5. Mol Cell Proteomics 6:1392-1405
76. **Conover CA, Kiefer MC** 1993 Regulation and biological effect of endogenous insulin-like growth factor binding protein-5 in human osteoblastic cells. J Clin Endocrinol Metab 76:1153-1159
77. **Firth SM, Baxter RC** 1999 Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3. J Endocrinol 160:379-387
78. **Marinaro JA, Neumann GM, Russo VC, Leeding KS, Bach LA** 2000 O-glycosylation of insulin-like growth factor (IGF) binding protein-6 maintains high IGF-II binding affinity by decreasing binding to glycosaminoglycans and susceptibility to proteolysis. Eur J Biochem 267:5378-5386
79. **Jones JI, Gockerman A, Busby WH, Jr., Camacho-Hubner C, Clemmons DR** 1993 Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J Cell Biol 121:679-687
80. **Coverley JA, Baxter RC** 1995 Regulation of insulin-like growth factor (IGF) binding protein-3 phosphorylation by IGF-I. Endocrinology 136:5778-5781
81. **Butt AJ, Dickson KA, Jambazov S, Baxter RC** 2005 Enhancement of tumor necrosis factor-alpha-induced growth inhibition by insulin-like growth factor-binding protein-5 (IGFBP-5), but not IGFBP-3 in human breast cancer cells. Endocrinology 146:3113-3122
82. **Cobb LJ, Salih DA, Gonzalez I, Tripathi G, Carter EJ, Lovett F, Holding C, Pell JM** 2004 Partitioning of IGFBP-5 actions in myogenesis: IGF-independent anti-apoptotic function. J Cell Sci 117:1737-1746
83. **Nam TJ, Busby WH, Jr., Rees C, Clemmons DR** 2000 Thrombospondin and osteopontin bind to insulin-like growth factor (IGF)-binding protein-5 leading to an alteration in IGF-I-stimulated cell growth. Endocrinology 141:1100-1106
84. **Salih DA, Tripathi G, Holding C, Szestak TA, Gonzalez MI, Carter EJ, Cobb LJ, Eisemann JE, Pell JM** 2004 Insulin-like growth factor-binding protein 5 (Igfbp5) compromises survival, growth, muscle development, and fertility in mice. Proc Natl Acad Sci U S A 101:4314-4319
85. **Donovan SM, Hintz RL, Rosenfeld RG** 1995 Investigation into the potential physiological sources of rat milk IGF-I and IGF-binding proteins. J Endocrinol 145:569-578
86. **Boutinaud M, Shand JH, Park MA, Phillips K, Beattie J, Flint DJ, Allan GJ** 2004 A quantitative RT-PCR study of the mRNA expression profile of the IGF axis during mammary gland development. J Mol Endocrinol 33:195-207
87. **Flint DJ, Boutinaud M, Tonner E, Wilde CJ, Hurley W, Accorsi PA, Kolb AF, Whitelaw CB, Beattie J, Allan GJ** 2005 Insulin-like growth factor binding proteins initiate cell death and extracellular matrix remodeling in the mammary gland. Domest Anim Endocrinol 29:274-282
88. **Phillips K, Park MA, Quarrie LH, Boutinaud M, Lochrie JD, Flint DJ, Allan GJ, Beattie J** 2003 Hormonal control of IGF-binding protein (IGFBP)-5 and

IGFBP-2 secretion during differentiation of the HC11 mouse mammary epithelial cell line. J Mol Endocrinol 31:197-208

89. **Beattie J, Allan GJ, Lochrie JD, Flint DJ** 2006 Insulin-like growth factor-binding protein-5 (IGFBP-5): a critical member of the IGF axis. Biochem J 395:1-19
90. **Tonner E, Barber MC, Allan GJ, Beattie J, Webster J, Whitelaw CB, Flint DJ** 2002 Insulin-like growth factor binding protein-5 (IGFBP-5) induces premature cell death in the mammary glands of transgenic mice. Development 129:4547-4557
91. **Marshman E, Green KA, Flint DJ, White A, Streuli CH, Westwood M** 2003 Insulin-like growth factor binding protein 5 and apoptosis in mammary epithelial cells. J Cell Sci 116:675-682
92. **Allan GJ, Tonner E, Barber MC, Travers MT, Shand JH, Vernon RG, Kelly PA, Binart N, Flint DJ** 2002 Growth hormone, acting in part through the insulin-like growth factor axis, rescues developmental, but not metabolic, activity in the mammary gland of mice expressing a single allele of the prolactin receptor. Endocrinology 143:4310-4319
93. **Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A** 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75:59-72
94. **Kiepe D, Ciarmatori S, Haarmann A, Tonshoff B** 2006 Differential expression of IGF system components in proliferating vs. differentiating growth plate chondrocytes: the functional role of IGFBP-5. Am J Physiol Endocrinol Metab 290:E363-371
95. **Olney RC, Mougey EB** 1999 Expression of the components of the insulin-like growth factor axis across the growth-plate. Mol Cell Endocrinol 156:63-71
96. **Birnbaum RS, Wiren KM** 1994 Changes in insulin-like growth factor-binding protein expression and secretion during the proliferation, differentiation, and mineralization of primary cultures of rat osteoblasts. Endocrinology 135:223-230
97. **Miyakoshi N, Richman C, Kasukawa Y, Linkhart TA, Baylink DJ, Mohan S** 2001 Evidence that IGF-binding protein-5 functions as a growth factor. J Clin Invest 107:73-81
98. **Devlin RD, Du Z, Buccilli V, Jorgetti V, Canalis E** 2002 Transgenic mice overexpressing insulin-like growth factor binding protein-5 display transiently decreased osteoblastic function and osteopenia. Endocrinology 143:3955-3962
99. **Schedlich LJ, Muthukaruppan A, O'Han MK, Baxter RC** 2007 Insulin-like growth factor binding protein-5 interacts with the vitamin D receptor and modulates the vitamin D response in osteoblasts. Mol Endocrinol 21:2378-2390
100. **Arai T, Arai A, Busby WH, Jr., Clemmons DR** 1994 Glycosaminoglycans inhibit degradation of insulin-like growth factor-binding protein-5. Endocrinology 135:2358-2363
101. **McCaig C, Perks CM, Holly JM** 2002 Intrinsic actions of IGFBP-3 and IGFBP-5 on Hs578T breast cancer epithelial cells: inhibition or accentuation of attachment and survival is dependent upon the presence of fibronectin. J Cell Sci 115:4293-4303

102. **Berfield AK, Andress DL, Abrass CK** 2000 IGFBP-5(201-218) stimulates Cdc42GAP aggregation and filopodia formationin migrating mesangial cells. Kidney Int 57:1991-2003
103. **Nam TJ, Busby W, Jr., Clemmons DR** 1997 Insulin-like growth factor binding protein-5 binds to plasminogen activator inhibitor-I. Endocrinology 138:2972-2978
104. **Tonner E, Barber MC, Travers MT, Logan A, Flint DJ** 1997 Hormonal control of insulin-like growth factor-binding protein-5 production in the involuting mammary gland of the rat. Endocrinology 138:5101-5107
105. **Radulescu RT** 1994 Nuclear localization signal in insulin-like growth factor-binding protein type 3. Trends Biochem Sci 19:278
106. **Schedlich LJ, Le Page SL, Firth SM, Briggs LJ, Jans DA, Baxter RC** 2000 Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin beta subunit. J Biol Chem 275:23462-23470
107. **Schedlich LJ, Young TF, Firth SM, Baxter RC** 1998 Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. J Biol Chem 273:18347-18352
108. **Amaar YG, Thompson GR, Linkhart TA, Chen ST, Baylink DJ, Mohan S** 2002 Insulin-like growth factor-binding protein 5 (IGFBP-5) interacts with a four and a half LIM protein 2 (FHL2). J Biol Chem 277:12053-12060
109. **Flint DJ, Boutinaud M, Whitelaw CB, Allan GJ, Kolb AF** 2006 Prolactin inhibits cell loss and decreases matrix metalloproteinase expression in the involuting mouse mammary gland but fails to prevent cell loss in the mammary glands of mice expressing IGFBP-5 as a mammary transgene. J Mol Endocrinol 36:435-448
110. **Boonyaratanakornkit V, Strong DD, Mohan S, Baylink DJ, Beck CA, Linkhart TA** 1999 Progesterone stimulation of human insulin-like growth factor-binding protein-5 gene transcription in human osteoblasts is mediated by a CACCC sequence in the proximal promoter. J Biol Chem 274:26431-26438
111. **Gregory CW, Kim D, Ye P, D'Ercole AJ, Pretlow TG, Mohler JL, French FS** 1999 Androgen receptor up-regulates insulin-like growth factor binding protein-5 (IGFBP-5) expression in a human prostate cancer xenograft. Endocrinology 140:2372-2381
112. **Huynh H** 1998 Suppression of uterine insulin-like growth factor binding protein 5 by estrogen is mediated in part by insulin-like growth factor I. Int J Oncol 12:427-432
113. **Huynh H, Yang XF, Pollak M** 1996 A role for insulin-like growth factor binding protein 5 in the antiproliferative action of the antiestrogen ICI 182780. Cell Growth Differ 7:1501-1506
114. **Okazaki R, Riggs BL, Conover CA** 1994 Glucocorticoid regulation of insulin-like growth factor-binding protein expression in normal human osteoblast-like cells. Endocrinology 134:126-132
115. **Allan GJ, Zannoni A, McKinnell I, Otto WR, Holzenberger M, Flint DJ, Patel K** 2003 Major components of the insulin-like growth factor axis are expressed early in chicken embryogenesis, with IGF binding protein (IGFBP) -5

expression subject to regulation by Sonic Hedgehog. Anat Embryol (Berl) 207:73-84

116. **Hwa V, Oh Y, Rosenfeld RG** 1997 Insulin-like growth factor binding protein-3 and -5 are regulated by transforming growth factor-beta and retinoic acid in the human prostate adenocarcinoma cell line PC-3. Endocrine 6:235-242
117. **Duan C, Hawes SB, Prevette T, Clemmons DR** 1996 Insulin-like growth factor-I (IGF-I) regulates IGF-binding protein-5 synthesis through transcriptional activation of the gene in aortic smooth muscle cells. J Biol Chem 271:4280-4288
118. **Fleming JM, Brandimarto JA, Cohick WS** 2007 The mitogen-activated protein kinase pathway tonically inhibits both basal and IGF-I-stimulated IGF-binding protein-5 production in mammary epithelial cells. J Endocrinol 194:349-359
119. **Ye P, D'Ercole J** 1998 Insulin-like growth factor I (IGF-I) regulates IGF binding protein-5 gene expression in the brain. Endocrinology 139:65-71
120. **Fleming JM, Leibowitz BJ, Kerr DE, Cohick WS** 2005 IGF-I differentially regulates IGF-binding protein expression in primary mammary fibroblasts and epithelial cells. J Endocrinol 186:165-178
121. **Allar MA, Wood TL** 2004 Expression of the insulin-like growth factor binding proteins during postnatal development of the murine mammary gland. Endocrinology 145:2467-2477
122. **Lochrie JD, Phillips K, Tonner E, Flint DJ, Allan GJ, Price NC, Beattie J** 2006 Insulin-like growth factor binding protein (IGFBP)-5 is upregulated during both differentiation and apoptosis in primary cultures of mouse mammary epithelial cells. J Cell Physiol 207:471-479
123. **Huynh HT, Robitaille G, Turner JD** 1991 Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp Cell Res 197:191-199
124. **Zavizion B, Gorewit RC, Politis I** 1995 Subcloning the MAC-T bovine mammary epithelial cell line: morphology, growth properties, and cytogenetic analysis of clonal cells. J Dairy Sci 78:515-527
125. **Zhao X, McBride BW, Politis I, Huynh HT, Akers RM, Burton JH, Turner JD** 1992 Receptor binding and growth-promoting activity of insulin-like growth factor-I in a bovine mammary epithelial cell line (MAC-T3). J Endocrinol 134:307-312
126. **Fleming JM, Desury G, Polanco TA, Cohick WS** 2006 Insulin growth factor-I and epidermal growth factor receptors recruit distinct upstream signaling molecules to enhance AKT activation in mammary epithelial cells. Endocrinology 147:6027-6035
127. **Gyrup C, Oxvig C** 2007 Quantitative analysis of insulin-like growth factor-modulated proteolysis of insulin-like growth factor binding protein-4 and -5 by pregnancy-associated plasma protein-A. Biochemistry 46:1972-1980
128. **Cheng HL, Shy M, Feldman EL** 1999 Regulation of insulin-like growth factor-binding protein-5 expression during Schwann cell differentiation. Endocrinology 140:4478-4485
129. **Duan C, Liimatta MB, Bottum OL** 1999 Insulin-like growth factor (IGF)-I regulates IGF-binding protein-5 gene expression through the phosphatidylinositol

3-kinase, protein kinase B/Akt, and p70 S6 kinase signaling pathway. J Biol Chem 274:37147-37153

130. **Xin X, Hou YT, Li L, Schmiedlin-Ren P, Christman GM, Cheng HL, Bitar KN, Zimmermann EM** 2004 IGF-I increases IGFBP-5 and collagen alpha1(I) mRNAs by the MAPK pathway in rat intestinal smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 286:G777-783
131. **Kuemmerle JF** 2000 Endogenous IGF-I regulates IGF binding protein production in human intestinal smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 278:G710-717
132. **Takaoka M, Smith CE, Mashiba MK, Okawa T, Andl CD, El-Deiry WS, Nakagawa H** 2006 EGF-mediated regulation of IGFBP-3 determines esophageal epithelial cellular response to IGF-I. Am J Physiol Gastrointest Liver Physiol 290:G404-416
133. **Burgermeister E, Chuderland D, Hanoch T, Meyer M, Liscovitch M, Seger R** 2007 Interaction with MEK causes nuclear export and downregulation of peroxisome proliferator-activated receptor gamma. Mol Cell Biol 27:803-817
134. **Lewis DA, Spandau DF** 2008 UVB-induced activation of NF-kappaB is regulated by the IGF-1R and dependent on p38 MAPK. J Invest Dermatol 128:1022-1029
135. **Mitsiades CS, Mitsiades N, Poulaki V, Schlossman R, Akiyama M, Chauhan D, Hideshima T, Treon SP, Munshi NC, Richardson PG, Anderson KC** 2002 Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene 21:5673-5683
136. **Pons S, Torres-Aleman I** 2000 Insulin-like growth factor-I stimulates dephosphorylation of ikappa B through the serine phosphatase calcineurin (protein phosphatase 2B). J Biol Chem 275:38620-38625
137. **Baumann B, Weber CK, Troppmair J, Whiteside S, Israel A, Rapp UR, Wirth T** 2000 Raf induces NF-kappaB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. Proc Natl Acad Sci U S A 97:4615-4620
138. **Couturier C, Brouillet A, Couriaud C, Koumanov K, Bereziat G, Andreani M** 1999 Interleukin 1beta induces type II-secreted phospholipase A(2) gene in vascular smooth muscle cells by a nuclear factor kappaB and peroxisome proliferator-activated receptor-mediated process. J Biol Chem 274:23085-23093
139. **Sun YX, Wright HT, Janciauskiene S** 2002 Alpha1-antichymotrypsin/Alzheimer's peptide Abeta(1-42) complex perturbs lipid metabolism and activates transcription factors PPARgamma and NFkappaB in human neuroblastoma (Kelly) cells. J Neurosci Res 67:511-522
140. **Schlezinger JJ, Jensen BA, Mann KK, Ryu HY, Sherr DH** 2002 Peroxisome proliferator-activated receptor gamma-mediated NF-kappa B activation and apoptosis in pre-B cells. J Immunol 169:6831-6841
141. **Baxter FO, Came PJ, Abell K, Kedjouar B, Huth M, Rajewsky K, Pasparakis M, Watson CJ** 2006 IKKbeta/2 induces TWEAK and apoptosis in mammary epithelial cells. Development 133:3485-3494

142. **Zaragoza R, Miralles VJ, Rus AD, Garcia C, Carmena R, Garcia-Trevijano ER, Barber T, Pallardo FV, Torres L, Vina JR** 2005 Weaning induces NOS-2 expression through NF-kappaB modulation in the lactating mammary gland: importance of GSH. Biochem J 391:581-588

143. **Kritikou EA, Sharkey A, Abell K, Came PJ, Anderson E, Clarkson RW, Watson CJ** 2003 A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland. Development 130:3459-3468