Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

INSULIN ANALOGUES: ANALYSIS OF PROLIFERATIVE POTENCY AND CHARACTERIZATION OF RECEPTORS AND SIGNALLING PATHWAYS ACTIVATED IN HUMAN MAMMARY EPITHELIAL CELLS

Presented by

Ashish Shukla

(Born in Allahabad, India)

Heidelberg, 2008

INSULIN ANALOGUES:

ANALYSIS OF PROLIFERATIVE POTENCY AND CHARACTERIZATION OF RECEPTORS AND SIGNALLING PATHWAYS ACTIVATED IN HUMAN MAMMARY EPITHELIAL CELLS

Referees: **Prof. Dr. Doris Mayer**

Prof. Dr. Lutz Gissmann

To my Maa, Pitashri and Anurag

For inspiration and wishes

TABLE OF CONTENTS

ZUSAMMENFASSUNG

Insulinanaloga wurden entwickelt mit dem Ziel den Blutzuckerspiegel bei Diabetikern besser zu kontrollieren. Insulinanaloga haben im Vergleich zu Normalinsulin eine veränderte Aminosäuresequenz. Dies hat Veränderungen in relevanten biochemischen Eigenschaften zur Folge, z.B. in der Affinität für den Insulinrezeptor (IR) und den Typ-I Insulin-like Growth Factor Receptor (IGF-IR), sowie in der Dissoziationsgeschwindigkeit vom IR. Das Ergebnis kann eine erhöhte mitogene Aktivität der Insulinanaloga im Vergleich zu Normalinsulin sein. Normales Brustdrüsenepithel zeigt eine starke Expression von IR und IGF-IR, und Brustkrebszellen zeigen häufig sogar eine Überexpression beider Rezeptoren. Aus diesem Grund ist das Brustdrüsenepithel ein empfindliches Zielorgan für Insulinanaloga und deren proliferationssteigernde Wirkung. In der Tat resultierte die Behandlung weiblicher Sprague-Dawley Ratten mit dem Insulinanalogon B10Asp in einer signifikanten Zunahme der Inzidenz von Mammakarzinomen im Vergleich zu Normalinsulin. Von allen Insulinanaloga, die heutzutage therapeutisch eingesetzt werden, wurde nur für Insulin Glargin (Lantus®) eine standardisierte zweijährige Karzinogenitätsstudie durchgeführt. In dieser Studie zeigten Insulin Glargin und Normalinsulin keine signifikanten Unterschiede in der Tumorinzidenz. Allerdings wurde eine extrem hohe Mortalitätsrate bei allen behandelten Tiergruppen berichtet, was die Schlussfolgerung, dass Insulin Glargin das Krebsrisiko nicht erhöht, infrage stellt. Es gibt nur wenige *in vitro*-Studien zur proliferativen Wirkung von Insulinanaloga auf Brustzelllinien und diese sind wenig aussagekräftig. Auch wurde der biochemische Mechanismus der proliferativen Wirkung von Insulinanaloga nicht geklärt. Die vorliegende Arbeit hatte das Ziel, die proliferative Potenz therapeutisch eingesetzter Insulinanaloga detailliert in insulinresponsiven epithelialen Brustzelllinien zu untersuchen und zu vergleichen und den molekularen und biochemischen Mechanismus der proliferativen Wirkung aufzuklären. Die Rolle des IR und des IGF-IR und der entsprechenden Signal-transduktionswege wurde analysiert für Normalinsulin und das Insulinanalogon, das den stärksten proliferativen Effekt zeigte.

Aus einer Gruppe von sieben Zelllinien zeigten MCF7 Zellen (eine Tumorzelllinie) und MCF10A Zellen (eine benigne Zelllinie) die stärkste proliferative Antwort auf Insulin. In Proliferationsassays mit MCF10A Zellen zeigten die vier untersuchten Insulinanaloga (Insulin Aspart, Insulin Lispro, Insulin Glargin und Insulin Detemir) gleich starke Wirkung wie Normalinsulin (menschliches Insulin und Rinderinsulin). In Proliferationsassays mit MCF7 Zellen induzierte Insulin Glargin jedoch eine signifikant stärkere Proliferation als Normalinsulin und die anderen drei Insulinanaloga. Dieser Befund wurde durch BrdU-Inkorporationstests an MCF7 Zellen bestätigt.

Die Aktivierung der beiden durch Insulin stimulierten Signalwege – PI3K und MAPK Signalweg – wurde durch Bestimmung des Phosphorylierungsgrads wichtiger Signalmoleküle (Akt und GSK3α/β für den PI3K-Weg und Erk1/2 für den MAPK-Weg) untersucht. In

MCF10A Zellen war die Phosphorylierung von GSK3α/β und Erk1/2 nach Behandlung mit allen Insulinanaloga und Normalinsulin gleich stark. Interessanterweise war jedoch die Phosphorylierung von Akt nach Insulin Glargin - Behandlung signifikant stärker als nach Behandlung mit Normalinsulin. In MCF7 Zellen verursachte Insulin Glargin eine sehr starke Phosphorylierung aller drei Signalproteine. Diese war signifikant stärker als bei Normal-insulin und den anderen Insulinanaloga. Die Verwendung spezifischer Inhibitoren in Proliferationsassays ergab, dass Insulin Glargin in MCF7 Zellen hauptsächlich den MAPK-Weg aktiviert.

Um die Rolle von IR und IGF-IR bei der starken mitogenen Aktivität von Insulin Glargin zu klären, wurden IR und IGF-IR durch RNAi-Technik jeweils spezifisch herunterreguliert. Die Aktivierung der Signalwege und Proliferation unter Knockdown-Bedingungen ergab eindeutig, dass Insulin Glargin den IGF-IR aktiviert, während die anderen Substanzen den IR aktivieren. Daraus folgt, dass die erhöhte proliferative Aktivität von Insulin Glargin auf der Aktivierung des IGF-IR beruht. Immunpräzipitation des IGF-IR und anschließende Analyse des Tyrosinphosphorylierungsgrades zeigten eine wesentlich stärkere Phosphorylierung in Zellen, die mit Insulin Glargin behandelt worden waren. Dies erhärtet die Befunde aus den Knockdown-Experimenten. Weiterhin wurden die Expressionsspiegel von Cyclin D1, eines IGF-I Zielgens, durch quantitative RT-PCR bestimmt. Sie ergaben eine höhere Expression von Cyclin D1 in Insulin Glargin - behandelten Zellen im Vergleich zu Normalinsulin. Dies bestätigt noch einmal die Aktivierung von IGF-IR durch Insulin Glargin.

Um zu klären, ob Insulinanaloga möglicherweise den bekannten Cross-talk zwischen IR/IGF-IR und Estrogenrezeptor-α (ERα) aktivieren, wurde sowohl die Zunahme des Phosphorylierungsgrads von ERα an Ser118 als auch die Induktion der ERE-abhängigen Luciferasegenexpression bestimmt. Im Vergleich zu Normalinsulin induzierte Insulin Glargin eine signifikant stärkere ERα-Phosphorylierung an Ser118, aber nur eine leichte Steigerung der Luciferase-Aktivität. Da der ERα durch Insulin Glargin nur schwach aktiviert wurde, ist es unwahrscheinlich, dass dieser Mechanismus wesentlich zur starken mitogenen Wirkung von Insulin Glargin beiträgt. Zum Schluss wurde durch Wundheilungs-, Transwell- und Matrigel- Assays geprüft, ob Insulin Glargin tumorpromovierendes Potential besitzt. Die Tests ergaben eine ähnliche migrationssteigernde Wirkung von Insulin Glargin und Normalinsulin in MCF7 Zellen.

Zusammenfassend lässt sich sagen, dass Normalinsulin und drei der untersuchten Insulinanaloga den IR und den PI3K-Signalweg aktivieren. Insulin Glargin aktiviert außerdem den IGF-IR und den MAPK-Signalweg und stellt ein starkes Mitogen in Brustkrebszellen dar, welche eine hohe IGF-IR Expression aufweisen. Möglicherweise birgt deshalb die Behandlung mit Insulin Glargin ein Risiko für Brustkrebspatientinnen und für Frauen mit bisher nicht entdeckten Tumoren oder Tumorvorstufen.

1 SUMMARY

Insulin analogues have been developed with the aim to provide better glycaemic control to diabetic patients. They are generated by modifying the insulin backbone which, however, may alter relevant biochemical characteristics such as the affinity to insulin receptor and type I insulin-like growth factor receptor (IGF-IR), and the insulin receptor dissociation rate. As a result insulin analogues may exhibit stronger mitogenic potency than regular insulin. Normal mammary epithelial cells show high expression of insulin receptor and IGF-IR and mammary cancer cells frequently even show overexpression of both receptors, thus suggesting mammary epithelial cells to be a sensitive target for insulin analogue - mediated proliferation. Indeed, treatment of female Sprague-Dawley rats with the insulin analogue B10Asp resulted in strong increase in the incidence of mammary tumours. Of all the insulin analogues available nowadays for therapeutical use, a standard two-year carcinogenicity study has been performed only for Insulin Glargine (Lantus®). The study showed similar incidence of mammary tumours in rats treated with Insulin Glargine or normal insulin. However, this study reported a very high mortality rate in all experimental groups thus raising questions on the conclusions drawn. *In vitro* studies on the effect of insulin analogues on mammary cell lines are scarce and lack comprehensiveness. In addition, the biochemical mechanism of the proliferative effect of the insulin analogues has not been clarified. This thesis aimed to study and compare in detail the proliferative potency of insulin analogues available for therapeutical use in insulin responsive mammary epithelial cell lines and to clarify the molecular and biochemical mechanism behind the proliferative potency. The role of insulin receptor, IGF-IR and related signalling pathways was analysed for the insulin analogue showing the strongest proliferative effect in comparison to regular insulin.

Among a panel of seven mammary epithelial cell lines, MCF7 (a tumour cell line) and MCF10A (a benign cell line) showed the strongest insulin response. Proliferation assays on MCF10A cells demonstrated equipotency of four insulin analogues (Insulin Aspart, Insulin Lispro, Insulin Glargine and Insulin Detemir) to regular insulins (human and bovine insulin). However, proliferation assays performed in MCF7 cells revealed that Insulin Glargine induced significantly stronger proliferation than regular insulin and the other three insulin analogues. This finding was corroborated by BrdU incorporation studies in MCF7 cells.

Activation of the two insulin-related signalling pathways - PI3K and MAPK pathway - was determined by studying the phosphorylation status of key signalling molecules (Akt and GSK3α/β for PI3K and Erk1/2 for MAPK pathway). In MCF10A cells, all insulin analogues

were equipotent to regular insulin in inducing phosphorylation of GSK3α/β and Erk1/2. Interestingly, Insulin Glargine induced significantly higher phosphorylation of Akt in comparison to regular insulin in MCF10A cells. On the contrary, in MCF7 cells, Insulin Glargine induced strong phosphorylation of the three signalling molecules studied. The signalling potency of Insulin Glargine was significantly stronger than that of regular insulin and all other insulin analogues. Use of specific inhibitors showed that MAPK is the major proliferation pathway activated by Insulin Glargin in MCF7 cells.

In order to determine the contribution of insulin receptor and IGF-IR to the strong mitogenic potency of Insulin Glargine, the RNAi technique was utilized to specifically target insulin receptor and IGF-IR. Study of signalling pathways and proliferation under knockdown conditions clearly demonstrated the activation of IGF-IR by Insulin Glargine whereas the other compounds activated the insulin receptor. Thus, the increased proliferative ability of Insulin Glargine in comparison to regular insulin is the result of IGF-IR activation. IGF-IR immunoprecipitated from cells treated with Insulin Glargine or regular insulin showed much higher tyrosine phosphorylation levels in the Insulin Glargine - treated cells, which substantiates the findings from the knockdown experiments. Moreover, analysis of expression levels of cyclin D1, an IGF-I responsive gene, by quantitative RT-PCR showed higher expression levels in MCF7 cells treated with Insulin Glargine than in cells treated with regular insulin, again corroborating the strong activation of IGF-IR by Insulin Glargine.

In order to clarify the potential activation of the established cross-talk between insulin receptor/IGF-IR and estrogen receptor-α (ERα) by insulin analogues, we determined the activation of ERα by analysing the phosphorylation status of Ser118 at ERα as well as ERE-dependent luciferase gene expression. In comparison to regular insulin, Insulin Glargine induced significantly stronger phosphorylation of ERα at Ser118 and slightly higher luciferase activity. However, since ERα was only weakly activated by Insulin Glargine, modulation of ERα activity is unlikely to play a strong role in rendering high proliferative ability to Insulin Glargine. Finally, the possible tumour-promoting potential of Insulin Glargine was studied by wound healing, transwell and matrigel assays. The assays demonstrated similar migration-inducing potential of Insulin Glargine and regular human insulin in MCF7 cells.

In summary, this study shows that different from regular insulin and other insulin analogues studied, which activate the insulin receptor and the PI3K pathway, Insulin Glargine activates the IGF-IR and the MAPK pathway too, and is a strong mitogen in breast cancer cells showing high IGF-IR expression. Insulin Glargine may therefore be of risk for patients with breast cancer or as yet undetected (pre-) cancerous lesions.

2 INTRODUCTION

2.1 Insulin

2.1.1 Function

Insulin is a ~ 6 kDa polypeptide hormone secreted by β-cells of the 'islets of Langerhans' in the pancreas. Together with glucagon, a hormone secreted by α-cells of pancreas, insulin maintains glucose homeostasis in the blood. Insulin has been demonstrated to have multiple biological effects in virtually all tissues. Its actions can be broadly categorised as metabolic actions and mitogenic actions. Metabolic actions of insulin comprise increased uptake of glucose, amino acids and fatty acids by target cells, increased expression and activity of enzymes involved in synthesis of glycogen (e.g., phosphofructokinase-2 and glycogen synthase), proteins and lipids, and decreased activity of enzymes involved in degradation of carbohydrates, proteins and lipids (Saltiel and Kahn, 2001). These varied metabolic effects make insulin the most potent anabolic hormone known. The main target tissues for metabolic actions of insulin are skeletal muscles, cardiac muscles, adipose tissue and liver. In muscles and adipose tissue, insulin induces translocation of the GLUT4, insulin-dependent glucose transporter, to the plasma membrane. Skeletal muscles are responsible for 75% of insulin-dependent glucose disposal whereas adipose tissue account for only a small fraction (Klip and Paquet, 1990). In liver cells, glucose is taken up by the activity of GLUT2, the high Km insulin-independent glucose transporter (Mueckler, 1994). Furthermore, in the liver insulin regulates the expression of glycolytic and gluconeogenic enzymes.

In addition to metabolic actions, insulin has also been demonstrated to elicit mitogenic response. In fact, results from knockout mice indicate that insulin receptor is not required for glucose metabolism in the embryo but rather for proper embryonic development. Insulin receptor knockout mice show 90% size of the control mice at birth. A few days after birth they die due to diabetic ketoacidosis indicating the importance of insulin in post-natal glucose metabolism (Accili *et al*., 1996; Joshi *et al*., 1996). Recent studies have additionally suggested a role of precursor insulin molecules (preproinsulin and proinsulin) along with insulin in emryonic development (Perez-Villamil, 1994; Hernandez-Sanchez, 2005). Furthermore, during early stages of retinal development, the physiological cell death is regulated by locally produced insulin/proinsulin, thus alluding a role for insulin as a survival factor (Diaz, 2000). Insulin is required for the optimal growth of several normal and cancer cell lines. Moreover,

insulin has been demonstrated to be involved in proliferation and differentiation of B-lymphocytes, T-lymphocytes and adipocytes (Spiegelman and Flier, 1996; Belfiore, 2007). The adipose tissue specific knockout mouse model of insulin receptor displays defective fat cell formation (Bluher *et al*., 2002). Interestingly, the recent study on fat cell-specific disruption of the insulin receptor gene demonstrated increased lifespan in these mice compared to control mice, suggesting a special role for insulin-signalling pathway of adipose tissue in longevity. Insulin and insulin receptor also play important roles in associative learning (Zhao and Alkon, 2001). Interruption of insulin production and insulin receptor activity results in learning and memory deficits. Hyperinsulinemia has been demonstrated to be one of the risk factors for Alzheimer's disease (Kidd, 2008). Insulin has been shown to be involved in modifying neurotransmitter release at synapses and in modulating the activities of both excitatory and inhibitory postsynaptic receptors such as NMDA and GABA receptors, respectively (Zhao and Alkon, 2001). In summary, insulin has pleiotropic effects on various tissues. This thesis is mainly concerned with the mitogenic effect of insulin.

2.1.2 Insulin synthesis

The primary product of the insulin gene is a 110 amino acid long preproinsulin molecule (Fig. 2.1). Preproinsulin contains an amino-terminal signal sequence that is required for the precursor hormone to pass through the endoplasmic reticulum (ER). After entering the ER, the signal sequence is proteolytically removed to form proinsulin. Proinsulin is first processed by ‘protein disulfide isomerase’ to form three disulfide bonds in the molecule. This process has been demonstrated to occur in ER (Dodson, 1998). Thereafter, proinsulin is sequestered within Zn^{2+} and Ca^{2+} rich secretory vesicles of the Golgi apparatus, wherein proinsulin forms hexameric complexes with Zn^{2+} and Ca^{2+} ions (Dunn, 2005). Proteolytic enzymes like Ca^{2+} dependent prohormone convertases (PC1 and PC2; Bailyes, 1991) and exoprotease (carboxypeptidase E) act on proinsulin molecule to form the mature insulin molecule of 51 amino acids. Like proinsulin, mature insulin is present in Golgi secretory vesicles as hexameric complexes containing Zn^{2+} and Ca^{2+} ions. These hexameric insulin complexes have very low solubility and are normally present as crystals within the mature secretory vesicles. Additionally, a catalytic activity of PC1 has been suggested to enhance targeting of processed insulin to the mature secretory granules (Kuliawat, 2000). The mature insulin molecule consists of 2 chains (A and B chain) linked together by two disulphide bonds (between A7 and B7, and A20 and B19). The A-chain consists of 21 amino acids including another disulfide bond (A6 and A11) and the B-chain consists of 31 amino acids.

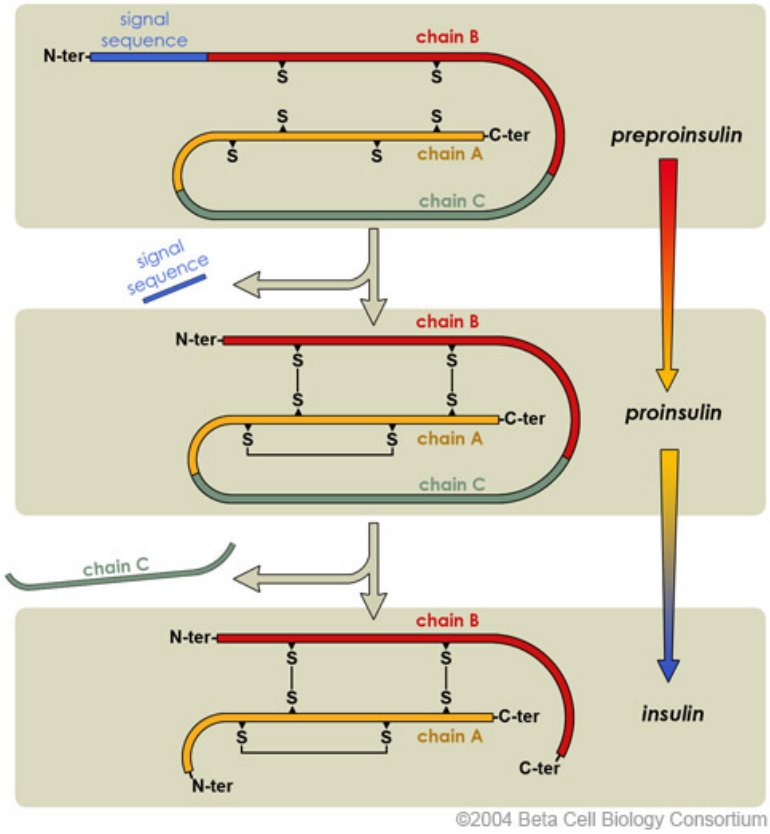


Figure 2.1 Post translational processing of preproinsulin molecule to generate the mature insulin molecule. (Taken from Beta Cell Biology Consortium website).

2.1.3 Insulin secretion

Insulin secretion from β-cells is a tightly regulated process (Fig. 2.2). The secretion of insulin can be induced by fatty acids, amino acids, and ionophores, but physiologically, the most important secretagogue is glucose (Hellman, 1975; McClenaghan *et al*., 1994; Henquin, 2000; Haber *et al*., 2003). After the meal, blood glucose level increases resulting in the entry of the glucose into the β-cells via GLUT2, an insulin-independent glucose transporter. Increased glycolysis and cellular respiration raises the level of ATP (or ATP/ADP ratio) in the cell. The high ATP/ADP ratio inactivates the ATP-dependent potassium channel. Consequently, potassium ions accumulate inside the cell and this causes depolarization of the cell membrane. The calcium channels on β-cells are voltage-gated. Depolarization of the membrane opens up these channels and allows calcium ions entry in the cell. Finally, the increase in calcium levels triggers fusion of insulin storage granules to the plasma membrane and thus exocytotic release of insulin.

Establishing the role of insulin itself in insulin secretion from β-cells has been a controversial issue. Insulin has either been shown to be not at all involved, or stated to be essential, or even

a negative regulator (Leibiger *et al*., 2008). However, recent conditional knockout mice models for proteins involved in insulin signalling pathway (like insulin receptor, IRS-1, IRS-2 and PKB) show impaired glucose tolerance due to defective insulin secretion.

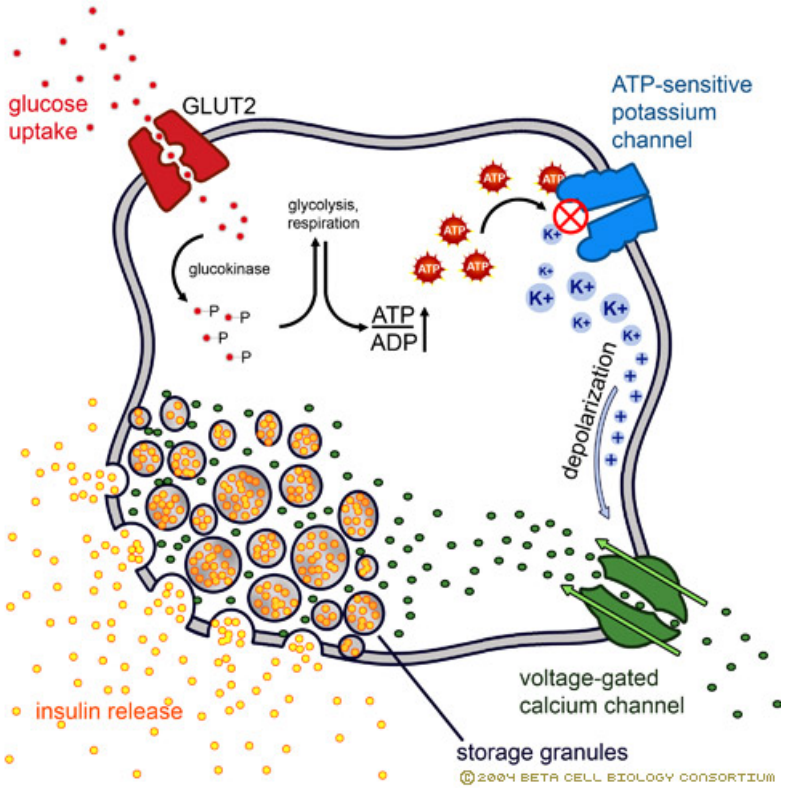


Figure 2.2 Schematic diagram demonstrating insulin secretion from a typical β-cell (Taken from Beta Cell Biology Consortium website).

Similar experiments on an established cell line of β-cell line, MIN6, yielded consistent results thus favouring the positive regulation of insulin secretion from β-cells by insulin (Da Silva-Xavier *et al*., 2004). Finally, effect of insulin on its own release from β-cells has been suggested to be dependent on the insulin concentration (Jimenez-Feltstrom *et al*., 2004). Low dosage of insulin (0.05 to 0.1nM) stimulates insulin release, concentrations between 1nM and 100nM have no effect on insulin release and higher concentrations result in inhibition of insulin exocytosis.

2.2 Insulin Receptor

2.2.1 General information

Insulin exerts its pleiotropic effects by binding to and activating its cognate receptor, insulin receptor (IR). Insulin receptor is a member of the tyrosine kinase receptor superfamily and is expressed by all tissues though the receptor numbers may widely differ. The insulin receptor has a heterotetrameric structure consisting of two α-subunits (MW of 110 kDa each) and two β-subunits (MW of 97 kDa each) and all subunits are held together by disulphide bonds (Fig. 2.3). Alternate splicing of insulin receptor gene can produce two isoforms – IR-A lacking exon 11 and IR-B with full transcript. IR-A has been shown to be the predominant isoform of insulin receptor in fetal life and in many cancer cells (Frasca *et al*., 1999). The α-subunit of insulin receptor is an extracellular subunit and possesses the insulin binding domain. Although both α-subunits of a $\alpha_2\beta_2$ holoreceptor may theoretically bind an insulin molecule, the receptor exhibits negative cooperativity and only one high affinity insulin binding site (Lee *et al*., 1993). Experiments have shown that the occupancy of one insulin binding domain decreases the affinity of the other insulin binding domain by 100-fold (Pang and Shafer, 1984).

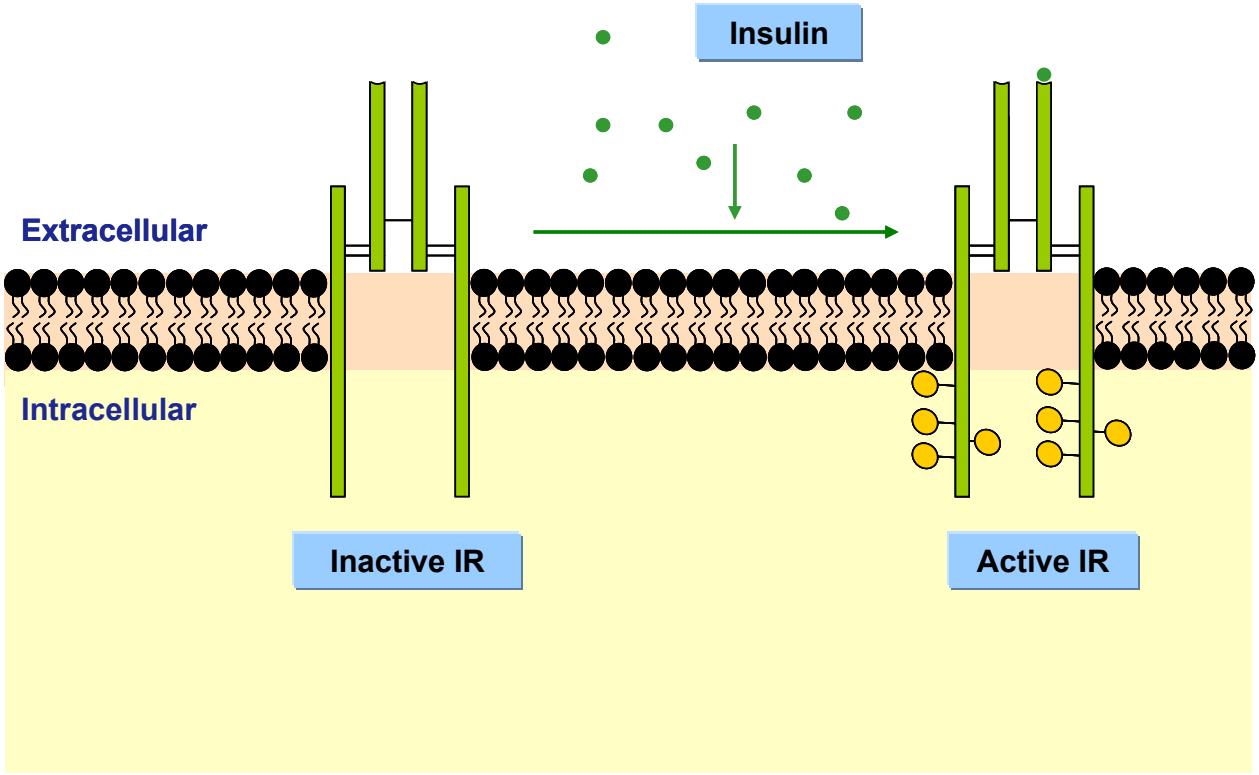


Figure 2.3 Insulin induced autophosphorylation of insulin receptor. Insulin (green circles) binds to the α-subunit of insulin receptor (IR) and activates it by inducing autophosphorylation at tyrosine residues in the β-subunit. The important tyrosine residues are at 950, 1158, 1162 and 1163 amino acid position.

The β-subunit consists of an extracellular part via which it is anchored to the α-subunit, a transmembrane region and an intracellular part which contains phosphorylation sites and intrinsic tyrosine kinase activity (Taylor, 1991). The α-subunit exerts inhibitory effect on the intrinsic kinase activity of the β-subunit. Proteolytic cleavage of the α-subunits or deletion mutants of α-subunits have been shown to relieve this inhibition (White and Kahn, 1994). Interestingly, a point mutation ($Val^{664} \rightarrow Glu$) in the transmembrane segment of insulin receptor also partially relieves the inhibitory effect of the α-subunit on the β-subunit thus suggesting multiple regulation of the tyrosine kinase activity of the β-subunit (Longo *et al*., 1992). The binding of insulin to the α-subunit causes a conformational change in the α-subunit. Consequently, the inhibitory effect of the α-subunit on the tyrosine kinase activity of the β-subunit is relieved. The first event in tyrosine kinase activation is binding of ATP to the β-subunits and subsequent autophosphorylation of the receptor.

The insulin receptor phosphorylates itself by a *trans* - mechanism. Structural studies reveal that the kinase domains of the two β-subunits are in juxtaposition so that one β-subunit can phosphorylate the other subunit (Lee *et al*., 1993). The most important autophosphorylation sites are tyrosine residues at positions 950 (intracellular juxtamembrane region of β-subunit), 1158, 1162 and 1163 (tyrosine kinase domain). Autophosphorylation of these tyrosine residues stimulates tyrosine kinase activity by 10-20 fold (White *et al*., 1988). Once autophosphorylated, the binding of insulin to the insulin receptor is no longer required and the receptor can be inactivated only by dephosphorylation or internalization (Taylor, 1991).

2.2.2 Insulin receptor signalling pathway

The activation of the β-subunit results in the phosphorylation of several intracellular substrates like insulin receptor substrate family of proteins (IRS), Src-homology-2-containing protein (Shc), Grb2-associated binder-1 (Gab1), Cbl, and adaptor protein containing PH and SH2 domains (APS). The most important and well characterized intracellular substrates of insulin receptor are IRS family of proteins (IRS 1-4). These proteins are involved in important insulin receptor function like glycogen synthesis, lipid synthesis, glucose uptake, cell growth and differentitation. IRS-1 and IRS-2 proteins are approximately 180kDa, IRS-3 is 60kDa and IRS-4 is approximately 160kDa. IRS-1 and IRS-2 are the major signalling molecules in the insulin signalling pathway and are widely distributed (Taniguchi *et al.,* 2006). Even though IRS-1 and IRS-2 are 43% identical in terms of amino acid sequence homology, their functions are not inter-changeable. IRS-1 knockout mice exhibit growth retardation, insulin resistance

and impaired glucose tolerance but do not develop overt diabetes (Araki *et al*., 1994; Tamemoto *et al*., 1994). IRS-2 knockout mice show defective growth in some tissues like certain regions of brain, retina and islets of Langerhans, insulin resistance, glucose intolerance and are severely diabetic (Sesti *et al*., 2001). IRS-1 and IRS-2 double knockout mice are non-viable. In contrast to IRS-1 and IRS-2, IRS-3 and IRS-4 are redundant in function and in mice these proteins were found to be expressed only in adipocytes, brain and embryonic tissues. IRS-3 knockout mice showed normal growth, glucose homeostasis, and glucose transport in adipocytes (Liu *et al*., 1999). Interestingly, a functional copy of IRS-3 has not been reported in humans raising questions regarding its importance in humans (Bjornholm *et al*., 2002). Targeted disruption of IRS-4 gene in mice resulted in only mild defects in growth, reproduction, and glucose homeostasis (Fantin *et al*., 2000). IRS-1/2 interact with the insulin receptor via their PH (pleckstrin-homology) domain and PTB (phosphotyrosine-binding) domain. These proteins have up to 20 tyrosine residues which can undergo phosphorylation by insulin receptor. These phosphorylated tyrosines serve as docking site for other intracellular molecules that contain Src-homology-2-domains (SH2 domains) (Taniguchi *et al.,* 2006).

Other intracellular substrates of active insulin receptor perform diverse functions. Cbl protooncogene is involved in insulin-stimulated glucose uptake. Shc activation is involved in induction of cell proliferation (Saltiel and Pessin, 2002). The detailed description of important signalling pathways activated by insulin receptor is presented below.

2.2.2.1 PI3K signalling pathway

The PI3K signalling pathway is the major pathway activated by insulin. This pathway elicits both metabolic and mitogenic responses in the cells. PI3K is the most upstream signalling molecule of this pathway. It consists of two subunits – p85 (regulatory subunit) and p110 (catalytic subunit). The regulatory subunit, p85, has been reported to have at least eight isoforms. The exact role of these different isoforms in the regulation of insulin action is not clear (Pons *et al.,* 1995). The p85 subunit interacts via its two SH2 domains with the phosphotyrosine motifs of IRS proteins (Ogawa, 1998). Binding of the p85 subunit to IRS results in the recruitment of p110 subunit to p85 and thus in the activation of the catalytic activity of PI3K (Fig. 2.4). The p110 subunit catalyzes phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) and generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Inhibitors against PI3K or transfection with dominant negative

constructs of the gene block insulin-mediated glucose transport, glycogen and lipid synthesis thus highlighting the importance of PI3K in the metabolic actions of insulin (Saltiel and Kahn, 2001). PIP3 binds to the pleckstrin homology (PH) domain of a variety of signalling molecules and thus influences glucose uptake, glycogen synthesis, and cell growth. The most important binding partner of PIP3 is phosphoinositide-dependent kinase 1and 2 (PDK1/2), for which PIP3 is an allosteric regulator.

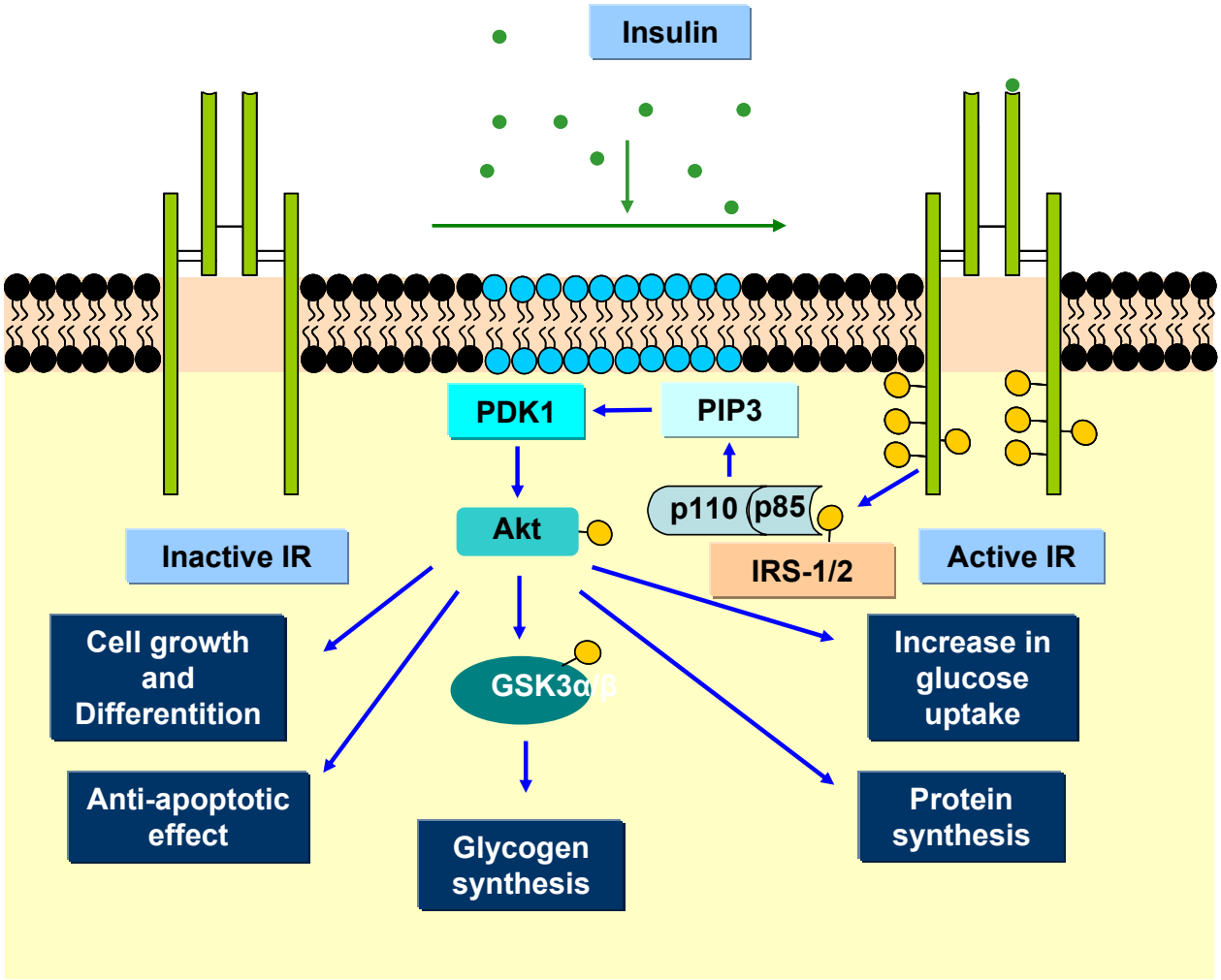


Figure 2.4 PI3K pathway activation by active insulin receptor. PI3K pathway is the major pathway activated by insulin receptor (IR). It induces glucose uptake, glycogen synthesis, protein synthesis, cell growth and differentiation. In addition, it has anti-apoptotic effect.

Akt, also known as protein kinase B (PKB), is a serine-threonine protein kinase. It has an amino-terminal PH domain, a central catalytic domain and a short regulatory domain at carboxy-terminus. The PIP3 recruits Akt to the plasma membrane by interacting with the PH domain of Akt protein. Once tethered to the membrane, Akt protein is phosphorylated at Thr308 and Ser473 by activated PDK1 and PDK2, respectively (Andjelkovic *et al*., 1997; Bellacosa *et al*., 1998). Activated Akt is another critical node in insulin signalling pathway. It

induces protein synthesis in the cell by interacting with mTOR (Nave *et al.,* 1999). In addition, activated Akt has also been demonstrated to be associated with GLUT4 vesicles suggesting its role in glucose uptake (Kupriyanova *et al.,* 1999). Furthermore, activated Akt directly phosphorylates FOXO1 at Ser256. FOXO1 are a class of transcription factors which upregulate expression of gluconeogenic genes. Phosphorylation of FOXO1 allows it to interact with 14-3-3, a cytoplasmic adapter protein. Consequently, it is sequestered to the cytoplasm, resulting in inhibition of gluconeogenesis (Tran *et al*., 2003). Activated Akt plays an important role in enhancing cell survival both directly and indirectly. Bad protein is a Bcl-2 family member protein and in unphosphorylated state is bound to anti-apoptotic factors like Bcl-2 and Bcl-xl. Activated Akt directly phosphorylates Bad protein on Ser136 which results in the sequestration of Bad protein to 14-3-3 and release of anti-apoptotic factors (Datta *et al*., 1997). Additionally, activated Akt has been demonstrated to enhance the degradation of IκB resulting in nuclear translocation of NF-κB and subsequent transcription of caspase inhibitors IAP1 and IAP2 and pro-survival Bcl-2 family members, Bcl-X_L and Bfl-1/A1 (Dutta *et al*., 2006). An important downstream target of Akt is GSK3α/β, a serine-threonine protein kinase which is a negative regulator of glycogen synthesis. Phosphorylation of GSK3α/β results in its inactivation. Inactivation of GSK3α/β restores the glycogen synthase activity which then catalyzes conversion of glucose to glycogen (Taniguchi *et al.,* 2006).

To summarise, PI3K pathway is the major mediator of the anabolic effects of insulin. Its activation results in increase in glucose uptake, glycogen synthesis, protein synthesis, cell proliferation and inhibition of gluconeogenesis and apoptosis.

2.2.2.2 CAP-Cbl signalling pathway

This signalling pathway is specifically involved in glucose uptake and collaborates with PI3K pathway for proper translocation of GLUT4 vesicles to the plasma membrane (Chiang *et al.,* 2003). The Cbl proto-oncogene is a direct intracellular substrate of the insulin receptor. Studies have shown that Cbl does not directly interact with insulin receptor and is recruited to insulin receptor by APS. APS binds to the phosphotyrosine residue of the activated insulin receptor via its SH2 domain. This results in its phosphorylation at tyrosine. Phosphorylated APS recruits Cbl to the insulin receptor where it is phosphorylated and activated. Activated Cbl forms a complex with CAP (Cbl Associated Protein) and activates TC10, a small GTPase. TC10 is activated specifically by insulin and disruption of its activation blocks insulin-stimulated glucose transport and GLUT4 translocation (Chiang *et al.*, 2001). The signalling

events downstream of TC10 are not very clear but the net effect of TC10 activation is GLUT4 translocation to the plasma membrane and thus increase in glucose uptake by the cell.

2.2.2.3 MAPK signalling pathway

MAPK pathway is another important pathway activated by insulin. This pathway is well-known for its proliferative effect on cells. The first major event in activation of the MAPK signalling pathway is activation of Ras protein (Fig. 2.5). Ras proteins (H-Ras, N-Ras and K-Ras) are 21 kDa GTPases that are attached to the cell membrane by prenylation. All the three isoforms are ubiquitously expressed. However, as all three isoforms have different lipid anchors, the cellular response can be modulated by selective targeting of the isoforms to different cellular compartments and signalling complexes (Hancock and Parton, 2005).

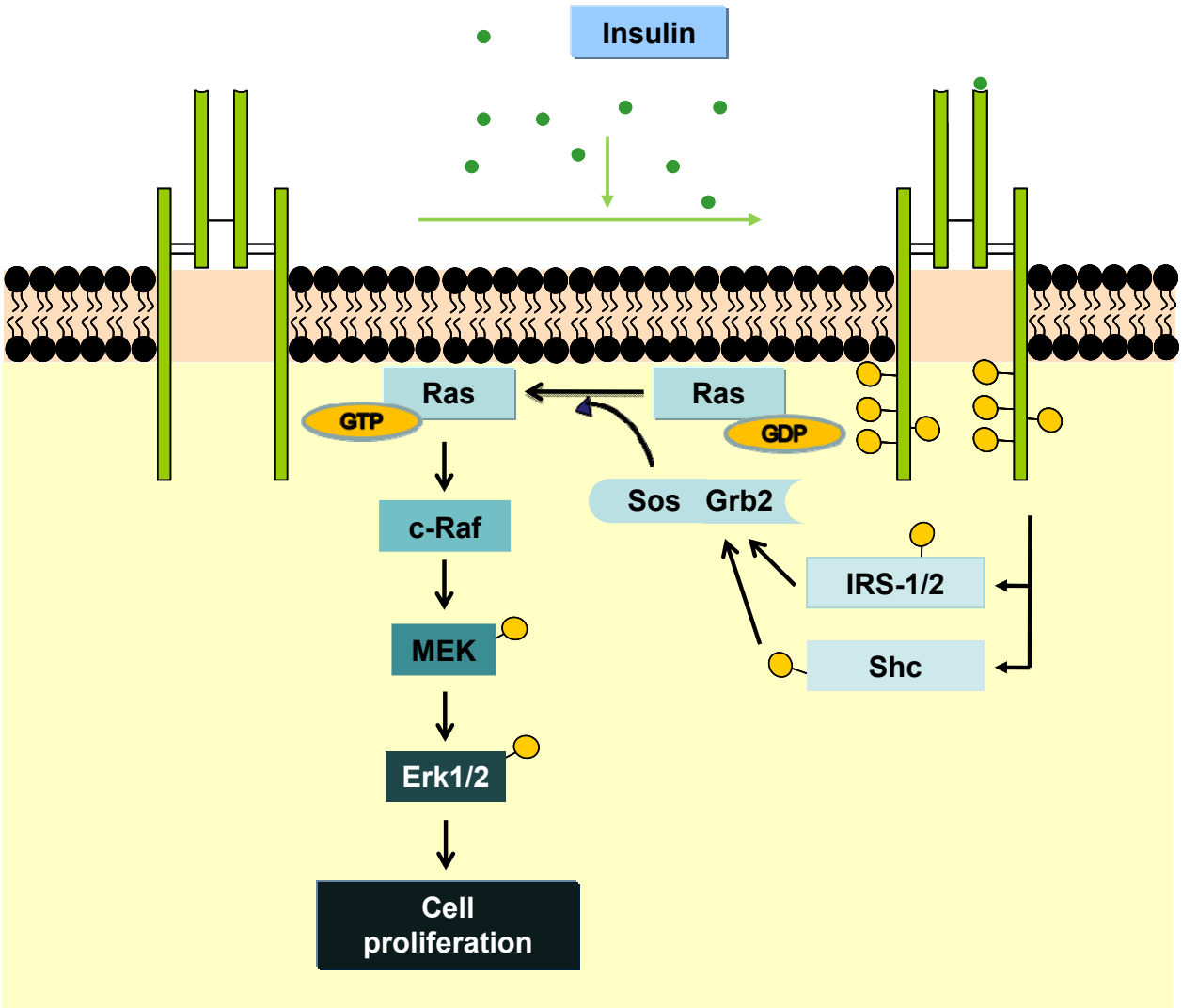


Figure 2.5 MAPK pathway activation by active insulin receptor. Insulin receptor (IR) can activate MAPK pathway by activation of Shc-Grb2-Sos complex. MAPK pathway induces cell proliferation.

Ras is active when it switches from the GDP-bound state to GTP-bound state (Lowy and Willumsen, 1993). Mutations in the Ras proteins resulting in constitutive activation of the Ras GTPase are found in 20% to 30% of human tumours.Activation of Ras involves activation of the Grb2-Sos complex. Grb2 (growth factor receptor bound protein 2) is an adapter protein and Sos (Son of sevenless) is a guanine nucleotide exchange factor which interacts with Grb2 by its SH3 domain. Studies have shown that the Grb2-Sos complex can be activated by at least two pathways.The phosphorylated IRS-1 protein serves as a docking site for Grb2 which then recruits Sos to the plasma membrane for activation of Ras. Alternatively, Grb2 can bind to Shc protein (another direct intracellular substrate of insulin receptor) and this leads to the activation of Grb2-Sos complex. Once recruited to the plasma membrane, Sos activates Ras protein by exchanging GDP with GTP. To this activated Ras binds c-Raf, a serine-threonine protein kinase also called Raf-1. c-Raf phosphorylates and activates mitogen activated protein kinase kinase (MEK) (Kyriakis *et al*., 1992). Activated MEK catalyses phosphorylation of mitogen activated protein kinase (MAPK) at threonine and tyrosine residues (Ahn *et al*., 1992). MAPK (also known as extracellular signal-regulated kinase, Erk) is a serine/threonine kinase and has two closely related isoforms, Erk1 and Erk2.The phosphorylated Erk1/2 enter the nucleus and has been demonstrated to activate transcription factors like FOS, MYC and JUN which are well known for their role in cell proliferation. Erk1/2 also increases expression of genes like Cyclin D1 that are required for cell cycle entry. Additionally, Erk1/2 are involved in a negative-feedback loop of insulin action by phosphorylating IRS-1 on serine residues (Bouzakri *et al.,* 2003). In summary, MAPK pathway is primarily involved in mediating mitogenic actions of insulin.

2.2.2.4 Inhibition of insulin receptor signalling

Once insulin dissociates from insulin receptor, insulin signalling is rapidly attenuated by various mechanisms. The insulin receptor and its substrates like IRS proteins are rapidly dephosphorylated by protein tyrosine phosphatases (PTPases). The most important PTPase in this regard is PTP1B. The disruption of PTP1B gene in mice results in increased insulin induced tyrosine phosphorylation of insulin receptor and IRS-1 and thus increased insulin receptor signalling (Drake and Posner, 1998). As a result, nowadays, PTP1B is being pursued as a drug target for the treatment of type II diabetes and obesity (Taylor and Hill, 2004). Another mechanism for attenuation of insulin signalling utilizes phosphorylation of serine and threonine residues of insulin receptor and IRS-1. Two serine/threonine kinases, ‘cAMP dependent protein kinase’ and ‘Protein kinase C’ have been implicated in this process

(Tanti *et al*., 1987; Karasik *et al*., 1990). Excessive serine phosphorylation of insulin receptor has been suggested as one of the reasons for insulin resistance. Additionally, insulin signalling is attenuated by internalization of the activated receptor. Studies have shown that after 5 min of insulin treatment approximately 30% of the insulin receptor is internalized (Marshall, 1985). Mutation of the kinase domain resulting in the loss of tyrosine kinase activity inhibits internalization of the receptor thus implicating a role of the kinase domain in the attenuation of insulin signalling. Interestingly, internalized insulin receptors may remain catalytically active as protein kinase (Khan *et al*., 1989).

2.3 Diabetes mellitus

Insulin being an important regulator of glucose homeostasis is highly required for healthy life. Impairment in insulin production or in insulin response causes a chronic disease called as 'Diabetes'. 'Diabetes mellitus' is characterized by sweet smelling urine due to excess of glucose in the urine. Its global incidence has been increasing sharply. According to WHO projection, the numbers of diabetic patients are expected to rise from current state of 170 million diabetic patients to approximately 300 million patients by 2025. Two major types of diabetes mellitus have been described – type I and type II.

Type I Diabetes

It is also referred to as 'insulin dependent diabetes'. This is an autoimmune disease in which the immune system targets the β-cells of the endocrine pancreas and results in the loss of insulin production. This disturbs glucose homeostasis and is reflected as 'hyperglycemia'. Excess glucose levels in the blood damages nerves (neuropathy), kidney (nephropathy) or eyes (retinopathy). For treating type I diabetic patients, efforts are being made to transplant pancreas or insulin producing β-cells. However, success has been limited as grafted tissues are rejected by the host immune system. Till date, the most reliable cure has been to take daily injections of insulin for controlling blood glucose level. Nowadays, insulin analogues have been developed which can mimic the insulin secretion profile of the body to a great extent and thus are proving to be of great help for diabetic patients.

Type II Diabetes

Type II diabetes or 'non-insulin dependent diabetes' comprises 90% of all cases. In this type, insulin target tissues do not respond to insulin. This is described as 'insulin resistance'. The development of insulin resistance is a complex and progressive event. Research so far has demonstrated that various molecular events can lead to insulin resistance. Defects related to insulin receptor like incomplete processing of insulin receptor, decrease in insulin receptor numbers on the cell surface, auto-antibodies to the insulin receptor or decreased kinase activity of insulin receptor have been implicated in insulin resistance (Becker and Roth, 1990). Other events like increased insulin degradation or defective downstream signalling from insulin receptor also result in insulin resistance. Furthermore, impairment in translocation of GLUT4 to the plasma membrane has been also shown to result in insulin resistance (James and Piper, 1994). Insulin resistance causes non-utilization of glucose and like in type I diabetes the patient suffers from 'hyperglycemia'. Initial body response to

counter insulin resistance is increased insulin production (hyperinsulinemia) by β-cells. This, however, on long term results in the destruction of β-cells and as a result insulin production ceases. Obesity is considered one of the major risk factors for type II diabetes (55% of patients are obese). The patients with early stage type II diabetes can be treated by giving drugs which increase insulin secretion (like sulphonylurea), or decrease glucose release from liver (like metformin). In addition, drugs which can increase insulin sensitivity like thiazolidinediones are very useful. The patients with late stage type II diabetes do not produce insulin. Like patients with type I diabetes, these patients also have to take daily insulin injections.

Gestational Diabetes

Gestational diabetes mellitus develops in the pregnant women. It is very similar to type II diabetes in terms of inadequate insulin secretion and responsiveness. It is fully treatable but chances are that approximately 20%–50% of affected women may develop type II diabetes later in their life.

2.4 Insulin Analogues

2.4.1 Basis for developing insulin analogues

The treatment of diabetic patients with insulin started only from the 1920s. At that time animal insulins derived from pancreas of pigs or cows were used to treat diabetes. Though it was of great help to save the life of diabetic patients, many of them reported allergic response towards animal insulins. The production of human insulin became possible only after sufficient knowledge was gained to genetically manipulate microorganisms for producing proteins of interest. In the 1980s, first time human insulin was produced from genetically engineered bacteria (Chance *et al.*, 1981). It was marketed as 'humulin' (insulin isophane) by Genentech and was a boon in treating diabetic patients. Additionally, unlike animal insulin it did not evoke immune response.

The most effective way, till date, to give insulin is by injecting it subcutaneously from where insulin diffuses into the blood and acts on the target tissues. Insulin administered subcutaneouly reaches blood in 30-60 min (onset time), the blood level reaches maximum in 2-3 h (peak time) and it stays in blood for total of 5-8 h (effective duration). The onset time of normal insulin is 30-60 min and effective duration is only 5-8 h, a patient may have to take injections twice a day. In order to reduce the dependency of diabetic patients on insulin injections, attempts were made to design insulin preparations with increased effective duration.

Insulin forms hexamers in presence of zinc ions (Dunn, 2005). By increasing or decreasing zinc ion concentration insulin release from the site of injection can be varied. Protamines are added to the insulin preparations. They are highly basic proteins and their concentration in the insulin preparation determines the dissociation rate of insulin hexamers. In order to improve the effective duration of normal insulin, insulin preparations are modified by altering concentrations of zinc ions or protamines. NPH (neutral protamine Hagedorn), lente and ultralente are such insulin preparations which have delayed insulin action duration and have been shown to remain in the blood for 10-16 h (NPH) or 10-24 h (lente and ultralente). These preparations have improved the efficacy of insulin treatment but still they do not mimic optimal timing of insulin action. For example, by modifying insulin preparations the onset time of normal insulin cannot be decreased below 30-60 min. In addition, lente and ultralente insulin preparations do not provide constant basal insulin level for a longer duration.

To encounter these inadequacies, pharmaceutical companies are generating artificial insulin molecules, also called insulin analogues, by modifying the insulin backbone. Depending upon the modification, insulin analogues can be of two types : rapid-acting and long-acting insulin analogues.

2.4.2 Rapid-acting insulin analogues

Insulin molecules exhibit self-association property in the presence of zinc and form stable hexamers which dissociate slowly. Rapid-acting insulin analogues are artificial insulin molecules which have been modified in a way that they are present mostly in monomeric form. As a result, they have much lower onset time in comparison to normal insulins. At present there are three rapid acting insulin analogues that have been approved for therapeutical use –Insulin Aspart (Novorapid®, produced by Novo Nordisk), Insulin Lispro (Humalog®, produced by Eli Lilly) and Insulin Glulisine (Apidra®, produced by Sanofi-Aventis).

Insulin Aspart

Regular human insulin has the amino acid proline at 28th position in the B-chain of insulin. Insulin Aspart is generated by introducing a mutation at this position which codes for aspartic acid (Fig. 2.6). Aspartic acid being a negatively charged amino acid increases the charge-charge repulsion between two monomers and thus has reduced tendency to form hexamers.

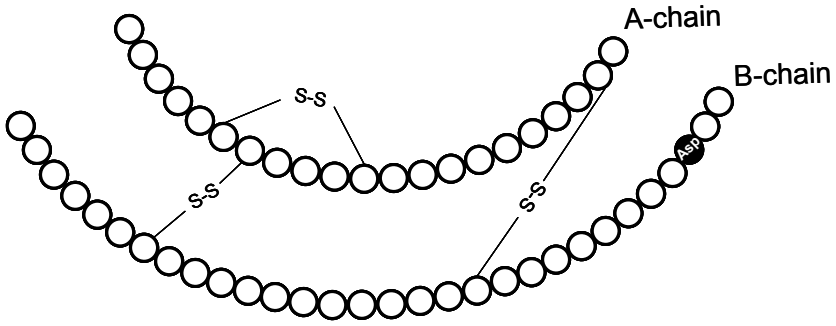


Figure 2.6 Schematic presentation of modification made in the insulin backbone to generate Insulin Aspart. Proline at 28th position in B-chain of regular insulin molecule is replaced with aspartic acid to generate Insulin Aspart.

It is thus more rapidly absorbed from the subcutaneous tissue at the site of injection compared to regular human insulin. Insulin Aspart is available for action within 5-15 min of taking the injection and the concentration peaks within 40-50 min. However, the total duration of action is less than 5 h (Rolla, 2008).

Insulin Lispro

Insulin Lispro is generated by interchanging amino acids at 28th and 29th position of the B-chain of insulin molecule to lysine and proline respectively (Fig. 2.7). It has been reported that because of this inversion Insulin Lispro does not form dimers and hexamers. As a result it is available within 5-15 min for the action. Like Insulin Aspart, its concentration also peaks within 30-90 min and it is eliminated from the body in less than 5 h (Rolla, 2008).

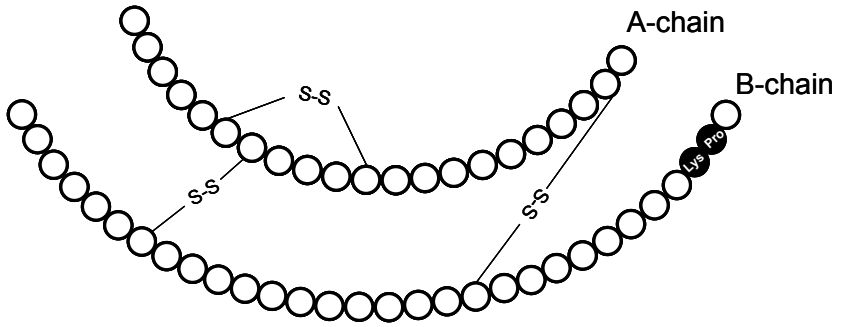


Figure 2.7 Schematic presentation of modifications made in the insulin backbone to produce Insulin Lispro. Proline at 28th position and Lysine at 29th position in B-chain of regular insulin molecule is interchanged to generate Insulin Lispro.

Insulin Glulisine

Insulin Glulisine differs from human insulin in that the amino acid asparagine at position B3 is replaced by lysine and the lysine in position B29 is replaced by glutamic acid (Fig. 2.8). Insulin Glulisine acts fast and it can be found in the blood within 15-20 minutes after the injection. Its concentration peaks within 34-91 minutes (Rolla, 2008).

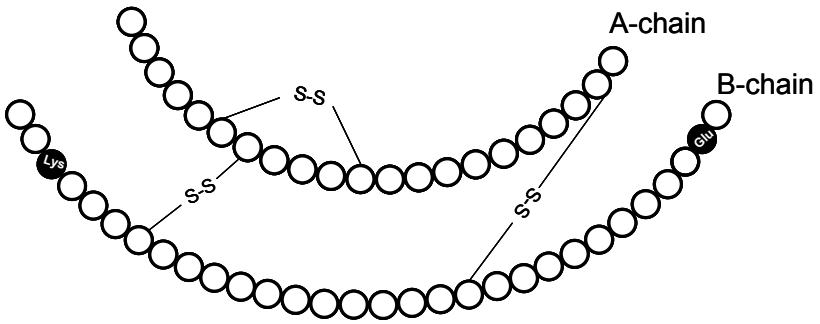


Figure 2.8 Schematic presentation of modifications made in the insulin backbone to produce Insulin Glulisine. Asparagine at 3rd position and lysine at 29th position in B-chain of regular insulin molecule are replaced by lysine and glutamic acid to generate Insulin Glulisine.

2.4.3 Long-acting insulin analogues

Currently there are two long-acting insulin analogues approved for clinical use – Insulin Glargine (Lantus®, produced by Sanofi-Aventis) and Insulin Detemir (Levemir®, produced by Novo Nordisk).

Insulin Glargine

Insulin Glargine is generated by replacing asparatic acid at 21st position in the A-chain by glycine and introducing two additional arginine amino acids in the B-chain of insulin molecule (Fig. 2.9).

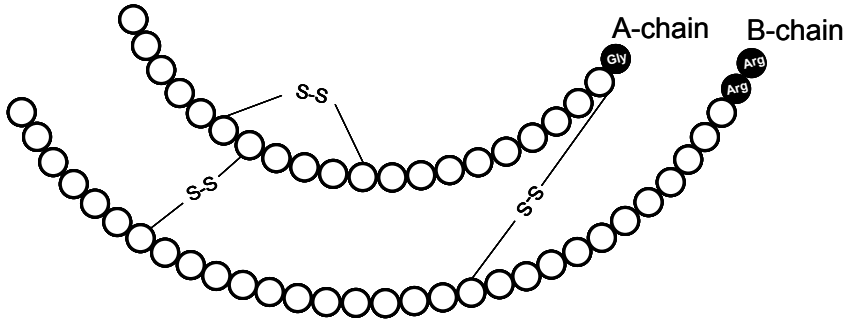


Figure 2.9 Schematic presentation of modifications in the insulin molecule to generate Insulin Glargine. A-chain of insulin Glargine has glycine instead of aspartic acid at 21st position and B-chain contains two extra amino acids (31st and 32nd position) which code for arginine.

Due to these modifications, Insulin Glargine has low aqueous solubility at neutral pH. When injected into the subcutaneous tissue, it forms microprecipitates and slowly diffuses into the blood. The onset time is approximately two hours. In contrast to rapid acting insulin analogues, there is no pronounced peak and the concentration in the blood remains relatively constant over a period of 24 h (Gerich *et al*., 2006).

Insulin Detemir

Insulin Detemir is produced by deleting threonine at the 30th position of the B-chain and adding a 14-carbon fatty acid chain to lysine in the 29th position (Fig. 2.10). The fatty acid chain enables insulin detemir to bind to albumin, the most prominent blood protein. This results in slow release of the insulin molecule and thus lengthens the time of action. Its action profile is similar to that of Insulin Glargine (Heise, 2007).

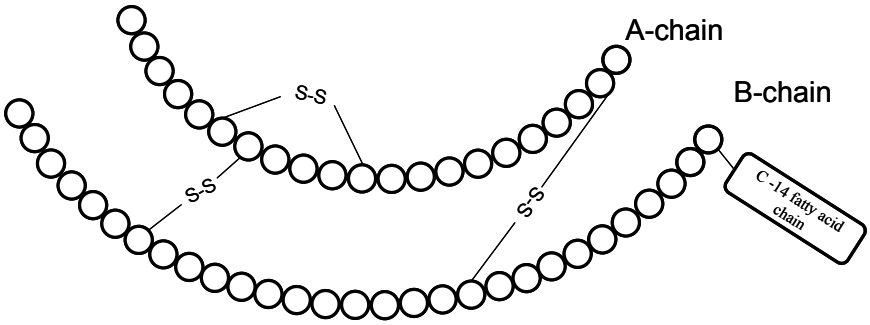


Figure 2.10 Schematic presentation of modifications in the insulin molecule to generate Insulin Detemir. Amino acid at the 30th position of the B-chain has been omitted and a 14-carbon fatty acid chain is added to the 29th position.

These insulin analogues are gradually replacing the conventional insulin preparations. Nowadays, insulin-dependent diabetic patients are being prescribed rapid-acting insulin analogues to prevent blood glucose spikes after meal together with long-acting insulin analogues which can maintain basal insulin level (Garber, 2006).

2.4.4 Mitogenic effects of insulin analogues

The modification of the insulin backbone to alter the pharmacodynamics (onset time, peak time and effective duration) of insulin has been a big step in improving the life-quality of diabetic patients. However, modification of the insulin molecule may alter its biochemical properties like affinity to insulin receptor and insulin-like growth factor – I receptor (IGF-IR) or insulin receptor dissociation rate contant (K_d) (Table 2.1). These factors may lead to higher proliferative potency of insulin analogues in comparison to normal insulin. For example, insulin receptor activation is predominantly related to metabolic control, but it also elicits a mitogenic response in the cell. The sustained activation of insulin receptor enhances its mitogenic effect. Hansen *et al.* (1996) showed that insulin analogues having lower insulin receptor dissociation rate constant (K_d) exhibited higher mitogenicity than regular insulin. This mitogenicity was disproportionately higher for insulin analogues that had a K_d value less than 40% of normal insulin (analogues H2 and X97, Table 2.1). IGF-IR is a growth factor receptor which shows a high degree of homology with insulin receptor. The activation of IGF-IR induces strong proliferation in the cells (Sachdev and Yee, 2001). Due to modifications, insulin analogues may activate the IGF-IR resulting in higher proliferative potency compared to regular insulin and thus can pose a health hazard. Kurtzhals *et al.* (2000) demonstrated that in a human osteosarcoma cell line, B10Asp insulin induced six-fold higher proliferation in comparison to regular insulin. B10Asp is an insulin analogue generated by replacing glutamic

acid at 10th position of B-chain with asparatic acid. This modification increases its affinity for insulin receptor by two-fold and for IGF-IR by approximately six-fold in comparison to regular human insulin (Table 2.1). Slieker *et al.* (1997) reported that modifications of the carboxy-terminus of the B-chain in insulin may increase the affinity for IGF-IR. B31ArgB32Arg insulin was approximately 20-fold stronger than regular insulin in stimulating proliferation in human osteosarcoma cell line (Kurtzhals *et al.,* 2000).

Analogue	**Insulin Receptor affinity [%]**	**Insulin Receptor off-rate or relative K_d [%]**	**IGF-I receptor affinity [%]**
Human insulin	100	100	100
H2 (A8HisB4HisB10GluB27His)	N.D.	**1.5 ± 0.1***	N.D.
X97 (B10GluB30desThe)	N.D.	**24 ± 4***	N.D.
B10Asp	**205 ± 20**	14 ± 1	**587 ± 50**
B31ArgB32Arg	**120 ± 4**	75 ± 8	**2,049 ± 202**
Insulin Aspart (Novorapid)	92 ± 6	**81 ± 8**	81 ± 9
Insulin Lispro (Humalog)	84 ± 6	100 ± 11	**156 ± 16**
Insulin Glargine (Lantus)	86 ± 3	152 ± 13	**641 ± 51**
Insulin Detemir (Levemir)	46 ± 5	204 ± 9	16 ± 1

Table 2.1 Insulin analogues differ from regular human insulin. Modifications performed to generate insulin analogues may result in altered insulin receptor affinity, insulin receptor off-rate (lower values mean longer duration of occupancy) and IGF-I receptor affinity. Data shown here are means ± SE (adapted from Kurtzhals *et al.,* 2000; * Hansen *et al.,* 1996; N.D means 'not determined').

Another analogue similar to B31ArgB32Arg namely Insulin Glargine (A21GlyB31ArgB32Arg), showed up to eight-fold higher mitogenic potency compared to regular insulin. Insulin Glargine was also demonstrated to have higher mitogenicity in primary cultures of normal fibroblasts and smooth muscle cells which expressed high levels of IGF-IR and IRS-1 proteins (Eckardt *et al.,* 2007).

2.4.5 Effect of insulin analogues on human mammary epithelial cell lines

Insulin receptor and IGF-IR show high expression levels in epithelial cells of the normal human mammary gland and have been shown to be significantly overexpressed in breast cancer and in malignant mammary cell lines (Papa *et al.,* 1990; Milazzo *et al.,* 1992; Schnarr *et al.,* 2000; Sachdev and Yee, 2001; Frasca *et al.,* 2008). Thus the mammary gland may represent a sensitive target for growth stimulation by insulin analogues. Indeed, treatment of MCF10A cells (a non-malignant human breast cell line) with B10Asp caused increased focus formation (Milazzo *et al.,* 1997). B10Asp was also more potent than regular insulin in stimulating colony formation in MCF7 cells (a malignant human breast cell line). Furthermore, treatment of female Sprague-Dawley rats with B10Asp led to strong increase in the incidence of mammary tumours (Dideriksen *et al.,* 1992). Studies on human mammary epithelial cells (HMEC) by Slieker *et al.* (1997) revealed that B31ArgB32Arg insulin stimulated proliferation ten-fold higher than regular insulin. Taken together, these studies demonstrate that insulin analogues may induce strong proliferation in mammary epithelial cells.

2.5 Breast Cancer

2.5.1 General information

The mammary gland is a unique organ in that it undergoes the majority of its development after birth and mostly during puberty. A female child has a rudimentary mammary gland. The onset of puberty results in increase of estrogen, progesterone and growth factors in blood. Under the effect of these hormones proper development of the mammary gland takes place (Sternlicht, 2006). In addition, during every menstrual cycle, estrogen and progesterone are secreted from ovary in a cyclical pattern. These hormones in combination with growth factors induce recurring rounds of proliferation and apoptosis of mammary cells (Ramakrishnan, 2002). It is suggested that this cyclical proliferation of mammary cells can result in the gradual accumulation of genetic alterations which may ultimately culminate into a breast cancer (Fig. 2.11).

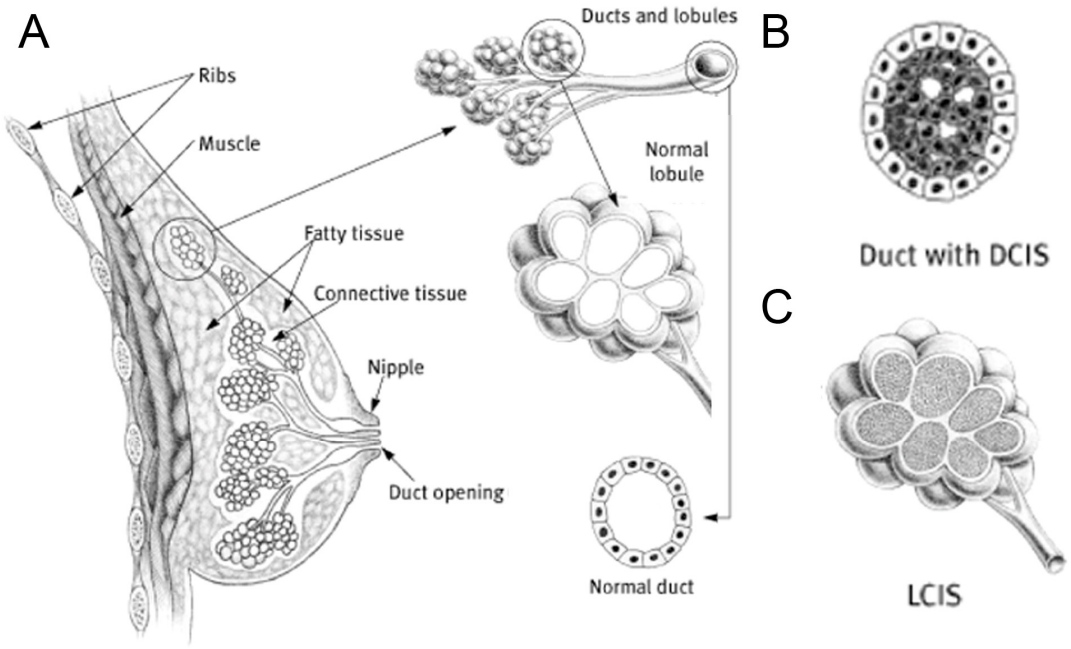


Figure 2.11 Schematic presentation of the organisation of normal breast tissue and two major types of breast cancer. A. Anatomically, a mature breast tissue is organised in ducts and lobules. Lobules contain milk producing glands. These lobules are linked together by tiny tubes called ductules, which join to form ducts and transport milk to the nipple. The lobules and ducts are surrounded by connective tissue and adipose tissue. Whole breast tissue is attached to the chest wall by the help of pectoral muscles. **B.** Appoximately 80% of breast cancer develop from ducts. The ductal carcinoma in situ (DCIS) is a clinically pre-cancerous state characterized by rapidly dividing ductal cells located within the duct. When aberrantly dividing ductal cells gain invasive property and spread to adjoining areas it is called 'invasive ductal carcinoma'. **C.** Nearly 10% of breast cancer originates from lobular cells. The uncontrolled division of lobular cells give rise to lobular carcinoma in situ (LCIS). When these cells become invasive they give rise to 'Lobular carcinoma' (adapted from National Cancer Institute website).

Breast cancer is the most frequently detected cancer in women and its incidence has been gradually increasing. Annually, about one million new breast cancer cases are detected with 320,000 cases only in Europe. Breast cancer incidence rates increase with age, more rapidly among pre-menopausal and peri-menopausal women than among post-menopausal women (Bray *et al*., 2004). This pattern of flattening after menopause is observed only for breast cancer. These findings again suggest that hormones play an important role in the development of breast cancer. Indeed, events which are related with hormonal changes like onset of puberty, menopause, pregnancy, lactation and estrogen replacement therapy have been reported by various epidemiological studies to be associated with breast cancer risk. For example, women who have first pregnancy before the age of 30 years have only half of the risk to develop breast cancer than nulliparous women (Dumitrescu and Cotarla, 2005). Studies also suggest that breast feeding women have low breast cancer risk. For every 12 month of breast feeding there is approximately 4% reduction in the breast cancer risk (Dumitrescu and Cotarla, 2005).

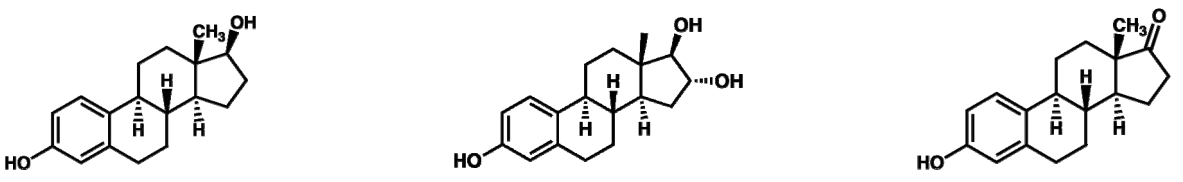
Besides influence of hormones, various other factors have been identified that increase the risk to develop breast cancer. Lifestyle choices can have strong impact on the development of breast cancer. For example, every 10 g increment in alcohol consumption increases breast cancer risk by 9% (Dumitrescu and Cotarla, 2005). Furthermore, diets which may result in the generation of high amounts of free oxygen radicals increase breast cancer risk. Anti-oxidant rich diet shows a negative correlation with breast cancer risk (Linos and Willett, 2007). In addition to lifestyle choices, the genetic background of women can also predispose her to develop breast cancer. The hereditary breast cancer comprises approximately 10% of total breast cancer cases (McPherson *et al*., 2000). The breast cancer susceptibility genes can be grouped into two categories: high-penetrance genes like BRCA1, BRCA2, PTEN, ATM or p53 and low-penetrance genes like ERα, alcohol dehydrogenase (ADH) and CYP1A1 (an enzyme that catalyses 2-hydroxylation of estradiol). Any deleterious mutation in high penetrance genes can increase breast cancer risk significantly. For example, women who are carriers of BRCA1 or BRCA2 mutations have approximately 80% increased risk for developing breast cancer (Oesterreich and Fuqua, 1999). Likewise, PTEN germline mutation results in 25-50% lifetime breast cancer risk (Dumitrescu and Cotarla, 2005). However, polymorphisms in low penetrance genes do not increase breast cancer risk drastically but in combination with various environmental factors they can have significant effects.

This thesis mainly aims to investigate and elucidate the mechanism behind the proliferative ability of insulin, insulin analogues and IGF-I on estrogen dependent and independent mammary cell lines. Therefore, the role of insulin, IGF-I and estrogen in breast cancer will be presented in detail in the following sections.

2.5.2 Role of estrogen in breast cancer

Epidemiological studies have shown that the prolonged exposure to estrogen increases breast cancer risk (Clemons and Goss, 2001). Estrogen exposure may be increased as a consequence of early onset of puberty, late menopause or estrogen replacement therapy. Girls who reach puberty before 12 years of age have 10-20% more risk to develop breast cancer than girls who reach puberty after the age of 14 years (Dumitrescu and Cotarla, 2005). Similarly, women with late onset of menopause show approximately 3% increase in breast cancer risk with every 1-year increase in the onset age of menopause (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). In contrast, the breast cancer risk was 40% lower in women who underwent surgical operations to induce menopause ovariectomy at or below 35 years of age in comparison to women who had menopause at normal age (McPherson *et al*., 2000). Conversely, post-menopausal women who develop breast cancer have approximately 15% higher circulating estradiol level than post-menopausal women without breast cancer (Dumitrescu and Cotarla, 2005). Considering results of all these studies, it can be concluded that higher estrogen level increases breast cancer risk. A brief description of estrogen and its mode of action are presented below.

Estrogens like other steroid hormones are derived from cholesterol. There are three major types of estrogens in females - estradiol (17β-estradiol), estrone and estriol (Fig. 2.12). Estradiol is the primary estrogen in females when they are reproductively active. After menopause, estradiol levels decrease and estrone becomes the major estrogen. Estriol is primarily produced during pregnancy by placental tissues.



A **B** **C**

Figure 2.12 Chemical structure of three major estrogens – estradiol (A), estriol (B) and estrone (C).

The major estrogen, estradiol, is pre-dominantly synthesised in the ovaries during follicular phase of the menstrual cycle. It plays a key role in the development of normal breasts and is also involved in the sexual maturation of uterus. Studies show that it can also be synthesised at other sites like adipose tissue, brain and arterial walls where it may have localized effects (Simpson, 2003). 95% of estradiol circulating in the blood is bound to plasma carrier proteins like sex hormone-binding globulin (SHBG) thus seriously limiting bioavailability of estradiol. Epidemiological studies show that in few breast cancer cases, the expression of these carrier proteins are downregulated resulting in increased bioavailability of estradiol (Calle and Kaaks, 2004). The free estradiol, being a steroid hormone (hydrophobic property), passes through the cell membrane and nuclear membrane and binds to its cognate receptor, estrogen receptor (ER) which pre-dominantly resides in the nucleus (classical mechanism of estradiol actions). Additionally, estradiol may also interact with ER in the cytoplasm and this liganded ER enters the nucleus.

Two subtypes of estrogen receptor have been reported: estrogen receptor – alpha (ERα) and estrogen receptor – β (ERβ). Both, ERα and ERβ, belong to the steroid/thyroid hormone superfamily of nuclear receptors and share similar structures and modes of action. They show modular structure with various functional domains. These are the NH_2-terminal or A/B domain that modulates gene transcription in cell specific manner through its Activation Function-1 (AF-1); the C-or DNA-binding domain which contains two zinc fingers for interaction with DNA helix; a D domain or hinge region which contains the NLS; an E domain that contains ligand binding domain and ligand-dependent Activation Function-2 (AF-2); and F domain which may distinguish between estrogen agonists and antagonists by interacting with other cell specific factors (Klinge, 2000; Ali and Coombes, 2002). ERα or ERβ knockout mice have life span equal to wild type mice but exhibit reproductive abnormalities (Couse and Korach, 1999). ERα is required for most of the estrogenic responses. Both, ERα or ERβ, are overexpressed in the majority of breast cancer cases. About 70% of primary breast cancers are ERα positive. ERα has been found to be a valuable prognostic factor in the clinical management of breast cancer (Sommer and Fuqua, 2001). Numerous studies have shown that ERα positive breast tumours respond better to therapy than ERα negative breast tumours (Allred *et al*., 2004).

In addition to the classical mechanism of estradiol actions, recent studies have demonstrated the existence of non-classical mechanisms. Estradiol has been suggested to be a ligand for GPR30, a membrane-associated ER localized to endoplasmic reticulum (Revankar *et al*.,

2005; Prossnitz *et al*., 2008). Activation of GPR30 by estrogen has been shown to result in intracellular calcium mobilization and synthesis of PIP3 in the nucleus. GPR30 knockout in female mice resulted in hyperglycemia, impaired glucose tolerance, reduced body growth and inhibition of estradiol-induced insulin release from β-cells of the pancreas suggesting regulation of insulin function by estradiol (Martensson *et al*., 2008). Additionally, estradiol has been shown to initiate rapid activation of signalling molecules like Akt and Erk1/2 via interaction of estradiol-activated ERα with a scaffold protein, MNAR (*m*odulator of *n*ongenomic *a*ction of estrogen *r*eceptor) (Greger *et al*., 2007). Both Akt and Erk1/2 are downstream effectors of EGFR and IGF-IR. This implies cross-talk of ERα with growth factor activated signalling pathways (Smith, 1998; Levin and Pietras, 2008). The cross-talk between ERα and IGF-IR is introduced in the following section.

2.5.3 Involvement of IGF signalling system in breast cancer

2.5.3.1 IGF signalling system

The IGF (insulin-like growth factor) system consists of IGF ligands (IGF-I and IGF-II), receptors (IGF-IR and IGF-IIR), IGF-binding proteins (IGFBPs; these bind to IGF ligands) and proteases of binding proteins (Sachdev and Yee, 2001). IGF-I and IGF-II are 79 and 60 amino acid long single chain polypeptide growth factors. Earlier, it was assumed that all IGF-I is produced by the liver under the tight control of GH (growth hormone) from pituitary gland and that the endocrine action of IGF-I is the major determinant for postnatal growth (Froesch *et al*., 1985; Daughaday and Rotwein, 1989). The liver specific knockout of the igf-1 gene by cre-lox recombination sytem decreased the circulating IGF-I level by 75% but this did not effect the postnatal growth. This indicated that an autocrine/paracrine action of IGF-I is the major determinant for the tissue growth (Sjögren *et al*., 1999; Yakar *et al*., 1999). Later, it was shown that IGF-I can be produced by other organs as well. Similarly, IGF-II is also expressed both in the liver and in extrahepatic sites, but unlike the igf-1 gene which lacks any imprinting, the igf-2 gene is paternally imprinted (Sasaki *et al*., 1992). Both, IGF-I and IGF-II show a high degree of homology to the insulin molecule. For example, the positions 1 to 29 in IGF-I are homologous to insulin B-chain and positions 42 to 62 to insulin A-chain (Rinderknecht and Humbel, 1978). However, IGFs mainly have a mitogenic effect while insulin mainly exerts metabolic effect.

Both IGF-I and IGF-II can bind to IGF-IR, which is a cell-surface tyrosine kinase receptor. It is expressed by all cell types with the exception of hepatocytes and T lymphocytes (Sachdev

and Yee, 2001). IGF-IR is highly homologous to insulin receptor, especially in the tyrosine kinase domain, in which they share 84% amino acid identities. Like insulin receptor, IGF-IR consists of two α-subunits and two β-subunits. The α-subunits are entirely extracellular and possess the ligand binding domain. The β-subunit has a small extracellular domain through which it is attached to the α-subunit by disulphide bonds, a membrane spanning domain and a large intracellular domain (Adams *et al*., 2000). The intracellular domain consists of the juxtamembrane domain, the tyrosine kinase domain and C-terminal domain. Under unstimulated condition, the catalytic activity of the IGF-IR is inhibited by the presence of an activation loop (a-loop; stretch of amino acids containing critical tyrosine residues 1131, 1135 and 1136) which acts as a pseudosubstrate and prevents the substrate access and ATP binding (Larsson *et al*., 2005). The β-subunits possess trans-phosphorylation activity by which one subunit phosphorylates the tyrosine residues in the other subunit.

The binding of ligand to the IGF-IR results in the trans-phosphorylation of the three critical tyrosine residues in the a-loop which changes the conformation of the a-loop. This conformational change releases the inhibitory effect of a-loop at the tyrosine kinase domain. Once activated, tyrosine kinase domain phosphorylates other tyrosine residues in the IGF-IR and exogenous substrates like IRS-1, IRS-2 and Shc which in turn result in the activation of PI3K, MAPK and 14-3-3 pathways (Baserga, 2000). Despite the high sequence homology, the functions of IGF-IR and insulin receptor are considerably different. The IGF-IR is mainly involved in regulation of cell proliferation, anti-apoptosis, differentiation and cell motility. Insulin receptor, as described in section 2.2 is mostly involved in control of glucose uptake and metabolism. Due to the high degree of homology, IGF-IR and insulin receptor are reported to form hybrids that basically behave as an IGF-IR (Frasca *et al*., 2003). IGF-IIR which is also referred to as mannose 6-phosphate receptor (M6PR), preferentially binds IGF-II. It has no intracellular kinase domain and does not act as a signalling molecule (Hebert, 2006). It is generally accepted that IGF-IIR functions as a sink for IGF-II and thus decreased expression of IGF-IIR can result in enhanced availability of IGF-II leading to increase in cell proliferation.

The IGFBPs are a family of six homologous proteins (IGFBP 1-6) which have high binding affinity for IGF-I and IGF-II (Binoux *et al*., 1991). Their binding affinity for IGF-I and IGF-II is comparable to the binding affinity of IGF-IR. These proteins are present in the circulation as well as extravascular fluids and sequester IGFs from circulation. In human serum > 95% of IGFs are bound to IGFBPs and thus unavailable for action. Nowadays, there is increasing

evidence that the IGFBPs may have growth regulatory actions that are independent of their normally suggested IGF binding activity (Gucev *et al.*, 1996). Cathepsins and matrix metalloproteinases cleave IGFBPs into smaller molecular forms that have reduced affinity for IGFs and this releases IGFs from the binding protein complex (Schmid *et al.,* 1991; Fowlkes *et al.*, 1994).

2.5.3.2 Cross-talk between IGF-IR and ERα

The IGF-IR signalling pathway has been reported to cross-talk with that of ERα (Fig. 2.13; Smith, 1998; Kato *et al.*, 2000). Several studies suggested synergistic effects of IGF-I and estradiol on MCF7 cell proliferation (Dupont and Roith, 2001).

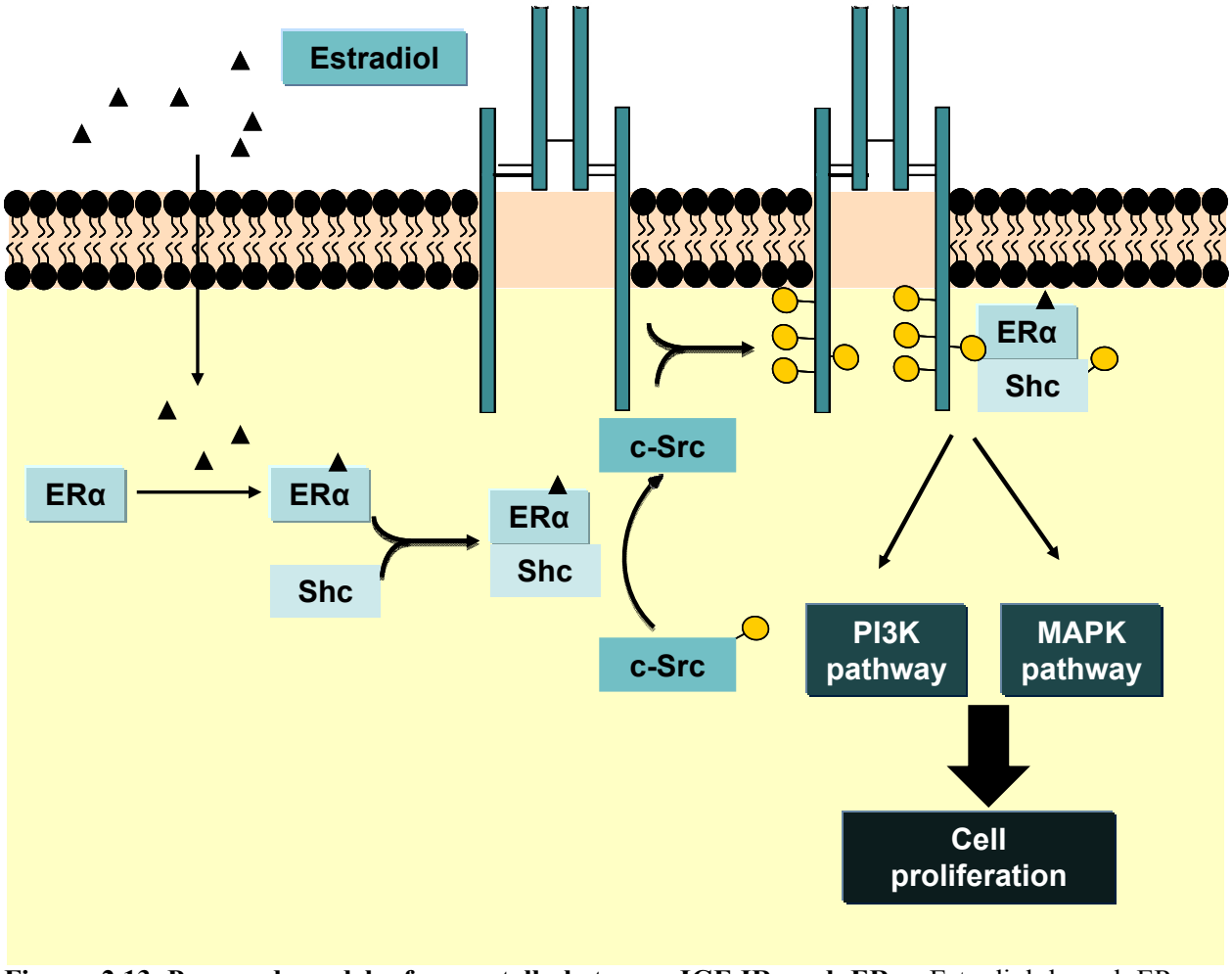


Figure 2.13 Proposed model of cross-talk between IGF-IR and ERα. Estradiol bound ERα interacts with Shc. This causes dephosphorylation and activation of c-Src. c-Src in turn phosphorylates IGF-IR, which phosphorylates and recruits Shc. The interaction of Shc to IGF-IR results in the translocation of ERα to the membrane. Activation of IGF-1R results in the activation of MAPK pathway (Song *et al.*, 2004) and probably also of PI3K pathway, overall leading to cell proliferation.

ERα has been shown to increase the expression of IGF-IR and IRS-1 (Mauro *et al.*, 2001; Maor *et al.*, 2006). Oesterreich *et al.*, (2001) generated MCF7 sublines (C4 and C4-12) that

were negative for ERα by prolonged estrogen withdrawal. The lack of ERα in these cell lines resulted in decrease in mRNA and protein of IGF-IR and IRS-1. Re-expression of ERα in these cell lines resulted in the increased expression of IGF-IR and IRS-1, thus confirming the direct role of ERα in regulating IGF-IR and IRS-1 gene expression.

Other studies have shown that ERα can directly interact with the signalling components of IGF-IR pathway. For example, ERα has been shown to interact with SH2 region of Src, the p85α regulatory subunit of PI3K, Shc and IGF-IR (Kahlert *et al.,* 2000, Migliaccio *et al.,* 2000, Simoncini *et al.,* 2000, Sun *et al.,* 2001, Song *et al.,* 2002). Recently, Song *et al*. (2004) demonstrated that estradiol can indirectly phosphorylate and activate IGF-IR. They suggested formation of a ternary complex between Shc, ERα, and IGF-IR in response to estradiol treatment. The downregulation of Shc, ERα, or IGF-IR with specific small inhibitory RNAs blocked E2-induced MAPK phosphorylation. In addition, our own study has shown that GSK3α/β, a downstream signalling molecule of PI3K pathway, is important for stabilization and full transcriptional activity of ERα (Grisouard *et al.*, 2007).

2.5.3.3 Role of IGF signalling system in breast cancer

Several studies have implicated the IGF system with increased breast cancer risk. The comparative analysis of IGF-I level in serum of breast cancer patients and matched controls revealed increased IGF-I level in the breast cancer patients (Pollak, 1998). Epidemiological studies have demonstrated high plasma levels of IGF-I as a potential risk factor for breast cancer (Hankinson *et al*., 1998). Schernhammer *et al*. (2005) reported that circulating IGF-I level is modestly associated with breast cancer risk among premenopausal women (RR, 1.6; 95% CI, 1.0-2.6), but not among postmenopausal women. The relative risk was higher for premenopausal women of age ≤ 50 years (RR, 2.5; 95% CI, 1.4-4.3). Studies implicate paracrine action of IGF-I in the development of breast cancer as *in situ* hybridization showed that IGF-I is mainly expressed in stromal cells and not in breast epithelium cells (Yee *et al*., 1989). Interestingly, transgenic mice overexpressing IGF-I show increased ductal hypertrophy in the lactating mouse (Sachdev and Yee, 2001). IGF-II has also been demonstrated to be positively associated with breast cancer (Singer *et al*., 2004). Transgenic mice overexpressing IGF-II developed mammary tumours (Bates *et al*., 1995). In breast cancer patients, free IGF-II serum concentrations and free IGF-II/total IGF-II ratio correlates with tumour size. Furthermore, *in situ* hybridization and immunohistochemical analysis of breast tissues revealed that, as observed with IGF-I expression, stromal cells adjacent to malignant

epithelial cells have higher IGF-II expression than stromal cells around benign or normal breast epithelium, suggesting paracrine action of IGF-II in breast cancer (Giani *et al*., 1996; Giani *et al*., 1998).

The analysis of primary breast tumours shows not only the increase in IGF-IR expression levels in comparison to benign tumours or normal breast epithelium (Pezzino *et al*., 1996), tyrosine kinase activity of the IGF-IR has also been reported to be enhanced in breast cancer (Resnik *et al.* 1998). Turner *et al*. (1997) demonstrated a positive association of IGF-IR levels and radioresistance of breast cancer cells. They also reported that tumours with elevated IGF-IR had a higher incidence of recurrency at the primary site. However, tumours with high IGF-IR levels have better prognosis than cancers with low level of IGF-IR (Yee *et al*., 1994). IGF-IR has been shown to be overexpressed in breast epithelial tumour cell lines. Studies of the IGF-IIR locus report significant loss of heterozygosity in breast cancer suggesting IGF-IIR as a breast tumour suppressor gene (Hankins *et al*., 1996). Mutations in the IGF-II binding domain of IGF-IIR have been reported in breast cancer cells (Byrd *et al*., 1999). The stable transfection of MDA-MB231 breast cancer cells with IGF-IIR cDNA not only markedly reduced their ability to form tumours but also reduced growth rate in nude mice, suggesting the importance of low expression of IGF-IIR in tumour formation (Lee *et al*., 2003). Other members of the IGF system like IGFBPs have also been studied in relation to breast cancer risk. Recent epidemiological studies suggested positive association between lower levels of IGFBP3 and increased breast cancer risk in premenopausal women (Bruning *et al*., 1995; Hankinson *et al*., 1998; Allen *et al*., 2005). Reduced expression of IGFBPs may be a reason for the reported increase in circulating levels of IGF-I and IGF-II in premenopausal women.

2.5.4 Involvement of insulin signalling pathway components in breast cancer

Several studies have implicated insulin and its cognate receptor in the development of cancer. Earlier studies in animal models suggested a direct role of insulin in the development of cancer. For example, pharmacological doses of insulin were shown to stimulate carcinoma formation and DNA synthesis and affect the squamous neoplastic cell differentiation in Swiss male mice treated with the chemical carcinogen 3-methylcholanthrene thus suggesting insulin as a strong co-carcinogen (Lupulescu, 1985). Dombrowski *et al*. (2003) demonstrated that high local insulin concentration in liver of diabetic BB/Pfd rats results in the formation of hepatocellular neoplasms, demonstrating the role of insulin as a potential carcinogen. Another study demonstrated that breast cancer induced in rats by the chemical carcinogen

dimethylbenz(a)anthracene regresses under insulin deficient conditions (pancreatic β-cells were destroyed by treating with alloxan). Tumour growth was shown to resume when insulin was provided exogenously (Heuson *et al*., 1972). Interestingly, tumour growth did not resume upon administration of exogenous estrogen. Similarly, MCF-7 human breast cancer cells formed tumours in insulin treated diabetic nude mice but not in untreated diabetic nude mice, again suggesting a direct role of insulin in tumour development (Nandi *et al*., 1995). Taken together, all these animal experiments reveal direct role of insulin in cancer formation in general and breast cancer in particular.

Epidemiological studies have linked increased insulin level with breast cancer. Circulating insulin levels were reported to be elevated in women with premenopausal breast cancer (Del Giudice *et al*., 1998). Data obtained from patients suffering from insulin resistance and/or hyperinsulinemia, two important characteristics of type II diabetes, show increased risk for breast cancer (Bruning *et al*., 1992; Yam *et al*., 1996; Gamayunova *et al*., 1997). The mechanism by which high insulin levels may result in breast cancer is presented in Fig. 2.14. Obesity which is an important risk factor for type II diabetes has been consistently shown to increase breast cancer rates in post-menopausal women by 30-50% (Carroll, 1998; Calle and Kaaks, 2004).

Insulin receptor has been reported to be overexpressed in majority of the breast cancer cases (Belfiore *et al*., 1996; Papa *et al*., 1996). Breast tumours with high expression level of insulin receptor showed significantly reduced disease free survival (Mathieu *et al*., 1997). Many well-established mammary epithelial tumourigenic cell lines like MCF7 and ZR75-1 also show increased expression of insulin receptor (Frasca *et al.*, 2003). Interestingly, breast cancer cells predominantly overexpress the fetal isoform of insulin receptor, IR-A (Frasca *et al*., 1999). The IR-A isoform differs from the B-isoform of insulin receptor by the absence of a 12–amino acid segment in the carboxy-terminus of the α-subunit. IR-A can bind both insulin and IGF-II and has enhanced mitogenic signalling relative to the IR-B (Frasca *et al.*, 2003).

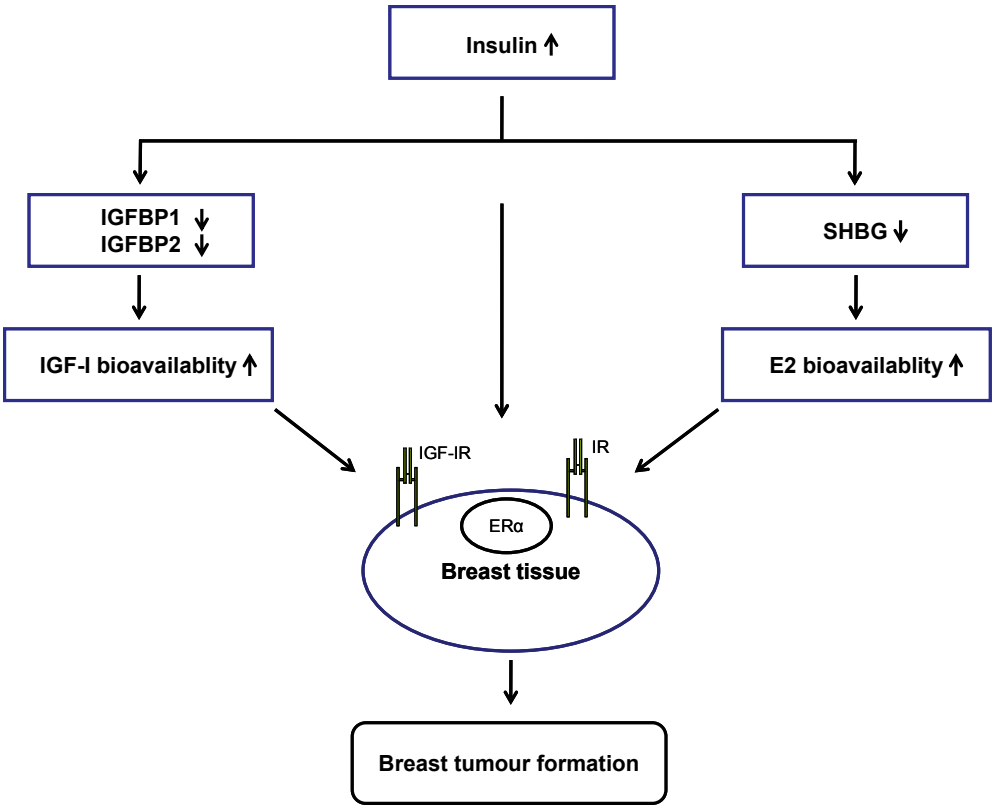


Figure 2.14 Diagram demonstrating the mechanism of breast tumour formation by high insulin levels. Elevated blood insulin levels result in activation of insulin receptor (IR) and in increase of mitogenic signal to mammary cells. In addition, elevated insulin levels lead to decrease in the synthesis of IGF-binding proteins (IGFBP) 1 and 2 and sex-hormone-binding globulins (SHBG) which results in increased bioavailability of IGF-I and estradiol (E2), respectively. Both, IGF-I and E2 are potent mitogens for mammary cells. The overall effect of all this may be breast tumour development (Calle and Kaaks, 2004).

2.6 Aims

Modification of the insulin backbone may alter the biochemical properties of the resulting insulin analogues like affinity for insulin receptor, dissociation rate from insulin receptor, and affinity for IGF-IR (Table 2.1). Consequently, the signalling and proliferative potencies of insulin analogues may be altered. This may pose a serious health risk as proliferation of cells which express these receptors may be stimulated by such insulin analogues. The findings demonstrating strong proliferative ability of B10Asp relative to human insulin in a osteosarcoma cell line (Kurtzhals *et al*., 2000) and the high incidence of mammary tumours in B10Asp treated female Sprague-Dawley rats (Dideriksen *et al.,* 1992) give credence to the health concern regarding insulin analogues. Normal breast tissue shows high expression of insulin receptor and IGF-IR and breast tumours show overexpression of both receptors (Papa *et al.,* 1990; Schnarr *et al.,* 2000; Frasca *et al.,* 2008). Since insulin analogues may activate IGF-IR in addition to insulin receptor, it can be concluded that the mammary gland represents a sensitive target for growth stimulation by insulin analogues.

The proliferative ability of insulin analogues that are currently approved for therapeutical use has been studied on various cell systems. However, there is lack of any comprehensive study in mammary epithelial cell lines. The few studies which have been published till date, are not comparable with each other as data has been acquired under dissimilar experimental conditions and estimated by different assay systems, thus leading to results which do not allow to draw general conclusions. In addition, all studies performed on mammary epithelial cell lines are limited in terms of the number of insulin analogues tested.

The initial aim of this work is to compare the proliferative potency of various insulin analogues with regular insulin in mammary epithelial cell lines. For this, we will screen a panel of mammary epithelial cell lines for their insulin responsiveness and select the strongly insulin responsive cell lines for the comparative analysis of insulin analogues proliferative potency.

Thereafter, we compare the signalling potency of insulin analogues with regular insulin. Specifically, we consider analysis of PI3K and MAPK pathways as these two pathways are activated by regular insulin and are involved in cell proliferation. The activation of these two pathways is studied by analysing the phosphorylation status of key signalling molecules after treatment with regular insulin or insulin analogues. For studying PI3K pathway activation, Akt and GSK3α/β phosphorylation status is studied. MAPK pathway activation is analysed by

studying phosphorylation status of Erk1/2. Specific inhibitors against both pathways are exploited to identify the pathway related to proliferation induced by insulin analogues.

In addition, we aim to study the role of insulin receptor and IGF-IR in the proliferative potency of insulin analogues that show significantly higher proliferation than regular insulin. We utilize RNAi technique to specifically target insulin receptor and IGF-IR. This will enable us to identify the receptor that is mainly activated by insulin analogues. In addition, it will also permit comparative analysis of PI3K and MAPK pathways activated by treatment with insulin, IGF-I and insulin analogues. The receptor identified to play the more important role in proliferation is further analysed. Immunoprecipitation is used to gain understanding of receptor activation by studying the phosphorylation status of the receptor after treatment with insulin, IGF-I and insulin analogue. Furthermore, the activation of this receptor is eventually corroborated by studying the expression of a downstream target gene at the transcriptional level using quantitative RT-PCR.

In order to clarify the potential activation of the cross-talk between insulin receptor/IGF-IR and ERα by insulin analogues, we determine the activation of ERα by analysing the phosphorylation status of Ser118 at ERα as well as ERE-dependent gene expression.

Moreover, we aim to extend our study to the possible tumour-promoting potential of those insulin analogues that demonstrate significantly higher proliferative potency than regular insulin. We assess this by employing the ‘wound-healing assay’ for studying cell migration.

To summarise, the objective of this study is to perform a detailed comparative analysis of proliferative and signalling potency of insulin analogues in insulin responsive mammary epithelial cell lines, and to elucidate the role of insulin receptor, IGF-IR and ERα in the mitogenic potency of insulin analogues.

3. RESULTS

3.1 Screening of mammary epithelial cell lines

In order to compare the mitogenic potency of insulin analogues with regular insulin we needed to identify 'insulin responsive' mammary epithelial cell lines. For this, we studied the proliferative response of a panel of mammary epithelial cell lines to treatment with regular insulin. In addition, we studied the expression pattern of insulin receptor, IGF-IR and ERα to gain more information about cell characteristics.

3.1.1 Proliferative response to the insulin treatment

To determine the insulin responsiveness of mammary epithelial cell lines, we treated cells with regular insulin (Actrapid or bovine insulin) for 72 hrs and measured proliferative response by colorimetric proliferation assay. Cells were treated with very high concentration (1.5μM) of regular insulin to stimulate maximum proliferation. Proliferation response (obtained as absorbance value) of insulin-treated cells was normalized with that of untreated cells so to obtain the 'fold of control'.

Cell line	Tumor	Type of normal insulin used	Fold of Control
MCF10A	No*	Actrapid	**1.7** ± 0.20
BT474	Yes*	Bovine Insulin	0.9 ± 0.05
MCF7	Yes*	Actrapid	**2.2** ± 0.28
T47D	Yes*	Bovine Insulin	1.1 ± 0.04
ZR75-1	Yes*	Actrapid	1.4 ± 0.20
MDA-MB231	Yes*	Bovine Insulin	1.3 ± 0.12
HCC1937	Yes*	Bovine Insulin	1.1 ± 0.05

Table 3.1 MCF10A and MCF7 exhibited highest insulin induced proliferation among the panel of cell lines tested. Cells were starved for 24 h in medium containing 2% charcoal-stripped serum and then were treated with 1.5μM insulin for 48 h (MCF10A) or for 72 h (all other cell lines). Thereafter, cells were fixed with 3% paraformaldehyde in PBS, stained with crystal violet and absorbance was read at 595nm. Fold of control was calculated by comparing the growth achieved in absence or presence of insulin. *, information regarding tumorigenicity of the cell lines was obtained from the ATCC website.

HCC1937, BT474 and T47D demonstrated poor proliferative response to the insulin treatment (Table 3.1). MDA-MB231 and ZR75-1 showed relatively moderate proliferation response to the insulin treatment, demonstrating approximately 1.3 to 1.4-fold increase in proliferation. In MCF10A cells, a benign mammary epithelial cell line, insulin treatment induced approximately 1.7-fold increase in proliferation. The strongest response to insulin treatment (approximately 2.2-fold) was observed in MCF7 cells, a malignant mammary epithelial cell line.

As MCF10A and MCF7 showed the maximum proliferative response to the insulin treatment among the cell lines tested, we utilized these two cell lines for carrying out comparative analysis of proliferative and signalling potency of regular insulin and insulin analogues (Humalog, Novorapid, Lantus and Levemir) approved for therapeutical use.

3.1.2 Expression pattern of Insulin Receptor, IGF-IR and ERα

The above mentioned seven mammary epithelial cell lines were studied for the expression pattern of insulin receptor, IGF-IR and ERα (Fig. 3.1). The MELN cells, a sub-cell line of MCF7 which are stably transfected with ERE-dependent firefly luciferase reporter gene, were also included in this analysis. All the cell lines analyzed, showed moderate levels of insulin receptor. IGF-IR expression level varied among the cell lines. It was found to be strongly expressed in MCF7 and weakly expressed in HCC1937. T47D, BT474, MDA-MB231, ZR75-1, and MCF10A cells showed comparatively moderate expression of IGF-IR.

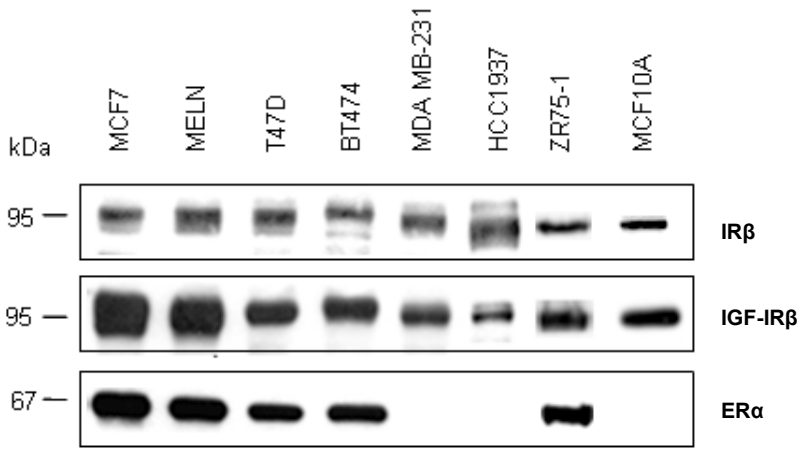


Figure 3.1. Expression profile of insulin receptor, IGF-IR and ERα in mammary epithelial cell lines. Lysates from the mammary cells were resolved by SDS-PAGE and immunoblots were probed for the expression of insulin receptor and IGF-IR by antibodies against beta-subunit of insulin receptor (IR) and IGF-IR, respectively. Anti- ERα was used for detecting ERα.

Regarding ERα expression pattern, three cell lines, MCF10A, MDA-MB231 and HCC1937, did not show any expression of ERα protein. All other cell lines expressed ERα and like IGF-IR, ERα was also found to be strongly expressed in MCF7 cells.

3.2 Dose-dependent proliferative response to insulin and insulin analogues in MCF10A and MCF7 cells

In order to study the dose-dependence of the proliferative response of MCF10A and MCF7 cells to the regular insulins and insulin analogues, cells were treated with increasing concentrations (1.5nM, 15nM, 150nM and 1.5µM) of each compound and subjected to colorimetric proliferation assay. Fold of control was calculated for each dosage of treatment. For estimating the mitogenic potency of insulin analogues, the proliferation response obtained with insulin analogues was compared with the proliferation response obtained with regular insulin.

3.2.1 Study of MCF10A cell proliferation using colorimetric method

Actrapid treatment induced proliferation response in MCF10A cells at all concentrations tested (Fig. 3.2). At 1.5nM, proliferation response to Actrapid was merely 1.1-fold of untreated cells. Treatment of MCF10A cells with 15nM, 150nM or 1.5µM Actrapid resulted in approximately 1.7-fold increase in the proliferation response compared to untreated cells indicating a saturation in response at 15nM of Actrapid.

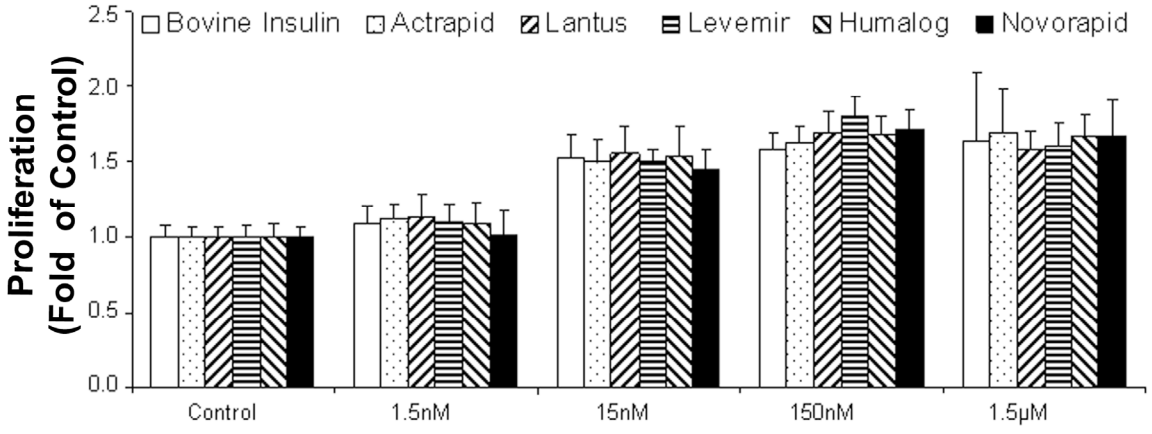


Figure 3.2 Proliferative ability of insulin and insulin analogues are similar in MCF10A cells. MCF10A cells were starved for 24 h in growth medium without insulin and then were treated with increasing concentrations (1.5nM to 1.5µM) of insulin and insulin analogues for 48 h. Thereafter, cells were fixed and stained with crystal violet dye. After air drying, stain was dissolved in 10% acetic acid and the absorbance was measured at 595nm in the plate reader. The values presented are the mean ± SD of n = 16.

The proliferation levels observed in MCF10A cells treated with bovine insulin were similar to the proliferation levels obtained with Actrapid treatment thus suggesting similar mitogenic potency of the two regular insulins in MCF10A cells. Treatment of MCF10A cells with any of the four insulin analogues resulted in proliferation levels that were comparable to the proliferation levels observed with regular insulins. Thus, regarding stimulation of proliferation in MCF10A cells, insulin analogues are equipotent to regular insulins.

3.2.2 Study of MCF7 cell proliferation using colorimetric method

In contrast to MCF10A cells, the treatment of MCF7 cells with increasing concentrations of Actrapid led to gradual increase in proliferation (Fig. 3.3a). With 1.5nM Actrapid, approximately 1.2-fold increase in proliferation level was observed and this reached to the maximum value of 2.2-fold with 1.5µM Actrapid treatment. Bovine insulin yielded proliferation levels comparable to Actrapid treatment in MCF7 cells, which again suggests equipotency of two regular insulins.

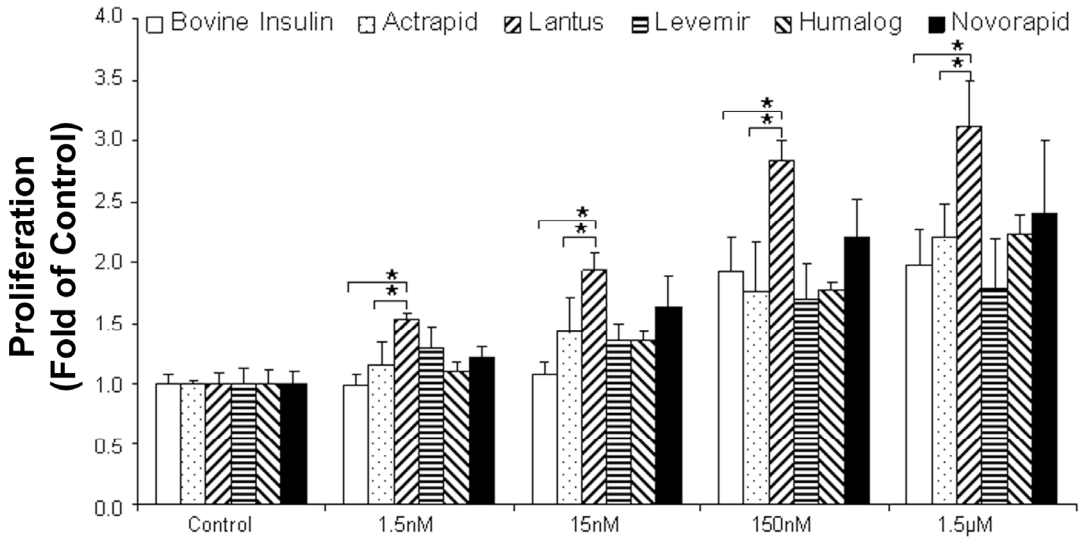


Figure 3.3a Lantus demonstrates highest proliferative ability in comparison to regular insulins and insulin analogues in MCF7 cells. MCF7 cells were starved for 24 h in medium containing 2% DCC-FBS and then were treated with increasing concentrations of insulin and insulin analogues for 72 h. Thereafter cells were fixed and stained with crystal violet dye. After air drying, the stain was dissolved in 10% acetic acid and the absorbance was measured at 595nm in a plate reader. Values presented are the mean ± SD of n = 20. The statistical analysis was done by using student's t-test. Asterisks (*) show statistically significant ($p < 0.05$, *t*-test) difference between two data points.

Humalog and Novorapid induced proliferation in MCF7 cells at all concentrations studied. The increase in proliferation observed with these two insulin analogues was similar to that obtained from regular insulins. In comparison to regular insulins, Novorapid induced slightly

stronger proliferation but the difference was not found to be statistically significant. Interestingly, MCF7 cell treatment with the two long-acting insulin analogues, Lantus and Levemir, yielded opposite effect. Treatment of MCF7 cells with 1.5nM and 15nM dosage of Levemir resulted in approximately 1.2-fold increase in proliferation which is comparable to the fold increase seen with Actrapid and bovine insulin at these concentrations. At higher concentrations (150nM and 1.5µM), Levemir was found to be slightly weaker in inducing MCF7 cell proliferation compared to the regular insulins.

However, treatment with Lantus elicited strong proliferative response in MCF7 cells at or above 1.5nM. At 1.5nM Lantus concentration, approximately 1.5-fold increase in proliferation was observed in comparison to untreated cells and this proliferation response increased to 1.8-fold at 15nM concentration. At higher dosages of 150nM and 1.5µM, Lantus induced even stronger proliferation of MCF7 cells, namely 2.7-fold and 3.1-fold in comparison to untreated cells, respectively. The statistical analysis showed that at concentrations ≥ 1.5nM, the fold of control obtained with Lantus are significantly higher compared to the fold of control obtained with Actrapid or bovine insulin.

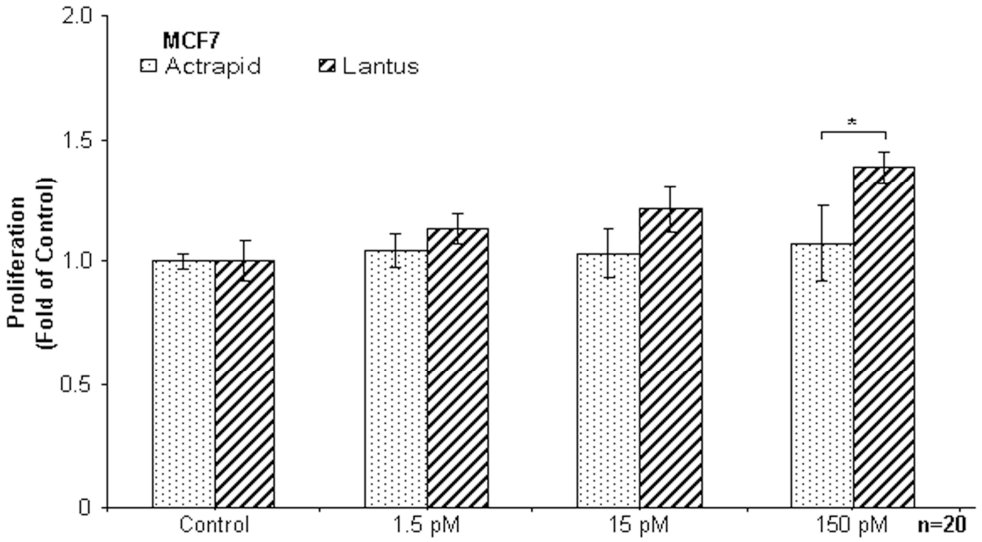


Figure 3.3b Lantus induces stronger proliferation at 1.5pM and 15pM and significantly higher proliferation at 150pM concentration compared to regular insulin in MCF7 cells. MCF7 cells were starved for 24 h in medium containing 2% DCC-serum and then were treated with increasing concentrations of Actrapid or Lantus for 72 h. Thereafter, cells were fixed and stained with crystal violet dye. After air drying, the stain was dissolved in 10% acetic acid and the absorbance was measured at 595nm in a plate reader. The values presented are the mean ± SD of n = 20. The statistical analysis was done by using student's t-test. Asterisk (*) shows statistically significant ($p < 0.05$, *t*-test) difference between two data points.

The observation that 1.5nM Lantus induces significantly stronger proliferative response of MCF7 cells than 1.5nM Actrapid motivated us to compare the proliferative ability of Lantus and Actrapid at lower concentrations (1.5pM, 15pM and 150pM). Our results show that even at 150pM concentration Lantus had significantly higher proliferative effect when compared to 150pM Actrapid (Fig. 3.3b). Also, at 1.5pM and 15pM concentration, Lantus treatment yielded stronger proliferation than Actrapid, though the difference was not statistically significant. The dose-response model showed initial statistically significant growth response (given by the ED10 estimation) of MCF7 cells to Actrapid at 5.2 nM (95% confidence intervals, lower: -1.0571e-09 mol l^{-1}, upper: 1.143e-08 mol l^{-1}) and to Lantus at 0.31 nM (95% confidence intervals: lower: -7.8890e-11 mol l^{-1}, upper: 7.059e-10 mol l^{-1}) concentrations.

To summarise, Humalog, Novorapid and Levemir had similar proliferative potency as normal insulins in MCF7 cells. Lantus, in comparison to the regular insulins, had significantly higher proliferative potency and was found to be approximately 1.5-fold stronger than Actrapid in eliciting proliferation of MCF7 cells at the concentrations ≥ 150pM.

3.2.3 Study in MCF7 cells by FACS analysis of BrdU incorporation

In order to gain more information regarding the proliferative ability of insulin and insulin analogues and to confirm our data regarding strong proliferative effect of Lantus in MCF7 cells, we chose FACS analysis of BrdU incorporation as a second readout.

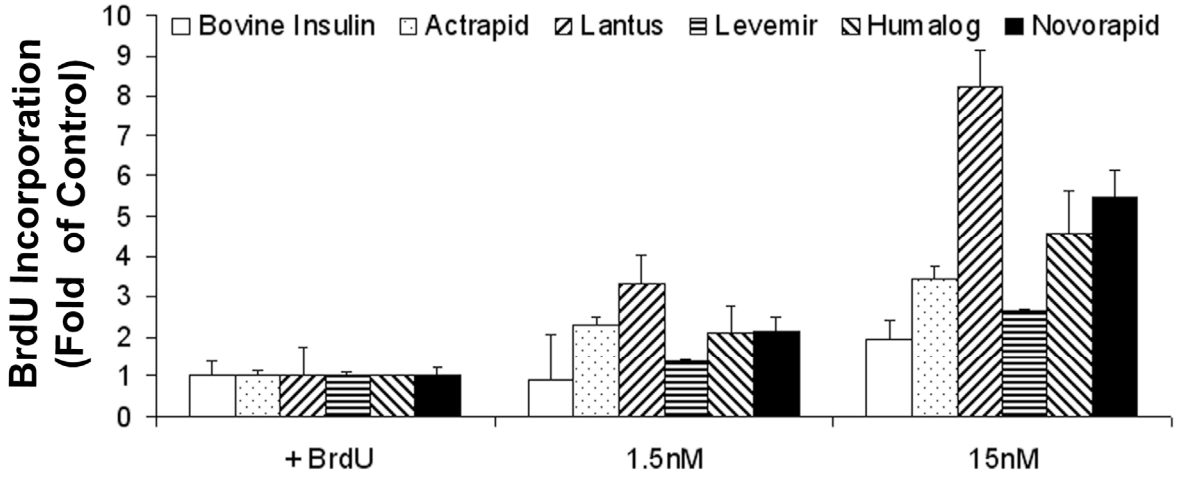


Figure 3.4 Lantus demonstrates strongest increase in BrdU incorporation in MCF7 cells when compared with insulin and insulin analogues. MCF7 cells were starved in medium containing 2% charcoal stripped serum for 24 h and then were stimulated with regular insulins or insulin analogues for 16 h. Cells were then incubated in 10µM BrdU for 1 hr and submitted to FACS analysis. Fold of control was calculated by comparing the BrdU incorporation in the absence or presence of insulin treatment. The values presented are the mean and range of duplicates.

Herein, we studied BrdU incorporation in MCF7 cells stimulated with regular insulins or insulin analogues for 16 h at two low concentrations (1.5nM and 15nM). MCF7 cells treated with Actrapid or bovine insulin exhibited increased BrdU incorporation in comparison to the untreated cells (Fig. 3.4). Treatment of MCF7 cells with 1.5nM Humalog or 1.5nM Novorapid demonstrated similar levels of BrdU incorporation as was observed with 1.5nM of regular insulins. 15nM Humalog or 15nM Novorapid however were approximately 2-fold stronger than Actrapid in stimulating BrdU incorporation. Treatment of MCF7 cells with 1.5nM or 15nM concentration of Levemir yielded slightly lower levels of BrdU incorporation in comparison to Actrapid.

In agreement with the data obtained from colorimetric proliferation assay (Fig. 3.3A), Lantus induced highest increase in BrdU incorporation in comparison to both regular insulins and any of the other insulin analogues. The comparative analysis of BrdU incorporation at 1.5nM and 15nM concentration of Lantus and Actrapid revealed that Lantus treatment led to approximately 1.5-fold (at 1.5nM) and 3-fold (at 15nM) higher BrdU incorporation than Actrapid. Taken together, the results from colorimetric proliferation assay (Section 3.2.2) and FACS analysis of BrdU incorporation clearly establish that in MCF7 cells Lantus has significantly higher proliferative potency than regular insulins or other insulin analogues studied.

3.3 Study of PI3K and MAPK signalling pathways in MCF10A and MCF7 cell lines

As described in section 2.4.4, biochemical properties of insulin analogues differ from regular insulin with respect to insulin receptor and IGF-IR binding affinity. This may result in differential activation of PI3K and MAPK pathways, the two insulin-activated pathways that are involved in cell proliferation. In this regard, it will be interesting as well as important to study the effect of insulin analogues on stimulation of both PI3K and MAPK pathways.

3.3.1 Activation of PI3K pathway by insulin and insulin analogues in MCF10A and MCF7 cells

In order to investigate the effect of regular insulin and insulin analogues on PI3K pathway, cells were treated with the compounds and phosphorylation status of Akt and GSK3α/β proteins, two important signalling molecules of the PI3K pathway, was studied. As insulin analogues may show altered affinity for IGF-IR, MCF7 cells treated with IGF-I, the physiological ligand of IGF-IR, were also analyzed. To determine the signalling potency of

insulin analogues, the phosphorylation status of signalling molecules were compared in cells treated for 10 min with 15nM of insulin analogue and regular insulin.

3.3.1.1 Study of Akt phosphorylation in MCF10A cells

The MCF10A cells were either left untreated or were treated with regular insulins (Actrapid or bovine insulin), insulin analogues or IGF-I.

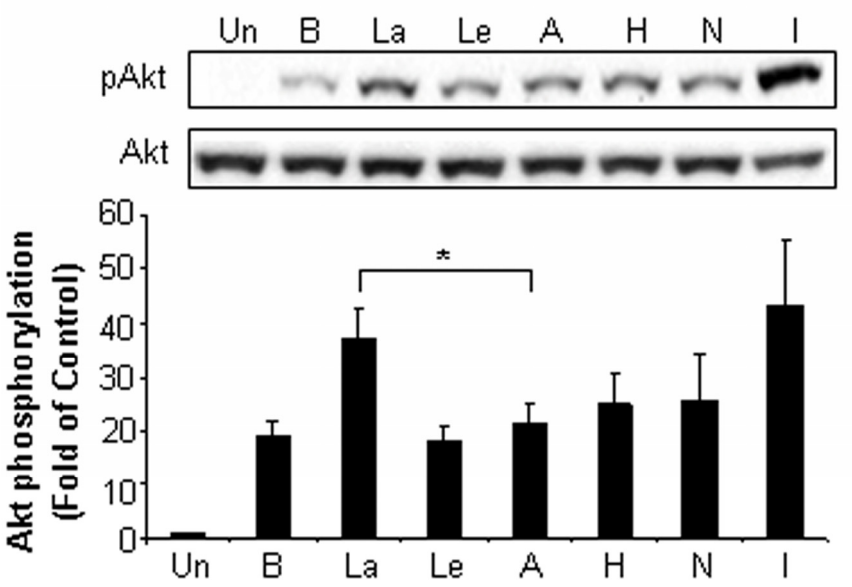


Figure 3.5 Only Lantus induced significantly stronger Akt phosphorylation in MCF10A cells in comparison to regular insulins. 5 x 10^5 MCF10A cells were plated and starved for 24 h in medium without insulin. Then cells were either processed untreated (Un) or after 10 min incubation with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). IGF-I (I; 15nM) was studied for comparison. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for phosphorylated Akt and total Akt. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. Statistical analysis was done by using student's t-test with at least three independent experiments and asterisk shows the significant difference ($p<0.05$) between Lantus and Actrapid.

The basal Akt phosphorylation in untreated MCF10A cells was almost undetectable in the immunoblots suggesting that endogenous Akt activity is very low (Fig. 3.5 lane; 'Un'). Treatment of MCF10A cells with bovine insulin (lane 'B') or Actrapid (lane 'A') increased phosphorylated Akt level substantially. However, the strongest increase in Akt phosphorylation level was observed after IGF-I treatment (lane 'I'). It was approximately two times higher than that observed with regular insulin. The Akt phosphorylation levels after Humalog (lane 'H'), Novorapid (lane 'N') or Levemir (lane 'Le') treatment was similar to the Akt phosphorylation level from regular insulin treatment. Treatment of MCF10A cells with

Lantus (lane 'La') showed strong phosphorylation of Akt. It was found to be approximately 1.7-fold stronger than Actrapid. Statistical analysis revealed that Lantus-induced Akt phosphorylation levels were significantly higher compared to the levels observed with regular insulins.

Thus, with respect to Akt phosphorylation, signalling potency of Humalog, Novorapid and Levemir was similar to regular insulin in MCF10A cells. However, Lantus was significantly stronger than regular insulins in inducing Akt phosphorylation.

3.3.1.2 Study of GSK3α/β phosphorylation in MCF10A cells

The phosphorylation levels of GSK3α/β in untreated MCF10A cells were very low (Fig. 3.6; lane 'Un'). Short term incubation of cells with bovine insulin or Actrapid, resulted in strong increase of GSK3α/β phosphorylation levels (lanes 'B' and 'A').

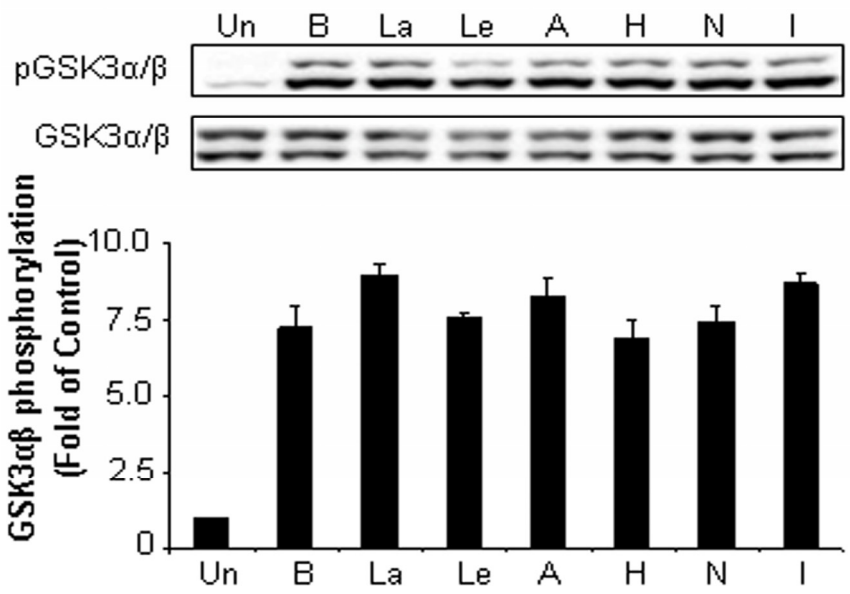


Figure 3.6 All insulin analogues are similar to regular insulins in inducing GSK3α/β phosphorylation in MCF10A cells. $5x10^5$ MCF10A cells were plated and starved for 24 h in medium without insulin. Then cells were either processed untreated (Un) or after 10 min incubation with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). IGF-I (I; 15nM) was studied for comparison. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for phosphorylated GSK3α/β and total GSK3α/β. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. Statistical analysis was done by using student's t-test with at least three independent experiments.

Surprisingly, IGF-I was equi-potent to regular insulins in inducing GSK3α/β phosphorylation (lane 'I') which is in contrast to the observed stronger effect of IGF-I in inducing Akt phosphorylation (refer to section 3.3.1.1). Treatment of MCF10A cells with Humalog,

Novorapid or Levemir caused similar increase in GSK3α/β phosphorylation levels as was obtained after treatment with regular insulins (compare lane 'H', 'N' and 'Le' with the lane 'A' or 'B'). Unexpectedly, Lantus treatment did not induce significantly higher phosphorylation levels of GSK3α/β in comparison to Actrapid treatment. To sum up, all insulin analogues were equi-potent to regular insulin in inducing GSK3α/β phosphorylation in MCF10A cells. Combining these results with the Akt phosphorylation results, it can be suggested that Humalog, Novorapid and Levemir are equi-potent to regular insulins in activating PI3K pathway. Lantus may be stronger than regular insulins in this regard.

3.3.1.3 Study of Akt phosphorylation in MCF7 cells

The endogenous Akt phosphorylation level in MCF7 cells was very low (Fig. 3.7 ; lane 'Un'). Treatment with bovine insulin or Actrapid led to increase in Akt phosphorylation levels (lanes 'B' and 'A'). Actrapid had slightly stronger effect than bovine insulin but it was not found to be significantly higher.

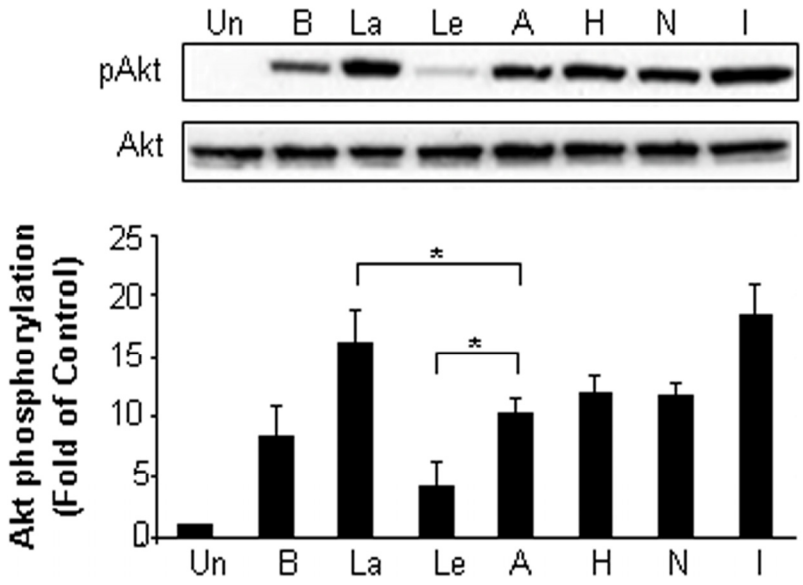


Figure 3.7 Of all insulin analogues studied, Lantus induces significantly stronger and Levemir induces significantly weaker phosphorylation of Akt in comparison to regular insulins in MCF7 cells. $5x10^5$ MCF7 cells were plated and starved for 24 h in serum free medium. Then cells were either processed untreated (Un) or after 10 min incubation with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). IGF-I (I; 15nM) was studied for comparison. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for phosphorylated Akt and total Akt. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. Statistical analysis was done by using student's t-test with at least three independent experiments and asterisk shows the significant differences ($p<0.05$) between Lantus and Actrapid or Levemir and Actrapid.

As was observed with MCF10A cells, IGF-I treatment to MCF7 cells induced strong phosphorylation of Akt (lane 'I'). IGF-I was approximately 2.5 times stronger than regular insulins. Both Humalog and Novorapid induced similar Akt phosphorylation levels as was achieved with Actrapid treatment (refer to the lanes marked as 'H' and 'N' and compare with lane 'A'). However, treatment of MCF7 cells with Levemir (lane 'Le') and Lantus (lane 'La') yielded contrasting results on Akt phosphorylation. Levemir was significantly weaker than Actrapid in inducing Akt phosphorylation (approximately 2-fold). On the contrary, Lantus treated MCF7 cells exhibited significantly higher Akt phosphorylation levels (approximately 2.2-fold) relative to Actrapid treated MCF7 cells (compare lanes 'La' and 'A').

In summary, Humalog and Novorapid were equi-potent to regular insulin in inducing Akt phosphorylation in MCF7 cells. Levemir induced significantly weaker effect while Lantus induced significantly stronger effect than regular insulin in inducing Akt phosphorylation.

3.3.1.4 Study of GSK3α/β phosphorylation in MCF7 cells

Basal level of GSK3α/β phosphorylation in MCF7 cells was low (Fig. 3.8; refer to the lane marked as 'Un'). Treatment with bovine insulin or Actrapid induced approximately 2.2-fold higher phosphorylation of GSK3α/β when compared with endogenous GSK3α/β phosphorylation levels (refer to the lanes marked as 'B' and 'A' for treatment with bovine insulin or Actrapid respectively and compare with lane marked as 'Un'). IGF-I (lane 'I') treatment induced strongest increase in GSK3α/β phosphorylation levels (approximately 3.5-fold of endogenous level). MCF7 cells treated with Humalog (lane marked as 'H') or Novorapid (lane marked as 'N') also showed approximately 2.2-fold increase in GSK3α/β phosphorylation levels as was observed with Actrapid treatment (compare lanes 'H' and 'N' with lane 'A'). Treatment of MCF7 cells with Levemir (lane marked as 'Le') and Lantus (lane marked as 'La') showed opposite effect. Levemir, in comparison to Actrapid, was significantly weaker in inducing GSK3α/β phosphorylation (approximately 0.5-fold weaker). The treatment with Lantus, however, resulted in 1.5-fold increase in GSK3α/β phosphorylation levels in comparison to Actrapid (refer to the lane marked as 'La' and compare with lane 'A'). Statistical analysis showed the difference to be significant.

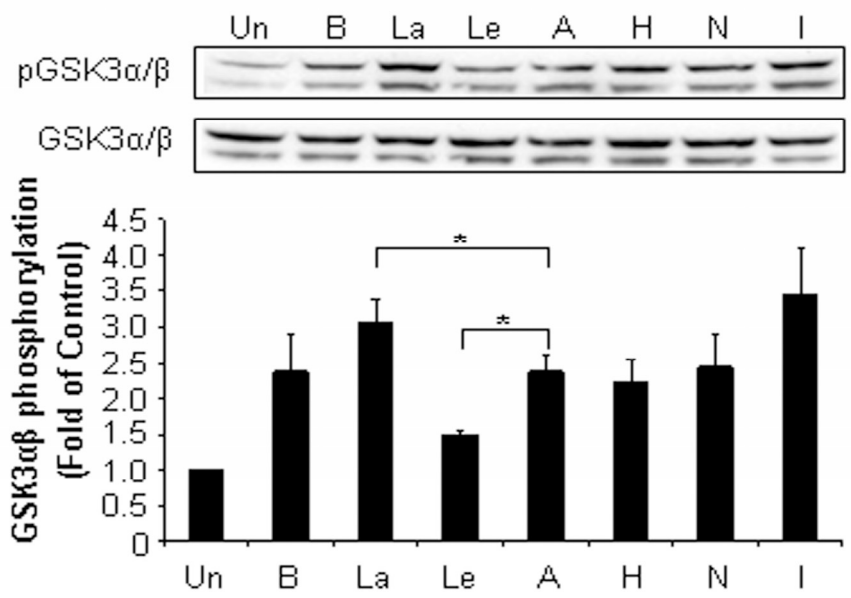


Figure 3.8 Of all insulin analogues studied, Lantus induces significantly stronger and Levemir induces significantly weaker phosphorylation of GSK3α/β in comparison to regular insulins in MCF7 cells. 5 x 10^5 MCF7 cells were plated and after 24 h were starved for another 24 h in serum free medium. Then cells were either processed untreated (Un) or after 10 min incubation with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). IGF-I (I; 15nM) was studied for comparison. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for phosphorylated GSK3α/β and total GSK3α/β. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. Statistical analysis was done by using student's t-test with at least three independent experiments and asterisk shows the significant difference ($p<0.05$) between Lantus and Actrapid or Levemir and Actrapid.

In summary, the two short acting insulin analogues, Humalog and Novorapid, were equi-potent to normal insulins in inducing GSK3α/β phosphorylation. However, the two long acting insulin analogues were opposite in their effect. Levemir was weak and Lantus was strongest of all insulin analogues in stimulating GSK3α/β phosphorylation. From the results on GSK3α/β phosphorylation and Akt phosphorylation, it may be concluded that Humalog and Novorapid are similar to regular insulins in activating PI3K pathway. Levemir is weaker while Lantus is significantly stronger than regular insulin in activating PI3K pathway.

3.3.2 Activation of MAPK pathway by insulin and insulin analogues in MCF10A and MCF7 cells

Erk1/2 represent the down most effector molecules of the **M**itogen **A**ctivated **P**rotein **K**inase (MAPK) pathway. The phosphorylation levels of these signalling molecules were compared in cells treated with insulin analogues and regular insulin in order to gain knowledge of the signalling potency of insulin analogues at the MAPK pathway level.

3.3.2.1 Study of Erk1/2 phosphorylation in MCF10A cells

The untreated MCF10A cells showed low levels of Erk1/2 phosphorylation (Fig. 3.9; lane 'Un'). Short term incubation of MCF10A cells with regular insulin (bovine insulin - 'B' and Actrapid - 'A') resulted in 10 to 12-fold higher Erk1/2 phosphorylation in comparison to untreated cells. IGF-I being a strong mitogen induced highest increase in Erk1/2 phosphorylation levels (about 35-fold of untreated sample; lane 'I'). Treatment of MCF10A cells with Levemir (lane 'Le') and Humalog (lane 'H') yielded similar Erk1/2 phosphorylation levels as observed after treatment with the regular insulins (compare 'Le' and 'H' with lanes 'A' or 'B').

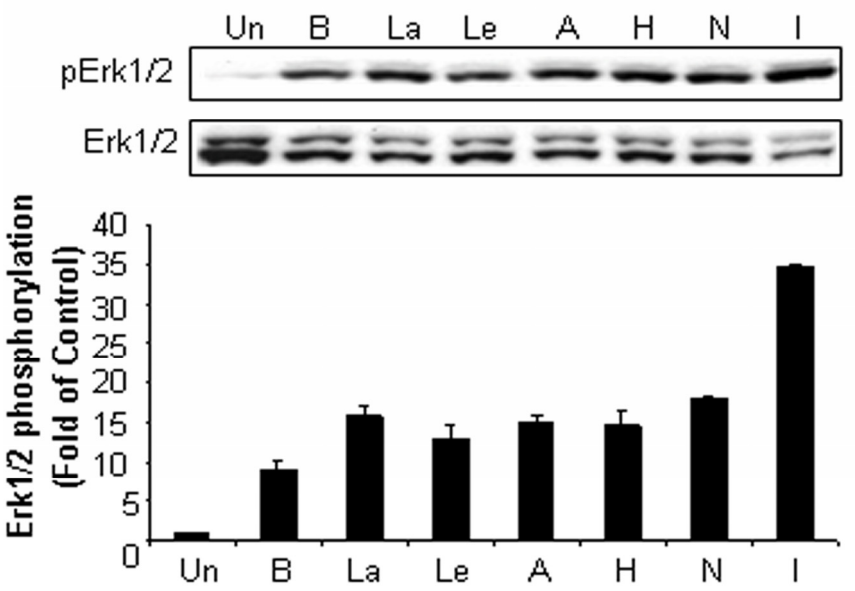


Figure 3.9 All insulin analogues tested were equi-potent to regular insulins in inducing Erk1/2 phosphorylation in MCF10A cells. 5 x 10^5 MCF10A cells were plated and after 24 h were starved for another 24 h in insulin deprived medium. Then cells were either processed untreated (Un) or after 10 min incubation with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). IGF-I (I; 15nM) was studied as a positive control. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for phosphorylated Erk1/2 and total Erk1/2. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. Statistical analysis was done by using student's t-test with at least three independent experiments.

Interestingly, short term treatment with Lantus (lane 'La') or Novorapid (lane 'N') led to slight increase in the Erk1/2 phosphorylation status in comparison to regular insulins but the differences observed were not significant (compare lanes 'La' and 'N' with 'A' or 'B'). Thus, it can be concluded that in MCF10A cells all insulin analogues were equi-potent to regular insulin in inducing Erk1/2 phosphorylation.

3.3.2.2 Study of Erk1/2 phosphorylation in MCF7 cells

The endogenous Erk1/2 phosphorylation levels in MCF7 cells were also very low (Fig. 3.10; lane 'Un'). Treatment with regular insulins and IGF-I led to increase in Erk1/2 phosphorylation levels (bovine insulin - 'B', Actrapid - 'A' or IGF-I - 'I'). Bovine insulin and Actrapid induced approximately three fold increase over the endogenous levels. IGF-I being a strong mitogen, showed the strongest effect (approximately 12-fold increase in comparison to endogenous levels).

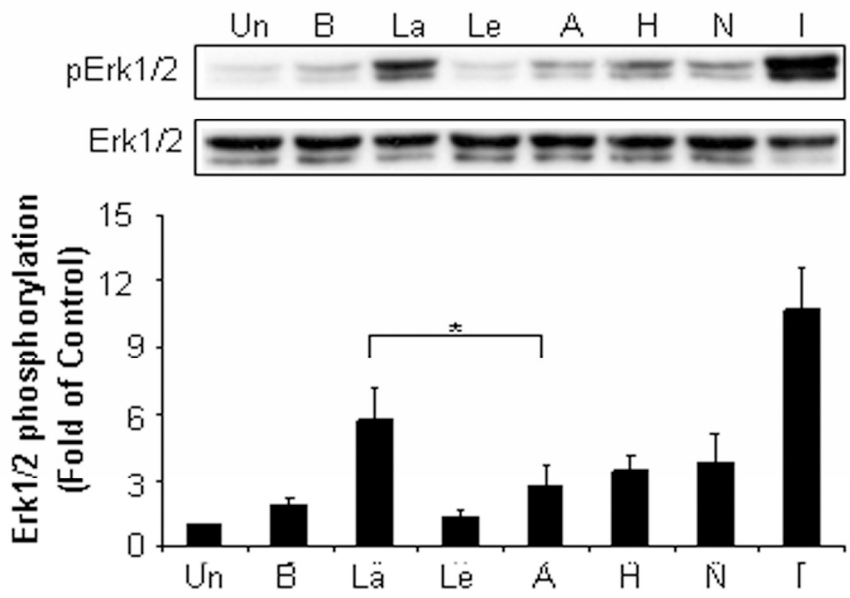


Figure 3.10 Lantus induced significantly stronger Erk1/2 phosphorylation in comparison to Actrapid in MCF7 cells. 5 x 10^5 MCF7 cells were plated and after 24 h were starved for another 24 h in serum free medium. Then cells were either processed untreated (Un) or after 10 min incubation with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). IGF-I (I; 15nM) was studied as a positive control. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for phosphorylated Erk1/2 and total Erk1/2. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. Statistical analysis was done by using student's t-test with at least three independent experiments and asterisk shows the significant difference ($p<0.05$) between Lantus and Actrapid.

Treatment of MCF7 cells with either Humalog (lane 'H') or Novorapid (lane 'N') induced slightly but not significantly stronger phosphorylation of Erk1/2 compared to Actrapid or bovine insulin (compare lanes 'H' and 'N' with 'A' or 'B'). Again, as observed with the phosphorylation of Akt and GSK3α/β, treatment of MCF7 cells with Levemir or Lantus yielded opposite results on induction of Erk1/2 phosphorylation. In comparison to Actrapid, Levemir was approximately 1.5-fold weaker in inducing Erk1/2 phosphorylation (compare 'Le' with 'A'). On the contrary, Lantus induced very strong phosphorylation of Erk1/2 and

showed approximately 2-fold stronger effect than Actrapid. The statistical analysis showed that phosphorylation is significantly stronger than with regular insulins (compare lane 'La' with lane 'A' and 'B').

To summarise, in MCF7 cells, Humalog and Novorapid were equi-potent to the regular insulins in inducing Erk1/2 phosphorylation. Levemir was weaker than regular insulins and Lantus strongly induced Erk1/2 phosphorylation thus suggesting strong activation of MAPK pathway by Lantus.

3.3.2.3 Study of p38 phosphorylation in MCF7 cells

The p38 MAPK when activated by phosphorylation leads to inhibition of proliferation (Zhang and Liu, 2002). Phosphorylated p38 has been reported to negatively regulate expression of cyclin D1 gene which is important for cell cycle progression. Thus, its effect is opposite to that of Erk1/2 MAPK. To gain further insights into the activation of MAPK pathway by insulin analogues in MCF7 cells, we studied phosphorylation status of p38 protein.

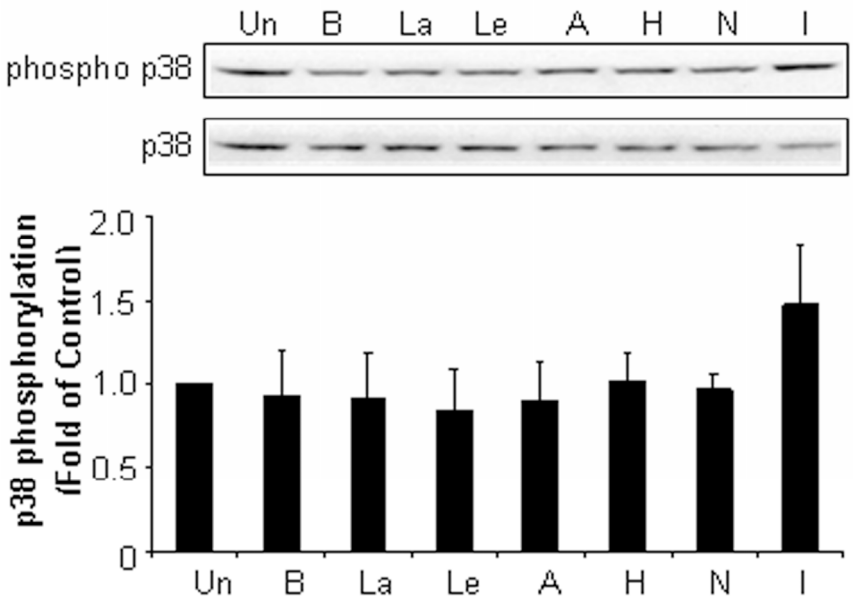


Figure 3.11 Insulin analogues did not differ from regular insulins regarding their effect on p38 phosphorylation in MCF7 cells. $5x10^5$ MCF7 cells were plated and after 24 h were starved for another 24 h in serum free medium. Then cells were either processed untreated (Un) or after 10 min incubation with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). IGF-I (I; 15nM) was studied as a positive control. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for phosphorylated p38 and total p38. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. The figure represents mean ± SD of datapoints from at least three independent experiments.

Interestingly, the basal phosphorylation level of p38 was very high suggesting growth inhibiting experimental conditions (Fig. 3.11; lane 'Un'). The treatment with normal insulin did not lead to any significant alteration in the phosphorylation levels of p38 (refer to the lanes marked as 'B' and 'A' for treatment with bovine insulin or Actrapid respectively). However, IGF-I treatment resulted in clear although not significant increase in p38 phosphorylation (refer to the lane marked as 'I' and compare with 'Un'). This is at variance with the anti-proliferative effect of phospho-p38 and suggests other roles of phospho-p38 in cell functions. Treatment of MCF7 cells with insulin analogues did not show any significant change in the p38 phosphorylation levels in comparison to regular insulin (refer to the lanes marked as 'H', 'N', 'Le' and 'La' for Humalog, Novorapid, Levemir and Lantus respectively and compare with lane 'A' and 'B'). Thus, unlike Erk1/2 MAPK pathway, p38 MAPK pathway remains unaffected by insulin analogues in comparison to regular insulins.

3.3.2.4 Role of MAPK in Lantus induced MCF7 cell proliferation

The Erk1/2 MAPK pathway is a well established mitogenic pathway in MCF7 cells (Lu and Campisi, 1992; Jhun *et al*., 1994). However, some studies show that the PI3K signalling pathway can also transduce proliferation signals in MCF7 cells (Dufourny *et al*., 1997).

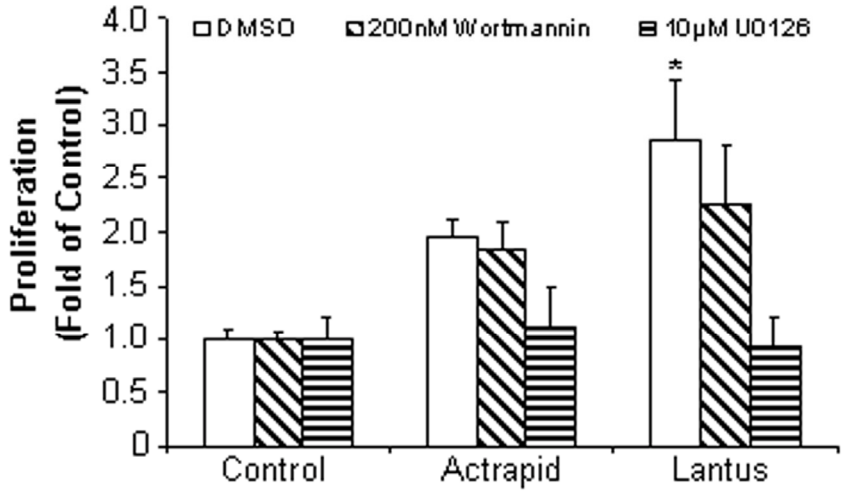


Figure 3.12 MAPK pathway is the major proliferation pathway in Lantus-induced MCF7 cell proliferation. 1 x 10^4 MCF7 cells were plated per well of a 96-well plate. After 24 h, cells were starved for another 24 h in growth medium containing 2% DCC stripped serum. The specific inhibitors for PI3K pathway (Wortmannin) and MAPK pathway (U0126) were added in combination with 150nM Actrapid or 150nM Lantus for another 72 h. The medium was changed every 24 h. After 72 h of incubation cells were PBS washed and fixed by 3% PFA and stained with crystal violet dye. The dye was dissolved in 10% acetic acid and absorbance was measured at 595nm in the plate reader. Values presented are the mean ± SD of n = 8. The statistical analysis was done by using student's t-test. Asterisk (*) shows statistically significant ($p < 0.05$, *t*-test) difference between Lantus and Actrapid data points.

Our proliferation assays demonstrated that Lantus induced MCF7 cell proliferation significantly stronger than regular insulins. As Lantus stimulated both PI3K and MAPK pathways strongly (Section 3.3.1 and Section 3.3.2) it remained to be determined which pathway was related to the strong proliferation of MCF7 cells in our experiments. To achieve this, MCF7 cells were treated with specific inhibitors of PI3K or MAPK pathways and stimulated with Actrapid or Lantus. The proliferation levels were assessed by colorimetric proliferation assays. Wortmannin, which inhibits the catalytic subunit of PI3K (p110), was used for inhibiting PI3K pathway. For inhibition of MAPK pathway, U0126 was used which binds to MAPKK, an upstream effector of Erk1/2. The colorimetric proliferation assay was performed on MCF7 cells treated with Actrapid or Lantus in combination with either Wortmannin or U0126. As both inhibitors were dissolved in DMSO, proliferation levels were analysed in cells treated only with DMSO or treated with DMSO in combination with Actrapid or Lantus for proper comparison.

Lantus induced stronger proliferation than Actrapid in presence of DMSO indicating negligible effect of DMSO (Fig. 3.12). Wortmannin treatment slightly diminished Actrapid and Lantus-induced proliferation. Nevertheless, Lantus induced proliferation was still stronger in comparison to Actrapid. Interestingly, U0126 treated MCF7 cells did not proliferate in response to Actrapid and Lantus treatment. Thus, the above inhibitor experiment clearly demonstrates that in MCF7 cells the MAPK pathway is the major proliferation pathway activated by Actrapid and Lantus.

3.4 Understanding the molecular mechanism behind increased proliferative effect of Lantus in MCF cells

Sections 3.3.1.3, 3.3.1.4 and 3.3.2.2 clearly demonstrated that Lantus has significantly higher signalling potency in comparison to all other insulin analogues tested and, most importantly, to regular insulin in MCF7 cells. This higher signalling potency is reflected in stronger proliferative potency of Lantus in comparison to regular insulin and other insulin analogues tested in MCF7 cells. The strong proliferative potency of Lantus may be due to strong activation of insulin receptor or may involve IGF-IR. ERα activation also induces proliferation of MCF7 cells and as described in section 2.5.3.2, ERα can cross-talk with IGF-IR. As ERα is abundantly expressed by MCF7 cells, there is a possibility that it may contribute to the proliferative potency of Lantus in MCF7 cells. Therefore, we investigated the role of insulin receptor, IGF-IR and ERα in the proliferative ability of Lantus.

3.4.1 Investigating the role of insulin receptor in the strong proliferative potency of Lantus

MELN cells are MCF7 cells stably transfected with ERE dependent firefly luciferase reporter gene. We utilised MELN cells for siRNA studies as MELN cells are easier to transfect than MCF7 cells. In order to study the involvement of insulin receptor in the strong signalling potency of Lantus, MELN cells were transfected with non-targeting siRNA (CT2 siRNA) or insulin receptor targeting siRNA. Thereafter, cells were stimulated for short term (10 min) with 15nM Actrapid, Lantus or IGF-I and phosphorylation status of Akt and Erk1/2 (important molecules of PI3K and MAPK signalling pathways, respectively) was studied.

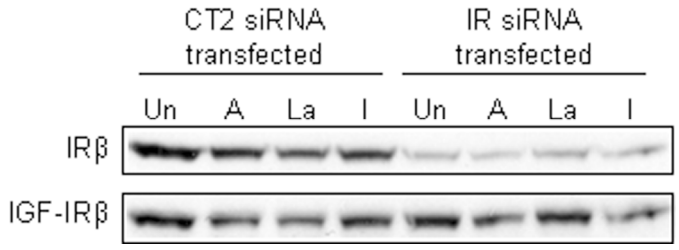


Figure 3.13a Insulin receptor knockdown in MELN cells. 3 x 10^5 MELN cells were plated per well of a 6-well plate. After 12 h, cells were transfected with either 25nM of non-taregting siRNA (CT2 siRNA) or 25nM of insulin receptor targeting siRNA. After 48 h of transfection, cells were starved for next 24 h in serum-free medium. Cells were then left untreated ('Un') or were treated for 10 min with 15nM Actrapid ('A'), 15nM Lantus ('La') or 15nM IGF-I ('I'). At the end of the experiment, medium was aspirated, cells were washed with PBS and cell lysates were prepared as mentioned in the Methods section. Equal amount of cell lysate was loaded and samples were resolved by 10% SDS-PAGE. After membrane transfer, blots were probed first for insulin receptor (IR) and then stripped and probed for IGF-IR.

MELN cells treated with siRNA targeting insulin receptor showed 70% decrease in the insulin receptor protein level (Fig. 3.13a; compare insulin receptor protein levels in samples treated with non-targeting siRNA or with insulin receptor targeting siRNA). The siRNA against insulin receptor did not target IGF-IR (refer to the immunoblot probed with antibody against the beta-subunit of IGF-IR) thus demonstrating the specificity of the siRNA.

3.4.1.1 Study of Akt phosphorylation under insulin receptor knockdown conditions

Under non-targeting conditions, MELN cells treated with 15nM Actrapid demonstrated increased Akt phosphorylation levels in comparison to untreated cells (Fig. 3.13b; compare lanes 'Un' and 'A' under CT2 siRNA label). Lantus treatment induced even higher Akt phosphorylation levels (compare lanes 'La' and 'A'). The highest Akt phosphorylation levels were obtained after treatment with IGF-I (compare lane 'I' with lanes 'A' and 'La'). This is in

agreement with the Akt phosphorylation data obtained under untransfected conditions in MCF7 cells (Sec 3.3.1.3).

Figure 3.13b Actrapid, Lantus or IGF-I induced Akt phosphorylation level in MELN cells transfected with insulin receptor siRNA. 3 x10^5 MELN cells were plated per well of a 6-well plate. After 12 h, cells were transfected with either 25nM of non-targeting siRNA (CT2 siRNA) or 25nM of insulin receptor targeting siRNA. After 48 h of transfection, cells were starved for next 24 h in serum-free medium. Cells were then left untreated ('Un') or were treated for 10 min with 15nM Actrapid ('A'), 15nM Lantus ('La') or 15nM IGF-I ('I'). At the end of the experiment, medium was aspirated, cells were washed with PBS and cell lysates were prepared as mentioned in Method section. Equal amount of cell lysate was loaded and samples were resolved by 10% SDS-PAGE. After membrane transfer, blots were probed first for phospho-Akt and then stripped and probed for total Akt protein.

Insulin receptor knockdown diminished Actrapid potency in inducing Akt phosphorylation by approximately 70%, thus confirming the well established fact that normal insulin at 15nM concentration acts mostly via insulin receptor (compare lanes marked as 'A' under the two transfection conditions). In comparison to Actrapid treatment, knockdown of insulin receptor did not significantly compromise Lantus ability to induce Akt phosphorylation suggesting the involvement of other receptors for Lantus induced signalling. Furthermore, IGF-I ability to induce Akt phosphorylation was also not compromised under insulin receptor knockdown conditions. In fact, it seemed to be increased under insulin receptor knockdown conditions. This is an interesting observation and indicates a 'quenching' effect of insulin receptor on IGF-IR which is abolished after silencing of the insulin receptor.

3.4.1.2 Study of Erk1/2 phosphorylation under insulin receptor knockdown conditions

Treatment of CT2siRNA transfected MELN cells with Actrapid induced phosphorylation of Erk1/2 (Fig. 3.13c; compare lanes marked as 'Un' and 'A'). This effect corresponds to the effect of Actrapid on untransfected MCF7 cells (Section 3.3.2.2). In addition, similar to the results obtained on untransfected MCF7 cells, Lantus and IGF-I treatment induced stronger phosphorylation of Erk1/2 than Actrapid in CT2siRNA transfected cells.

Figure 3.13c Actrapid, Lantus or IGF-I induced Erk1/2 phosphorylation level in insulin receptor siRNA transfected MELN cells. 3 x 10^5 MELN cells were plated per well of a 6-well plate. After 12 h, cells were transfected with either 25nM of non-taregting siRNA (CT2 siRNA) or 25nM of insulin receptor targeting siRNA. After 48 h of transfection, cells were starved for next 24 h in serum-free medium. Cells were then left untreated ('Un') or were treated for 10 min with 15nM Actrapid ('A'), 15nM Lantus ('La') or 15nM IGF-I ('I'). At the end of the experiment, medium was aspirated, cells were washed with PBS and cell lysates were prepared as described in the Methods section. Equal amount of cell lysate was loaded and samples were resolved by 10% SDS-PAGE. After membrane transfer, blots were probed first for phosphorylated forms of Erk1/2 and after stripping for total Erk1/2.

Under insulin receptor knockdown conditions, the ability of Actrapid to induce Erk1/2 phosphorylation was decreased by approximately 80%. However, under these conditions, Lantus ability to induce Erk1/2 phosphorylation was not decreased to the same extent (compare lanes marked as 'La' under both transfection conditions). Furthermore, as reported for Akt phosphorylation, insulin receptor knockdown resulted in increased IGF-I ability to induce Erk1/2 phosphorylation (compare lanes marked as 'I' under two transfection conditions). This again suggests a quenching effect of insulin receptor on IGF-IR.

3.4.2 Investigating the role IGF-IR in the strong proliferative potency of Lantus

In order to determine the involvement of IGF-IR in the stronger signalling and proliferative potency of Lantus in comparison to Actrapid, a similar approach was used as described in the above section. MELN cells were either treated with non-targeting siRNA (CT2 siRNA) or with siRNA targeting the IGF-IR and then signalling and proliferative potencies of Lantus were compared to Actrapid. IGF-I treatment was used as positive control for IGF-IR activation.

MELN cells transfected with IGF-IR targeting siRNA showed approximately 90% decrease in IGF-IR protein level when compared with the cells transfected with non-targeting siRNA (Fig. 3.14a; compare IGF-IR protein levels in samples treated with non-targeting siRNA or with IGF-IR targeting siRNA). The downregulation of the IGF-IR did not lead to decrease in the insulin receptor protein content, demonstrating the specificity of siRNA (refer to the immunoblot probed with antibody against beta subunit of insulin receptor).

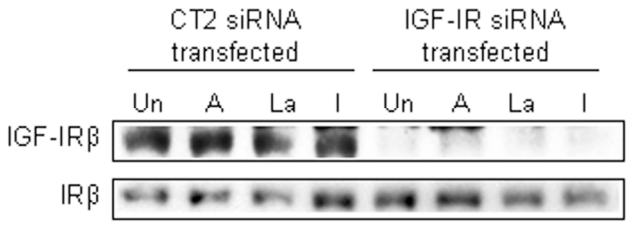


Figure 3.14a IGF-IR knockdown in MELN cells. 3 x 10^5 MELN cells were plated per well of a 6-well plate. After 12 h, cells were transfected with either 25nM of non-taregting siRNA (CT2 siRNA) or 25nM of insulin receptor targeting siRNA. After 48 h of transfection, cells were starved for next 24 h in serum-free medium. Cells were then left untreated ('Un') or were treated for 10 min with 15nM Actrapid ('A'), 15nM Lantus ('La') or 15nM IGF-I ('I'). At the end of the experiment, medium was aspirated, cells were washed with PBS and cell lysates were prepared as mentioned in the Methods section. Equal amount of cell lysate was loaded and samples were resolved by 10% SDS-PAGE. After membrane transfer, blots were probed first for IGF-IR and then stripped and probed for insulin receptor.

3.4.2.1 Study of Akt phosphorylation under IGF-IR knockdown conditions

Under non-targeting conditions, treatment of MELN cells with IGF-I induced strong phosphorylation of Akt (Fig. 3.14b, compare lanes marked as 'Un' and 'I' under non-targeting conditions). Furthermore, treatment with Actrapid and Lantus induced Akt phosphorylation with Lantus showing a significantly stronger effect than Actrapid (compare lanes marked as 'A' and 'La' under non-targeting conditions). This is in agreement with the Actrapid and Lantus induced Akt phosphorylation levels obtained in untransfected cells (refer to Fig. 3.7).

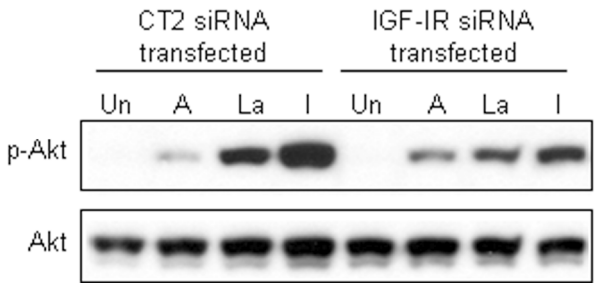


Figure 3.14b Actrapid, Lantus or IGF-I induced Akt phosphorylation level in MELN cells transfected with IGF-IR siRNA. 3 x 10^5 MELN cells were plated per well of a 6-well plate. After 12 h, cells were transfected with either 25nM of non-targeting siRNA (CT2 siRNA) or 25nM of insulin receptor targeting siRNA. After 48 h of transfection, cells were starved for next 24 h in serum-free medium. Cells were then left untreated ('Un') or were treated for 10 min with 15nM Actrapid ('A'), 15nM Lantus ('La') or 15nM IGF-I ('I'). At the end of the experiment, medium was aspirated, cells were washed with PBS and cell lysate were prepared as mentioned in the Methods section. Equal amount of cell lysate was loaded and samples were resolved by 10% SDS-PAGE. After membrane transfer, blots were probed first for phospho-Akt and then stripped and probed for total Akt protein.

Knockdown of IGF-IR decreased IGF-I ability to induce Akt phosphorylation by approximately 60% (compare lanes marked as 'I' under the two transfection conditions). Interestingly, IGF-IR knockdown also decreased Lantus ability to induce Akt phosphorylation (compare lanes marked as 'La' under the two transfection conditions). On the contrary, Actrapid was observed to be slightly more potent in inducing Akt phosphorylation after IGF-IR knockdown (compare lanes marked as 'A' under the two transfection conditions). This suggests that IGF-IR also exerts a 'quenching' effect on insulin receptor activity, which is abolished after knockdown of the IGF-IR. Thus, from the results on the receptor signalling after insulin receptor knockdown or IGF-IR knockdown it may be concluded that both receptors regulate each others activity.

3.4.2.2 Study of Erk1/2 phosphorylation under IGF-IR knockdown conditions

In MELN cells transfected with non-targeting siRNA, the phosphorylation status of Erk1/2 in response to the treatment with Actrapid, Lantus or IGF-I was comparable to the results obtained under untransfected conditions (compare Fig. 3.14c with Fig. 3.10). IGF-I induced strongest phosphorylation of Erk1/2 compared to Actrapid and Lantus. Lantus induced Erk1/2 phosphorylation level was significantly higher than the level induced by Actrapid.

Figure 3.14c Actrapid, Lantus or IGF-I induced Erk1/2 phosphorylation level in MELN cells transfected with IGF-IR siRNA. 3 x 10^5 MELN cells were plated per well of a 6-well plate. After 12 h, cells were transfected with either 25nM of non-taregting siRNA (CT2 siRNA) or 25nM of insulin receptor targeting siRNA. After 48 h of transfection, cells were starved for next 24 h in serum-free medium. Cells were then left untreated ('Un') or were treated for 10 min with 15nM Actrapid ('A'), 15nM Lantus ('La') or 15nM IGF-I ('I'). At the end of the experiment, medium was aspirated, cells were washed with PBS and cell lysates were prepared as described in the Methods section. Equal amount of cell lysate was loaded and samples were resolved by 10% SDS-PAGE. After membrane transfer, blots were probed first for phosphorylated forms of Erk1/2 and after stripping for total Erk1/2.

In MELN cells transfected with IGF-IR-targeting siRNA, IGF-I ability to induce Erk1/2 phosphorylation was compromised and was reduced to approximately 60% (compare lane 'I' under the two transfection conditions). The knockdown of IGF-IR severely decreased Lantus

ability to induce Erk1/2 phosphorylation thus suggesting that Lantus-induced strong activation of MAPK pathway involves IGF-IR activation (compare lanes marked as 'La' under the two transfection conditions). In the absence of IGF-IR, Actrapid was more potent in inducing Erk1/2 phosphorylation compared to non-targeting conditions (compare lanes marked as 'A' under two types of transfections). This observation demonstrates again that IGF-IR has a 'quenching' effect on insulin receptor.

The results demonstrate that IGF-IR plays an important role in Lantus induced strong phosphorylation of Akt and Erk1/2. In addition, IGF-IR exerts a quenching effect on insulin receptor as was evident from increased signalling potency of Actrapid under IGF-IR knockdown conditions.

3.4.2.3 Study of the proliferative potency of Lantus under IGF-IR knockdown conditions

Finally, in order to ascertain the involvement of IGF-IR in the strong proliferative ability of Lantus, the proliferation assay was performed under knockdown conditions. For this, MELN cells were transfected with non-targeting siRNA or IGF-IR targeting siRNA and cells were treated with Actrapid, Lantus or IGF-I.

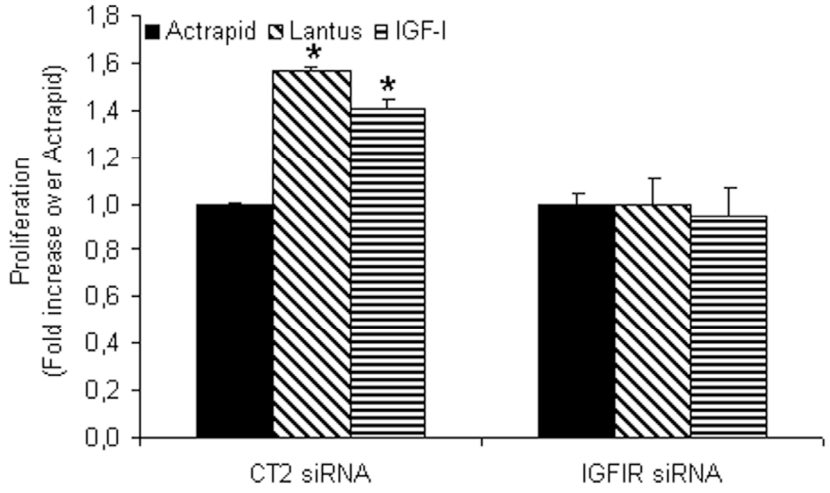


Figure 3.15 Lantus mediated strong proliferation in MELN cells is due to the activation of IGF-IR. 1 x 10^4 MELN cells were plated per well of a 96-well plate. After 12 h, cells were transfected using oligofectamine (2µl/well) with 25nM CT2 siRNA or 25nM IGF-IR targeting siRNA. After 48 h of transfection cells were starved for next 24 h in medium containing 2% DCC-FBS. Thereafter, cells were treated with 150nM Actrapid, 150nM Lantus or 15nM IGF-I for 72 h. At the end of the experiment, cells were fixed and stained with crystal violet dye. After air drying, the stain was dissolved in 10% acetic acid and the absorbance was measured at 595nm in a plate reader. Fold of control was determined with reference to Actrapid. Statistical analysis (student's *t*-test) was performed with at least 8 data points. Asterisks mean statistical significance at $p < 0.05$.

Under non-targeting conditions, treatment of MELN cells with both, Lantus or IGF-I yielded significantly higher proliferation of MELN cells compared to Actrapid (Fig. 3.15). As expected, IGF-IR knockdown decreased IGF-I ability to stimulate proliferation. Under IGF-IR knockdown conditions, proliferation levels achieved with Lantus treatment were similar to that of Actrapid treatment. This is a direct confirmation that the high proliferative potency of Lantus involves IGF-IR activation. Thus, it can be concluded that increased signalling and proliferative potency of Lantus compared to Actrapid involves activation of IGF-IR.

3.4.2.4 Study of Lantus induced IGF-IR phosphorylation

IGF-IR activation is characterized by the phosphorylation of specific tyrosine residues in the kinase domain of IGF-IR. Thus, Lantus induced activation of IGF-IR should result in increased phosphorylation of IGF-IR. To study this, we performed immunoprecipitation of IGF-IR followed by detection of phospho-tyrosine in the immunoprecipitates. Cell lysates from untreated MCF7 cells or MCF7 cells treated with Actrapid, Lantus or IGF-I were subjected to immunoprecipitation using a polyclonal antibody against the beta-subunit of IGF-IR (Fig. 3.16).

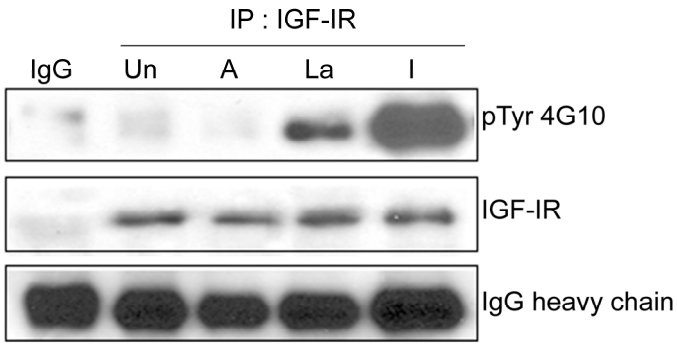


Figure 3.16 Lantus in comparison to Actrapid strongly phosphorylates IGF-IR in MCF7 cells. MCF7 cells were starved in medium containing 2% DCC-FBS for 24 h and then were treated with 15nM Actrapid (A), 15nM Lantus (La) or 15nM IGF-I (I) for 10 min or were left untreated (Un). Cell lysates obtained were subjected to immunoprecipitation with antibody against IGF-IR. Precipitation with non-immune IgG was included to study the specificity of the immunoprecipitation. Immunoprecipitates obtained were resolved by 10% SDS-PAGE and the blot was first probed for the phosphorylated form of IGF-IR then stripped and probed for IGF-IR.

The IGF-IR could not be detected in the lysate subjected to immunoprecipitation using non-immune IgG thus confirming the specificity of the immunoprecipitation procedure (refer to lane marked ‘IgG’ and compare with other lanes of the immunoblot). The basal phosphorylation of IGF-IR was very low and almost undetectable in the immunoblot (refer to lane labeled ‘Un’). IGF-I being a cognate ligand of IGF-IR induced strong phosphorylation of

IGF-IR (refer to lane labeled 'I'). Importantly, treatment of MELN cells with Actrapid did not increase IGF-IR phosphorylation levels in comparison to untreated cells (refer to lane labeled 'A'). On the contrary, high phosphorylation levels of IGF-IR were detected in MELN cells treated with Lantus thus confirming the activation of IGF-IR by Lantus (refer to the lane labeled 'La').

3.4.2.5 Study of induction of a IGF-IR responsive gene by Lantus treatment

The activation of IGF-IR results in increased expression of genes involved in cell proliferation like cyclin D1 (Sachdev and Yee 2001, Furlanetto *et al*.,1994). In order to investigate if IGF-IR activation by Lantus results in increase of cyclin D1 gene expression, we utilised quantitative RT-PCR and studied the cyclin D1 expression level in Lantus treated MCF7 cells after 1 h, 3 h or 6 h of treatment. The cyclin D1 gene expression level observed in Lantus treated MCF7 cells was compared with Actrapid treated MCF7 cells to determine the relative strength of the two compounds. IGF-I treatment was used as a positive control. At 1 h of treatment, no significant difference in cyclin D1 expression level was observed between any of the treatments (Fig. 3.17).

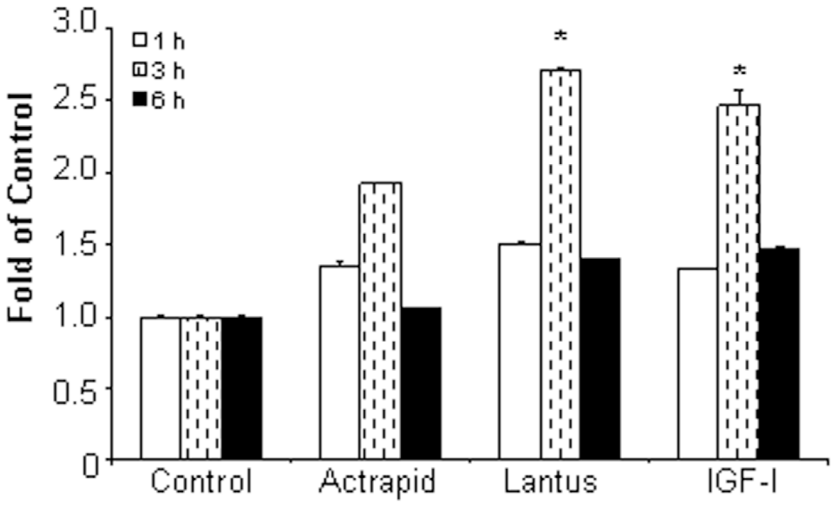


Figure 3.17 Lantus in comparison to Actrapid induced significantly higher expression of Cyclin D1 gene in MCF7 cells. MCF7 cells were serum-starved for 24 h and were either left untreated or were treated for 1 h, 3 h and 6 h with 15nM Actrapid or 15nM Lantus or 15nM IGF-I. Total RNA was extracted and reverse transcribed to obtain cDNA. Real time PCR was performed by using specific primers for cyclin D1. GAPDH was used as internal control. Standard curve for Ct values was obtained by plotting the number of molecules in serially diluted cDNA against their Ct value. This standard curve was then used to determine from each sample the number of molecules at the end of the experiment. After normalizing the cyclin D1 value against GAPDH, fold of control was calculated for each time point. Statistical analysis was done with at least two independent experiments and asterisks show data significantly different from Actrapid ($p<0.05$).

At the 3 h timepoint, Actrapid treatment induced approximately 1.8-fold higher cyclin D1 gene expression in comparison to untreated cells, thus confirming insulin to be a mitogen. Treatment of MCF7 cells with Lantus for 3 h resulted in approximately 2.6-fold increase in cyclin D1 gene expression. The increase was found to be significantly higher than with Actrapid treatment. Similarly, at the 3 h timepoint, IGF-I treatment induced approximately 2.4 fold increase in cyclin D1 gene expression and this increase was also significantly higher in comparison to Actrapid treatment. After 6 h of treatment, cyclin D1 gene expression was decreased in all samples, the Actrapid treated sample demonstrating basal levels of cyclin D1 gene expression and Lantus and IGF-I treated cells showing slightly higher than basal levels. This reduction may be explained by the cyclical expression pattern of cyclin D1 gene (Stacey, 2003).

To sum up, Lantus induced expression levels of cyclin D1, an IGF-I regulated gene. The expression levels are comparable with the expression levels obtained with IGF-I treatment. This experiment thus confirms that Lantus activates IGF-IR.

3.4.3 Investigating the role of ERα in the strong proliferative potency of Lantus

Several studies have suggested a cross-talk between IGF-IR and ERα signalling (Smith, 1998; Kato *et al.*, 2000). IGF-I induces stronger proliferation in MCF7 cells which expressed ERα compared to MCF7 cells which do not express ERα, suggesting an important role of ERα in IGF-IR mediated cell proliferation (Oesterreich *et al*., 2001). Results shown above clearly establish that Lantus induces strong proliferation in MCF7 cells via activation of IGF-IR. As MCF7 cells show a high expression level of ERα (Fig. 3.1), it was hypothesised that Lantus-induced high proliferation involves ERα activation. In order to investigate the potential role of ERα regarding the proliferative potency of Lantus, the following two approaches were used –

1. ERα activation involves phosphorylation of the Ser118 residue in ERα (Lannigan, 2003). This serine residue has been demonstrated to be phosphorylated by GSK3β and MAPK, two protein kinases related to IGF-I signalling pathway (Grisouard *et al.*, 2007; Kato *et al.*, 2000). Thus, to assess activation of ERα by Lantus, the phosphorylation status of Ser118 residue of ERα was studied in Lantus treated MCF7 cells. Regular insulin and IGF-I were studied for comparison. Other insulin analogues were also studied.

2. The potential ERα activation by regular insulin, Lantus and IGF-I was directly assessed by luciferase reporter assay system. Estradiol treated cells were the positive control.

3.4.3.1 Study of ERα phosphorylation and activation in MCF7 cells

MCF7 cells were treated with regular insulin, insulin analogue and IGF-I for 10 min and phosphorylation status of ERα at Ser118 residue was compared.

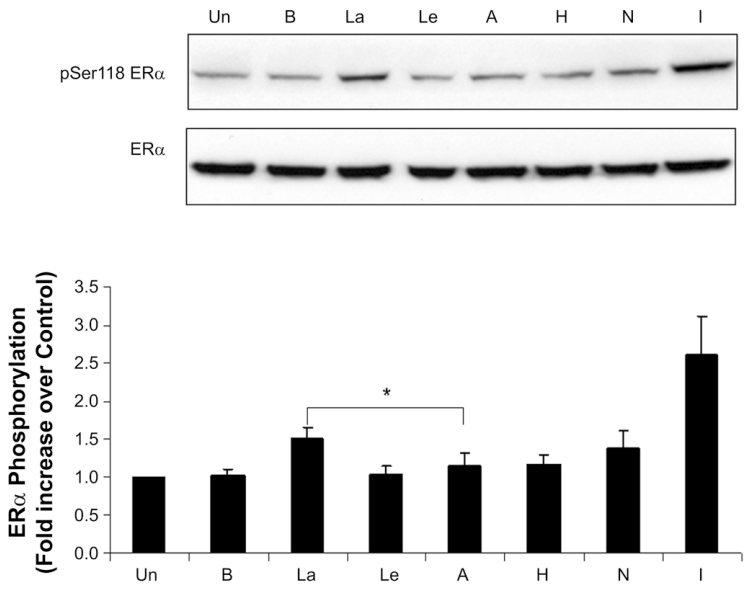


Figure 3.18 Lantus induced significantly higher phosphorylation of ERα in comparison to regular insulin and other insulin analogues in MCF7 cells. 5 x 10^5 MCF7 cells were serum starved for 24 h and then were processed without any treatment (Un) or after 10 min treatment with 15nM insulin (B – Bovine Insulin and A – Actrapid) or insulin analogues (La – Lantus, Le – Levemir, H – Humalog and N – Novorapid). 15nM IGF-I was studied for comparison. The cell lysates were resolved by 10% SDS-PAGE and immunoblots were probed for ERα phosphorylated at Ser118 residue and total ERα protein. Fold of control was obtained by normalizing phosphorylated forms over the protein levels and then comparing with the untreated sample. Statistical analysis was done with at least three independent experiments and asterisk shows the significant difference ($p<0.05$) between Lantus and Actrapid effects.

MCF7 cells exhibited a weak basal phosphorylation level of ERα (Fig. 3.18; lane 'Un'). Treatment with bovine insulin or Actrapid did not result in significant increase in phosphorylated ERα levels (compare lanes marked 'A' or 'B' with lane 'Un'). However, IGF-I treatment resulted in 2.5-fold increase in ERα phosphorylation levels in comparison to endogenous levels (compare lane 'I' with 'Un'). Interestingly, in comparison to regular insulin, Lantus treatment resulted in 1.5-fold increase in ERα phosphorylation levels. Statistical analysis revealed that the effect of Lantus is significantly stronger than that of Actrapid. There was no observable difference in ERα phosphorylation level in cells treated

with regular insulin and other insulin analogues (Levemir, 'Le'; Humalog, 'H' or Novorapid, 'N'). Thus, Lantus, like IGF-I, induces stronger phosphorylation of ERα at Ser118 residue in comparison to regular insulin and other insulin analogues tested, suggesting stronger activation of ERα by Lantus and IGF-I.

3.4.3.2 Study of ERα activation by Lantus using reporter assay system

Activated ERα dimerises and acts as a transcription factor. As a transcription factor it binds to estrogen response elements (ERE) in the promoter region of estrogen responsive genes and induces their transcription (McDonnell and Norris, 2002). In order to directly study the activation of ERα by Lantus, an ERE dependent luciferase reporter assay system was utilized. MELN cells (MCF7 cells stably transfected with ERE-luc reporter system) were treated with Actrapid, Lantus or IGF-I and the luciferase activity was measured.

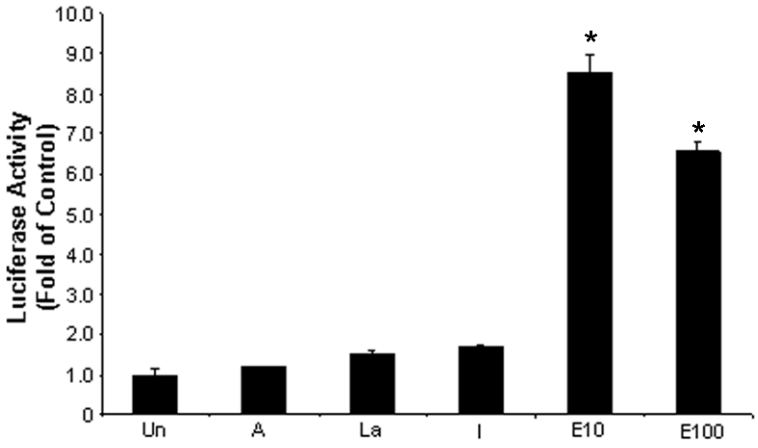


Figure 3.19 Lantus was slightly stronger than Actrapid in inducing luciferase activity in MELN cells. 3 x 10^5 MELN cells were plated and after 24 h cells were processed untreated (Un) or were processed after treatment with 15nM Actrapid (A), 15nM Lantus (La) or 15nM IGF-I for 6 h. MELN cells treated with 10nM or 100nM estradiol (E10, E100) were used as positive control. Specific luciferase activity was determined by normalizing the luminescence with the protein concentration. Fold of control was calculated by normalizing specific activity from treated cells with the specific activity of untreated cells. Values presented are the mean and range of four values. The statistical analysis was done by using student's t-test. Asterisks (*) show statistical significance ($p < 0.05$, *t*-test) in comparison to untreated cells.

Untreated MELN cells showed very low luciferase activity (Fig. 3.19). Treatment with 10nM and 100nM estradiol led to 8.5-fold and 6.8-fold increase over untreated cells, respectively. MELN cells treated with Actrapid, Lantus or IGF-I showed only little increase in luciferase activity compared to the untreated cells. Lantus and IGF-I treated cells demonstrated slightly higher luciferase activity than Actrapid treated cells, reflecting the ERα phosphorylation

described in section (3.4.3.1). Taken together, this suggests that Lantus modulates ERα activity slightly stronger than Actrapid. However, combining the results from ERα phosphorylation and luciferase assays, it can be concluded that ERα is only weakly activated by Lantus and thus ERα is unlikely to play a strong role in rendering high proliferative ability to Lantus.

3.5 Study of Lantus induced cell migration by wound healing assay in MCF7 cells

Cancer is characterised by metastasis which involves cell migration and cell invasion. We investigated the potency of Lantus in comparison to Actrapid in inducing migration in MCF7 cells. For this, cell monolayer was wounded and cells were treated with Actrapid or Lantus for 21 h (Fig. 3.20). At specific timepoints (3 h, 6 h, 12 h and 21 h), wound width was determined and wound healing was calculated as percentage of initial wound width. IGF-I, the cognate ligand of IGF-IR, is known to stimulate cell migration. As Lantus also activates IGF-IR (section 3.4), IGF-I was included in the study as a control.

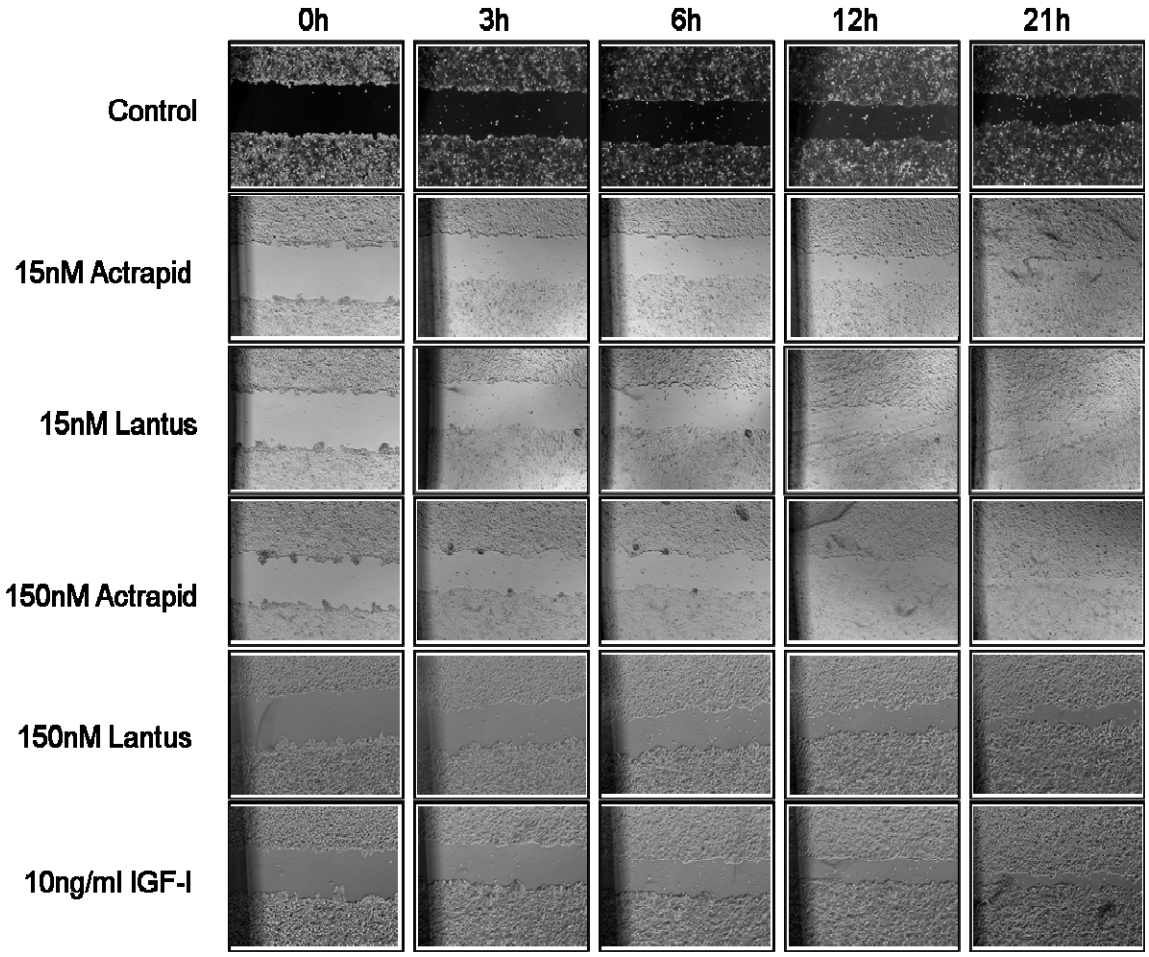


Figure 3.20 Lantus and Actrapid induced comparable MCF7 cell migration. Confluent monolayer of MCF7 cells was wounded using a yellow tip. Cells were treated with 2µg/ml mitomycin C for 1 hr. Thereafter, cells were either left untreated or treated with Actrapid (15nM or 150nM), Lantus (15nM or 150nM) or IGF-I (15nM) for 3 h, 6 h, 12 h or 21 h. At each timepoint wounds were photographed and percentage of wound healing was calculated. The picture presented here is a representative image from two independent wounds.

Untreated MCF7 cells demonstrated approximately 60% of wound healing by 21 h (Fig. 3.21). Treatment with 15nM IGF-I increased MCF7 cell migration as ~ 88% wound closure was observed at the 21 h timepoint in comparison to ~ 60% observed in untreated cells. At this timepoint, 15nM and 150nM Actrapid were also found to induce cell migration as reflected by ~ 75% of initial wound closure. Similarly, Lantus treatm ent (15nM and 150nM) of MCF7 cells resulted in ~ 80% closure of initial wound after 21 h which was 1.3-fold of wound closure observed in untreated cells at this timepoint. Comparison of Lantus and Actrapid-induced MCF7 cell migration reveals that both compounds stimulate migration to a similar extent.

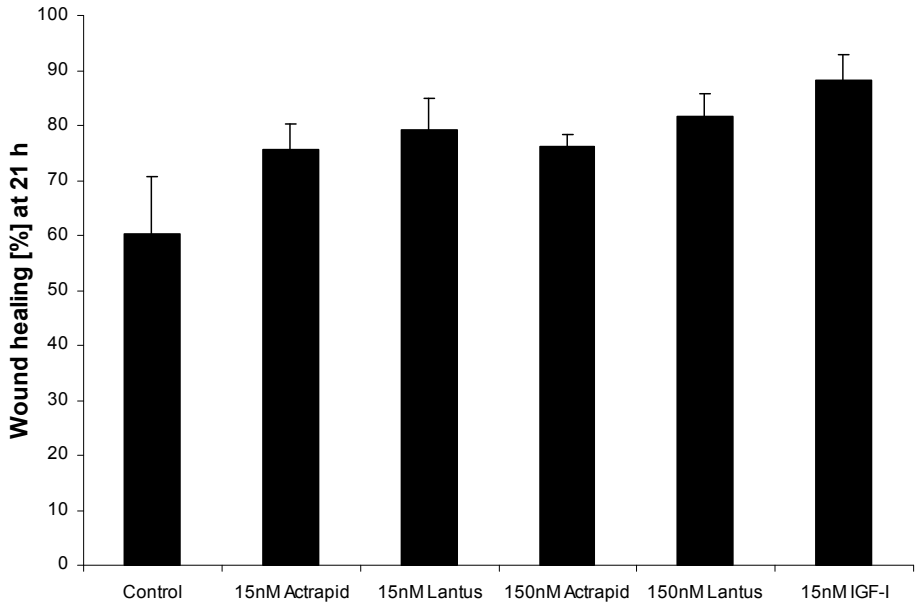


Figure 3.21 Graphical presentation of MCF7 cell migration under different treatments after 21 h. Distance between the two edges of the wound was measured at 0 h (initial wound) and 21 h. The extent of wound healing at 21 h was calculated by subtracting the distance between the two edges at 21 h and 0 h. These values were utilised to calculate 'wound healing [%]' by dividing wound width at 21h by the initial wound width. The data presented here are mean ± SD of two independent wounds.

In summary, the wound healing experiment demonstrates that the migration-inducing potential of Lantus and Actrapid in MCF7 cells are similar.

4 DISCUSSION

Insulin analogues have been a great achievement in maintaining normal glucose serum levels in diabetic patients. However, as insulin analogues are generated by modifying the insulin backbone which alters binding properties to insulin receptor and IGF-IR, serious concerns have been raised regarding the possible increase of mitogenic potential of insulin analogues. These concerns have been augmented by studies demonstrating high potential of B10Asp, an insulin analogue generated by replacing amino acid at 10^{th} position with asparagine, in causing proliferation, focus formation and colony formation in osteosarcoma cells, MCF10A cells and MCF7 cells, respectively. In addition to *in vitro* studies, B10Asp administration to female Sprague-Dawley rats resulted in increased mammary tumour incidence compared to regular insulin. The observation that insulin receptor and IGF-IR are highly expressed in normal mammary epithelial cells and even overexpressed in tumorigenic mammary epithelial cells, suggests high susceptibility of mammary epithelial cells toward the mitogenic effect of insulin analogues. Thus, a comprehensive and detailed study of the mitogenic potency of therapeutically available insulin analogues on mammary epithelial cells is strongly warranted.

In this regard, *in vitro* experiments reported till date, studying the mitogenic effects of therapeutically used insulin analogues on mammary epithelial cells, have been largely inconclusive owing to the experimental assays used and inconsistencies in the results. Furthermore, a standard two-year carcinogenicity test has not been performed for therapeutically used insulin analogues except for Insulin Glargine. In this study, the rats treated with Insulin Glargine and regular insulin showed equal incidence of mammary tumours. However, the study also reported a very high mortality rate in all treated animal groups thus raising questions on the experimental design and conclusions drawn. The aim of the present work was to compare the proliferative and signalling ability of insulin analogues that are currently therapeutically used, with the regular insulin in human mammary epithelial cells. In addition, we also aimed to unravel the molecular and biochemical mechanism behind the proliferative potency of those insulin analogue(s) which demonstrated higher proliferative ability than regular insulin.

Our study showed that in comparison to regular insulin, only Insulin Glargine (Lantus), of all four insulin analogues tested, elicited significantly higher proliferation in MCF7 cells, a cell line with high IGF-IR/IR ratio. In MCF10A cells, an insulin responsive mammary epithelial

cell line exhibiting low IGF-IR/IR ratio, Insulin Glargine had similar proliferative potency as other insulin analogues and regular insulin. At the molecular level, we observed that Insulin Glargine treatment resulted in strong activation of PI3K and MAPK signalling pathways in MCF7 cells. The strong activation of MAPK pathway by Insulin Glargine is reflected in the significantly increased proliferative ability of Insulin Glargine compared to regular insulin. Insulin Glargine exerts its strong mitogenic potency by strongly activating IGF-IR besides insulin receptor. ERα, by virtue of its cross-talk with IGF-IR, is also activated by Insulin Glargine but its overall contribution into the mitogenic potency of Insulin Glargine may be low. Finally, our preliminary experiments on stimulation of the migratory and invasive potential of the cells by Insulin Glargine revealed no significant difference from regular insulin.

We postulate that Insulin Glargine may rather be a tumour promoter than a tumour inducer and exerts a significantly stronger proliferative effect in comparison to regular insulin (Actrapid) in cells that demonstrate high IGF-IR/IR ratio. The findings presented in this work may be of clinical relevance for those diabetic patients who have breast cancer or as yet undetected (pre-) cancerous lesions.

4.1 Insulin responsiveness of mammary epithelial cell lines

It is well established that insulin is a mitogen. In order to find a suitable model to study the mitogenic effect of insulin and insulin analogues on mammary epithelial cells, a panel of tumorigenic and non-tumorigenic cell lines was screened for their responsiveness toward insulin treatment (Table 3.1). Although all cell lines analysed expressed insulin receptor moderately, only MCF7 and MCF10A cells showed a clear proliferative response to the insulin treatment. The other mammary epithelial cell lines, though expressing similar levels of insulin receptor as MCF10A and MCF7, exhibited weak or lack of proliferative response indicating the importance of signalling molecules downstream of insulin receptor for transduction of the mitogenic effect of insulin. For example, the lack of IRS-1 protein expression in ZR75-1 cells (Gliozzo *et al.*, 1998) may be the reason for ZR75-1 cells to demonstrate merely 1.4-fold increase in proliferation after insulin treatment. The poor proliferative response of BT474 cells to insulin treatment may also be explained by the low expression level of IRS-1 protein (Mayer *et al.*, 2008). Bartucci *et al.* (2001) showed that absence of sustained activation of the PI3K pathway results in the lack of mitogenic response to IGF-I treatment in MDA-MB231 cells. This may also be true for the weak mitogenic

response to insulin observed in our study, where MDA-MB231 cells show barely a 1.3-fold increase after insulin treatment. Two other cell lines, T47D and HCC1937, respond poorly to the insulin treatment. Although the reason for this is unclear, it may be hypothesized that signalling pathways leading to proliferative response after insulin treatment are interrupted in these long-term established cell lines.

Thus, our study suggests that insulin responsiveness of a cell line is not only dependent on the insulin receptor expression level but also on the expression and functional integrity of downstream signalling molecules.

4.2 Characterisation of proliferative and signalling potency of insulin analogues

Insulin analogues are commercially available as pharmaceutical preparations but not as the pure compounds from the companies. Since the pure compounds should be preferentially used for research purposes, we tried to obtain the pure insulin analogues from the respective companies. Unfortunately, formal agreement could not be made because they were either not willing to provide us the compounds or the requested conditions for signing the Material Transfer Agreement were not acceptable to us. Therefore, insulin analogues (Lantus, Levemir, Novorapid, Humalog) as well as regular human insulin (Actrapid), used in the present work, were bought from the pharmacy. We consider this justifiable, since in the end these pharmaceutical preparations are used for injection in diabetic patients.

MCF10A and MCF7, the two most insulin-responsive cell lines, were employed to characterize the proliferative and signalling potency of the insulin analogues in comparison with regular insulin. In order to analyse the possible mitogenic potency of the additives in the pharmaceutical preparations, we first inactivated regular insulin and insulin analogues in the pharmaceutical preparation by chemical reduction of S-S bridges and heating. Thereafter, we performed proliferation assays with the resultant solutions. There was no difference in the proliferative rate of untreated cells or cells treated with carrier solvents of pharmaceutical preparations after inactivation of regular insulin or insulin analogues. This indicates that additives did not have mitogenic potency (data not shown).

Purified bovine insulin is commercially available as zinc insulin while Actrapid is a pharmaceutical preparation of regular human insulin. Of importance is the fact that in all our experiments bovine insulin was studied for comparison and demonstrated similar proliferative

and signalling ability as Actrapid. This permits the conclusion that the pharmaceutical formulation does not alter the efficiency of insulin action significantly. In addition, similar proliferative potency of bovine insulin and Actrapid demonstrates that human insulin can be replaced with animal insulin without any safety concerns with regard to mitogenicity of the compound. Out of four insulin analogues studied, three insulin analogues (Insulin Lispro [Humalog], Insulin Aspart [Novorapid] and Insulin Detemir [Levemir]) demonstrated similar proliferative ability in both MCF10A and MCF7 cells. Only Insulin Glargine clearly induced significantly higher proliferation in MCF7 cells in comparison to Actrapid. However, in MCF10A cells, the proliferative effect of Insulin Glargine was similar to that of Actrapid. The proliferation and signalling potency of each analogue will be discussed in detail below.

4.2.1 Proliferative and signalling potency of Insulin Glargine

Several studies have attributed higher mitogenic potency to Insulin Glargine in comparison to regular insulin. Kurtzhals *et al.* (2000) by thymidine incorporation assay reported six-fold higher proliferative potency of Insulin Glargine than regular insulin in osteosarcoma cells while Eckardt *et al.* (2007) by measuring BrdU incorporation demonstrated three-fold higher proliferative potency of Insulin Glargine than regular insulin in fibroblasts and coronary artery smooth muscle cells. In agreement with the above mentioned studies, we found that in MCF7 cells, Lantus treatment led to approximately 1.5-fold (at 1.5nM) and 3-fold (at 15nM) higher BrdU incorporation than regular insulin. Furthermore, proper proliferation assay involving three days of treatment of MCF7 cells with Insulin Glargine or regular insulin, revealed that at concentrations ≥ 150pM, Insulin Glargine induced significantly stronger proliferation than regular insulin. Even at concentrations as low as 1.5pM and 15pM, which reflect lower than physiological insulin concentrations after overnight fasting, the Insulin Glargine effect was stronger than Actrapid though the difference was not statistically significant. The difference between Insulin Glargine and Actrapid was approximately 1.3-fold at most of the concentrations studied with the 1.5-fold difference at higher concentrations (150nM and 1.5µM). The difference between the data obtained by the BrdU incorporation assay and the colorimetric proliferation assay may be on the one hand due to the difference in the sensitivity of the two assays. On the other hand, BrdU incorporation detects only S-phase cells whereas the colorimetric assay represents the cells in all phases of the cell cycle.

The physiological insulin concentration in serum of healthy people varies between ~15pM and ~200pM after overnight fasting (Labor Lademannbogen, Labor Limbach). Postprandial

levels are about 500pM. The fact that within this concentration range, we did not find regular insulin to induce significant proliferation of MCF7 cells suggests that at low concentrations (1.5pM, 15 pM and 150pM) regular insulin is unlikely to be a strong mitogen. Regarding serum concentrations of Insulin Glargine only few data are available. Free insulin serum levels of healthy volunteers and of patients with type-1 diabetes who received Insulin Glargine injections for 1 to 11 days varied between 70pM and 90pM, with peak levels of about 220pM after injection (Heise *et al.,* 2002; Heise *et al.,* 2004; Gerich *et al.,* 2006; Becker *et al.,* 2008). However, at concentrations of 1.5pM and 15pM, Lantus clearly induced stronger proliferation than Actrapid and at 150pM the proliferation levels obtained with Lantus were significantly higher (approximately 1.3-fold) than with regular insulin. Our findings demonstrating higher proliferative ability of Insulin Glargine in comparison to regular insulin in MCF7 cells at physiological concentrations raise safety concerns regarding treating diabetic patients who have or are at risk to have breast cancer with Lantus.

However, in contrast to our data on MCF7 cells, Staiger et al. (2007) and Liefvendahl and Arnqvist (2008) did not observe higher mitogenic potency of Insulin Glargine compared to regular insulin in MCF7 cells. The dissimilarity in the results may be due to differences in the cell handling procedure or due to methodical differences. Additionally, proliferation values presented by Staiger et al. (2007) had very high error bars (in some cases SEM was 25% of the value) thus making these data difficult for reasonable analysis.

Our investigation of the molecular mechanism behind the strong proliferative potency of Insulin Glargine revealed that Insulin Glargine strongly activates both PI3K and MAPK pathways. We observed that Insulin Glargine compared to insulin and other insulin analogues, induced significantly higher phosphorylation of Akt and GSK3α/β proteins, two important downstream targets in the PI3K pathway. Likewise, Insulin Glargine also strongly activated the MAPK pathway in MCF7 cells as reflected by high Erk1/2 phosphorylation levels. Quantitative evaluation of the phosphorylation signals followed by the statistical analysis revealed that Insulin Glargine was approximately two times stronger than regular insulin in activating PI3K and MAPK pathways. There are contradictory reports regarding the major proliferation pathway in MCF7 cells. Lu and Campisi (1992) and Jhun *et al.* (1994) demonstrate MAPK to be the major proliferation-linked pathway. On the contrary, Dufourny *et al.* (1997) demonstrate PI3K to be the major proliferation-linked pathway. The results from our proliferation experiments in presence of specific inhibitors of PI3K and MAPK pathways, favours MAPK pathway as the major proliferation pathway in MCF7 cells. In addition to

activation of the signalling pathways, we also studied the role of IR, IGF-IR and ERα in Lantus-induced proliferation. These findings are elaborately discussed in Sections 4.4 and 4.5.

In contrast to MCF7 cells, the proliferative potency of Insulin Glargine was similar to regular insulin in MCF10A cells. This finding agrees with other reports demonstrating equi-potency of Insulin Glargine and regular insulin on benign rat-1-fibroblasts (Berti *et al.,* 1998), human coronary artery cells (Staiger *et al.,* 2005), muscle cells (Bähr *et al.,* 1997), and human skeletal muscle cells (Ciaraldi *et al.,* 2001). The molecular background for the differential proliferative ability of Lantus in MCF7 and MCF10A cells is due to differences in the IGF-IR/IR ratio in these cells (Section 4.4). The equi-potency of both Insulin Glargine and regular insulin in MCF10A cells is reflected in the comparable activation of the MAPK pathway as suggested by the similar levels of phosphorylated Erk1/2. Interestingly, analysis of PI3K pathway activation revealed that in comparison to regular insulin, Insulin Glargine induced significantly higher phosphorylation of Akt protein. Slightly higher levels of phosphorylated GSK3α/β, a downstream effector of Akt, were also observed with Insulin Glargine treatment compared to regular insulin treatment but the difference was not statistically significant. Surprisingly, treatment of the cells with IGF-I caused similar phosphorylation of GSK3α/β as seen with Insulin Glargine. The reason of this result is unclear. It may be hypothesised that in MCF10A cells, the signal downstream of Akt level diverges and GSK3α/β is not the main target of Akt. Conversely, it can be argued that signal transduction from Akt to GSK3α/β is so strong in MCF10A cells that differences between the two treatments cannot be detected by immunoblotting. To prove this, the specific kinase activity of Akt needs to be determined. However, the complexity of the findings requires a more detailed analysis. We did not study which signalling pathway is responsible for proliferation in MCF10A cells. Nevertheless, the similar proliferative ability of Insulin Glargine and Actrapid and similar activation of MAPK pathway but not PI3K pathway by Insulin Glargine and Actrapid indicate that like in MCF7 cells, in MCF10A cells MAPK pathway is the major proliferation pathway too.

Another interesting observation is that the ratio of phosphorylated to total proteins of signalling molecules (Akt, GSK3α/β and Erk1/2) in MCF10A cells treated with regular insulin or insulin analogue is much higher than the ratios observed in similarly treated MCF7 cells. However, the proliferation levels observed in MCF7 treated cells were significantly higher than in MCF10A treated cells. This suggests that care should be taken to present high phosphorylation levels of key signalling molecules as primary readout for proliferative potency of any compound.

4.2.2 Proliferative and signalling potency of Insulin Detemir

Addition of a 14-carbon fatty-acid chain to the amino acid at 29th position of the B-chain of insulin renders Insulin Detemir to have higher affinity for albumin and increased lipophilicity compared to human insulin. It binds to BSA with an affinity of ~ 4x10^5 l/mol (Kurtzhals *et al.*, 1996). Insulin Detemir also exhibits 50% lower binding affinity for the insulin receptor and 80% lower affinity for the IGF-IR than human insulin and dissociates almost two times faster than human insulin from insulin receptor (Kurtzhals *et al*., 2000). These differences in biochemical properties were shown by Kurtzhals *et al.* (2000) to be reponsible for the merely 11% (corrected for albumin binding) mitogenic potency of Insulin Detemir relative to human insulin.

However, under our experimental conditions, we observed that only at high concentrations (150nM and 1.5µM), Insulin Detemir-stimulated proliferation levels in MCF7 cells are lower than with regular insulins. At lower concentrations (1.5nM and 15nM) Insulin Detemir treatment of MCF7 cells yielded similar proliferation levels as obtained with regular insulin. This difference may be explained by the 80% lower affinity of Insulin Detemir for IGF-IR compared to regular insulin. At high concentrations, regular insulin can activate IGF-IR to some extent. On the contrary, Insulin Detemir with its 80% lower binding affinity for IGF-IR may not activate IGF-IR even at higher concentrations. Therefore, at high concentrations of regular insulin the proliferation levels achieved are higher than that achieved by Insulin Detemir treatment. However, at lower concentrations, regular insulin does not or only very weakly activate IGF-IR. Thus, under these conditions both, Insulin Detemir and regular insulin, are observed to induce similar levels of proliferation in MCF7 cells. Interestingly, although Insulin Detemir shows ~ 50% reduced binding affinity for insulin receptor and in comparison to regular insulin dissociates two times faster from insulin receptor (Kurtzhals *et al*., 2000), its proliferative ability is similar to regular insulin at low concentrations in MCF7 cells. These results strongly suggest that the binding affinity for insulin receptor is not a major determinant for the proliferative potency of insulin. This also explains the finding, that in MCF10A cells, a cell line with low IGF-IR/IR ratio (0.8:1), Insulin Detemir exhibited similar proliferative ability as regular insulin at all the concentrations studied.

At the molecular level, in MCF7 cells, the signalling potency of 15nM Insulin Detemir was approximately 50 % lower than 15nM regular insulin as demonstrated by the phosphorylation levels of Akt, GSK3α/β and Erk1/2. These experiments were performed under serum-free conditions, thus the results are solely based on the biochemical properties of Insulin Detemir

and not affected by the serum binding properties of Insulin Detemir. As at 15nM concentration, regular insulin only weakly activates IGF-IR, the relative difference in the phosphorylation levels of key signalling molecules induced by Insulin Detemir and regular insulin is most probably due to reduced insulin receptor affinity of Insulin Detemir and not to reduced affinity for IGF-IR. Interestingly, the ~ 50 % lower activation of the MAPK pathway (as reflected by the phosphorylation status of Erk1/2 proteins) by 15nM Insulin Detemir compared to 15nM regular insulin did not lead to corresponding decrease in the proliferative ability of Insulin Detemir vis-à-vis regular insulin. Most probably, the stronger Erk1/2 phosphorylation by 15nM Actrapid in comparison to 15nM Insulin Detemir is a transient effect and consequently there is no difference in proliferative potence between the two compounds. This assumption is supported by the study from Hansen *et al.* (1996) in which it was demonstrated that the sustained activation of insulin receptor is required for its mitogenic effect. However, the precise interpretation of this observation demands a more detailed study. In MCF10A cells, signalling potency of Insulin Detemir was similar to regular insulin. The difference in the signalling potency of Insulin Detemir between MCF7 cells and MCF10A cells is most likely due to differences in the cell characteristics.

Altogether, it can be concluded that Insulin Detemir does not exhibit higher mitogenic potency than human insulin and therefore can be assumed to have low safety risks. In fact, studies have associated Insulin Detemir therapy with positive effects in diabetic patients like loss of weight (Tibaldi, 2007). The weight loss is attributed to the increased concentration of Insulin Detemir in the brain, which results in elevated insulin signalling and thus lowering of the 'hunger signal'. The higher lipophilicity of Insulin Detemir in comparison to human insulin allows Insulin Detemir to cross the blood-brain barrier easily which results in higher concentration in brain (Rossetti *et al.,* 2007). As the albumin concentration in brain is 200-fold lower than in the blood (Seyfert *et al.*, 2002), there is a higher percentage of free Insulin Detemir in brain than in the blood. Hennige *et al.* (2005) determined the tyrosine phosphorylation status of the insulin receptor and IRS-2 protein in hypothalamic and cerebrocortical tissues of C57B1/6 mice and demonstrated that insulin signalling is faster and stronger in the mice subjected to Insulin Detemir treatment compared to those treated with regular insulin.

4.2.3 Proliferative and signalling potency of Insulin Aspart

Insulin Aspart is generated by replacing proline at the 28th position of B-chain with aspartic acid. Literature survey reveals that mitogenic response to Insulin Aspart is highly dependent

on the cell types under study and the mitogenic potency of Insulin Aspart can be weaker, similar or stronger than that of regular insulin. In Saos/B10 cells, Insulin Aspart showed only 58 ± 22 [%] of the potency of Actrapid in inducing thymidine incorporation (Kurtzhals *et al.*, 2000). However, in CHO-K1 cells Insulin Aspart induced thymidine incorporation to a similar level as regular insulin (Hansen *et al.*, 1996). Recently, Eckardt *et al.* (2007) also demonstrated equal potency of Insulin Aspart and regular insulin by studying BrdU incorporation into the DNA of human primary smooth muscle cells from different donors. Trüb *et al*. (1999) showed a slight increase in the percentage of S-phase cells (1.2-fold of regular insulin) after Insulin Aspart treatment in MCF7 cells.

We also observed a differential mitogenic response to Insulin Aspart in the two insulin-responsive mammary epithelial cell lines, MCF10A and MCF7. In agreement with the studies from Hansen *et al.* (1996) and Eckardt *et al.* (2007), Insulin Aspart - treated MCF10A cells demonstrated similar proliferation levels as Actrapid - treated MCF10A cells. This was reflected also at the signalling pathway level, where MCF10A cells treated with Insulin Aspart and Actrapid showed similar phosphorylation levels of Akt, GSK3α/β and Erk1/2. However, in contrast to MCF10A cells and in agreement with Trüb *et al*. (1999), treatment of MCF7 cells with 15nM Insulin Aspart resulted in ~ 1.5-fold higher BrdU incorporation in comparison to regular insulin. This higher potency of Insulin Aspart vis-à-vis regular insulin to stimulate BrdU incorporation was also reflected in the slightly higher proliferative effect of Insulin Aspart compared to Actrapid at all concentrations studied though the differences were not statistically significant. This trend was further corroborated by our results on Erk1/2 phosphorylation levels which were somewhat higher in Insulin Aspart - treated MCF7 cells than in Actrapid - treated MCF7 cells whereas the PI3K pathway was similarly activated by both Insulin Aspart and Actrapid in MCF7 cells.

The slightly higher proliferative potency of Insulin Aspart in MCF7 cells may be explained by the fact that Insulin Aspart has a lower insulin receptor dissociation rate and therefore stays for longer duration on the insulin receptor than regular insulin, which results in a stronger proliferative effect (Hansen *et al.*, 1996). However, it is interesting that the altered biochemical property of Insulin Aspart does not result in stronger proliferative ability in comparison to regular insulin in MCF10A cells. It may be argued that the high number of insulin receptors relative to IGF-IR in MCF10A cells (low IGF-IR/IR ratio) compensates the differences in the biochemical properties of Insulin Aspart and regular insulin, resulting in equipotency of the two compounds with respect to proliferative ability. Nevertheless, this

observation merits further analysis. In summary, our study demonstrates that under low IGF-IR/IR condition, Insulin Aspart may not pose a safety risk with regard to proliferation. However, the slightly increased mitogenic potency of Insulin Aspart in comparison to regular insulin in MCF7 cells demands further studies, preferably using animal models**.**

4.2.4 Proliferative and signalling potency of Insulin Lispro

Insulin Lispro is generated by interchanging the aminoacids at position 28 and 29 of the B-chain of insulin molecule to lysine and proline respectively. This modification results in slightly lower affinity for insulin receptor (0.8-fold of human insulin) and slightly higher affinity for IGF-IR (1.5-fold of human insulin). However, these altered binding properties of Insulin Lispro did not result in altered proliferative ability in comparison to regular insulin (Kurtzhals *et al.,* 2000; Slieker *et al*., 1997; Eckardt *et al*., 2007). In agreement with these studies, our results from the proliferation experiments on MCF10A and MCF7 cells also demonstrate similar proliferative potency of Insulin Lispro and regular insulin. Furthermore, at the molecular level, Insulin Lispro-induced phosphorylation levels of Akt, GSK3α/β and Erk1/2 were similar to regular insulin - induced levels in both cell lines. Taken together, we can conclude that the slightly higher IGF-IR affinity of Insulin Lispro does not result in higher proliferative potency in comparison to Actrapid and thus may have limited safety risks regarding mitogenicity.

4.3 Insulin receptor and IGF-IR quench each other

Our knockdown experiments yielded interesting results regarding the influences insulin receptor and IGF-IR exercise on each other. Surprisingly. under IGF-IR knockdown conditions, we observed an increase in the signalling and proliferative potency of regular insulin. Specifically, regular insulin clearly induced stronger Akt and Erk1/2 phosphorylation in MELN cells which exhibited low levels of IGF-IR compared to MELN cells exhibiting normal levels. The increased signalling potency of regular insulin also resulted in increased proliferative potency in MELN cells exhibiting low IGF-IR levels. Similarly, signalling and proliferative potency of IGF-I was observed to increase under insulin receptor knockdown conditions. IGF-I treatment of normal MELN cells yielded high phosphorylation levels of Akt and Erk1/2. These phosphorylation levels were found to increase strongly in MELN cells expressing low levels of insulin receptor.

These findings suggest that both IGF-IR and insulin receptor have inhibitory effect on each others ligand. Recent studies have shown that insulin receptor and IGF-IR, being highly homologous to each other, may form hybrid receptors (Frasca *et al*., 1999). As insulin receptor has two splice variants, IR-A (lacks exon 11) and IR-B (with full transcript), there can be two types of hybrid receptors - HR-A and HR-B. Regular insulin, compared to its binding affinity for the insulin receptor homodimer, demonstrates decreased binding affinity for HR-A, and has been suggested not to bind to HR-B (Frasca *et al*, 2003). Under conditions with reduced IGF-IR levels more homodimers of insulin receptors are present, which results in increased binding of regular insulin and in increased MAPK activation and proliferative potency of regular insulin. IGF-I does not exhibit strong differences in the binding affinities to HR-A or IGF-IR and binds to HR-B with lower affinity than to HR-A. The marked increase in signalling potency of IGF-I in cells which have elevated IGF-IR homodimer (due to reduction in insulin receptor) suggests towards the higher level of HR-B than HR-A in these cells. This is contradictory to the studies demonstrating higher levels of IR-A in mammary tumorigenic cells and thus higher levels of HR-A. This interesting observation will certainly need further investigations.

4.4 Proliferative ability of Insulin Glargine is due to the strong activation of IGF-IR

In comparison to regular insulin, Insulin Glargine induced significantly stronger proliferation at concentrations ≥ 150pM in MCF7 cells. By using RNA interference, we found that the higher signalling and proliferative potency of Insulin Glargine in comparison to regular insulin is due to its ability to activate IGF-IR besides the insulin receptor. Insulin did not induce phosphorylation of Akt and Erk1/2 when the level of its cognate receptor was significantly reduced. However, under these conditions, Insulin Glargine did induce phosphorylation of Akt and Erk1/2 suggesting involvement of IGF-IR. Futhermore, in MELN cells with significantly reduced IGF-IR expression level, the signalling and proliferative potency of Insulin Glargine and regular insulin were similar, which again suggests IGF-IR activation by Insulin Glargine and renders an explanation for the high proliferative ability of Insulin Glargine. The strong activation of IGF-IR by Insulin Glargine can be explained by the fact that Insulin Glargine possesses six-fold higher IGF-IR binding affinity than regular insulin (Kurtzhals *et al.,* 2000). Insulin Glargine most probably activates IGF-IR by directly binding to it as the immunoprecipitation analysis showed that Insulin Glargine treatment results in strong phosphorylation of IGF-IR. The activation of IGF-IR was also reflected in

the transcription of the IGF-I responsive gene cyclin D1 in Insulin Glargine-treated MCF7 cells.

The differences in the proliferative potency of Insulin Glargine and regular insulin in MCF10A and MCF7 cells can be explained by taking into consideration the nature of two cell lines and the biochemical properties of Insulin Glargine. MCF10A cells exhibit low IGF-IR/IR ratio (0.8:1; Gammeltoft et al. [1999]) in contrast to high IGF-IR/IR ratio (7:1; Liefvendahl *et al.* [2008] to 4:1; Gammeltoft *et al.* [1999]) observed in MCF7 cells. We postulate that in MCF7 cells, the IGF-IR plays the more important role in determining the proliferative potency of Insulin Glargine. However, in MCF10A cells, the insulin receptor probably plays a more important role in determining the proliferation response to Insulin Glargine. This may explain why Insulin Glargine with its slightly decreased affinity for insulin receptor and nearly 1.5 fold faster dissociation rate from insulin receptor does not elicit stronger proliferative response than Actrapid in MCF10A cells.

4.5 ERα does not play a major role in the proliferative potency of Insulin Glargine

ERα is a well established nuclear receptor and transcription factor and plays an important role in breast cancer development. About 70% of primary breast cancers are ERα-positive. In breast cancer cell lines like MCF7 and ZR75-1, ERα is abundantly expressed. Insulin Glargine induced strong proliferation in ERα-positive MCF7 cells but not in ERα-negative MCF10A cells. We described in the above section that IGF-IR activation plays an important role in the strong proliferative effect of Insulin Glargine. The IGF-IR signalling pathway has been reported to cross-talk with ERα signalling (Kato *et al*., 2000; Dupont and Le Roith, 2001; Zhang *et al*., 2005) in a way that IGF treatment of cells results in phosphorylation of ERα in the AF-1 domain. This phosphorylation modulates ERα activity in a positive manner. Therefore, there is a possibility that activation of ERα contributes to the growth stimulatory role of IGF-IR. In order to assess the involvement of ERα in IGF-IR signalling, we first tried to express ERα in MCF10A cells. This would have enabled us to compare the influence of IGF-IR activation on proliferation of ERα positive and ERα negative MCF10A cells. However, this approach was not successful as MCF10A cells are very resistant to transfection (transfection efficiency was less than 5% with any method chosen). Alternatively we attempted to knockdown ERα in MCF7 cells. ERα knockdown resulted in decrease of IGF-IR protein levels (data not shown). This can be explained by the positive regulation of IGF-IR

expression by ERα in MCF-7 cells (Maor *et al*., 2006). Finally, we studied activation of ERα by IGF-I and Insulin Glargine in MCF7 cells. Previous findings from our laboratory show that phosphorylation of ERα at Ser118 residue located in the AF-1 domain is required for full activation of ERα (Medunjanin *et al.,* 2005). The Ser118 residue can be phosphorylated by GSK3α/β (Medunjanin *et al.,* 2005) and by MAPK (Kato et al., 1995). Both enzymes are important components of signalling pathways activated by IGF-IR. Our results show that Insulin Glargine induced significantly higher phosphorylation of ERα at Ser118 residue in comparison to regular insulin and all other insulin analogues. However, Insulin Glargine increased ERα activity only slightly stronger than regular insulin as was evident from the results of ERE-dependent luciferase activity studies. Taken together, these results suggest that activation of ERα contributes only weakly to the Insulin Glargine mediated MCF7 cell proliferation.

4.6 Model for Insulin Glargine action in MCF7 cells

The results on Insulin Glargine-induced signalling are summarized in a model to explain the high proliferative ability of Insulin Glargine in MCF7 cells (Fig. 5.1 a, b). Under normal conditions (Fig 5.1a), regular insulin via insulin receptor activates mainly PI3K/Akt pathway and to low extent MAPK pathway. IGF-I, through the IGF-IR, activates both PI3K/Akt and MAPK pathways strongly.

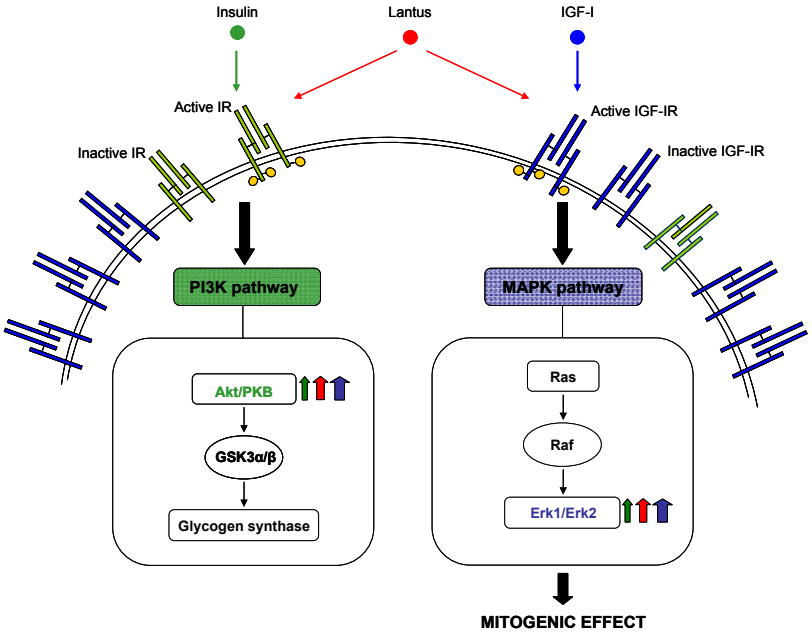


Figure 5.1a Illustration of the mode of action of Insulin Glargine in MCF7 cells under normal conditions. Insulin (green solid circle) under physiological conditions binds to the insulin receptor (IR; shown in green) and activates mainly PI3K pathway (shown by thick green arrow at Akt) and to low extent MAPK pathway (shown by thin green arrow at Erk1/2). IGF-I (blue solid circle) binds to

IGF-IR (shown in blue) and activates both PI3K and MAPK pathways strongly (shown by the thick blue arrows at Akt and Erk1/2). Insulin Glargine (depicted as Lantus in red solid circle) can bind to both receptors and activates PI3K and MAPK pathways equally strongly (shown by red arrows at Akt and Erk1/2).

Insulin Glargine stimulates both insulin receptor and IGF-IR resulting in activation of both PI3K and MAPK pathways at levels higher than observed with insulin. The strong activation of IGF-IR by Insulin Glargine is responsible for the strong proliferative potency of Insulin Glargine. This mechanism of action applies to cell lines characterized by a high IGF-IR/IR ratio such as MCF7.

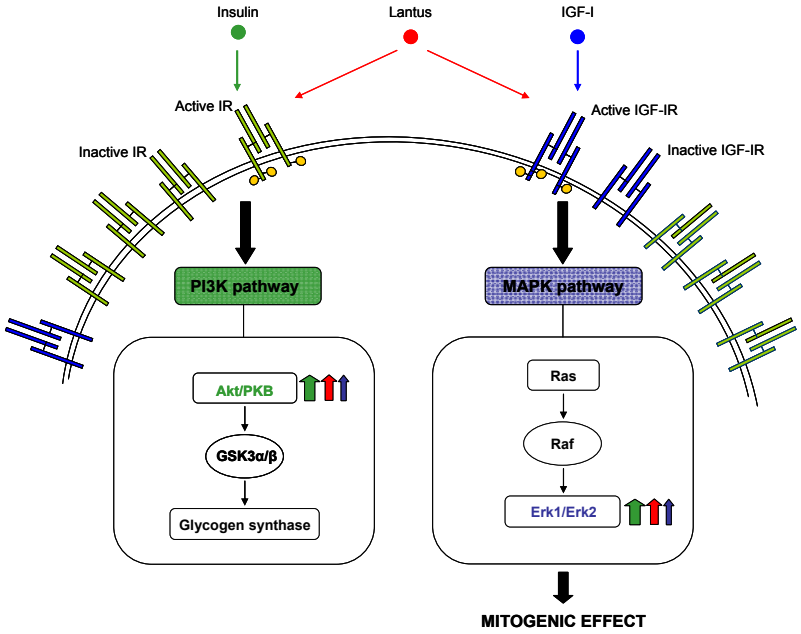


Figure 5.1b Illustration of the mode of action of Insulin Glargine in MCF7 cells under IGF-IR knockdown conditions. Under IGF-IR knockdown conditions, IGF-I mediated PI3K and MAPK activation decreases significantly (compare the blue arrows thickness under both the conditions) while insulin mediated activation of PI3K and MAPK pathway increases compared to normal condition (compare the thickness of green arrows). IGF-IR knockdown decreases Insulin Glargine mediated PI3K and MAPK pathway activation to the level stimulated by insulin (compare the thickness of green and red arrow).

Under IGF-IR knockdown conditions (Fig. 5.1b), the ability of IGF-I to activate PI3K and MAPK pathway is severely compromised. Insulin Glargine treatment elicits similar phosphorylation levels of Akt and Erk1/2 as obtained with regular insulin. Thus, under IGF-IR knockdown conditions Insulin Glargine behaves like normal insulin. This explains the observation that in cell lines with low IGF-IR/IR ratio, the proliferative ability of Insulin Glargine is similar to that of Actrapid. Interestingly, under IGF-IR knockdown conditions insulin treatment causes stronger stimulation of insulin receptor accompanied by stronger phosphorylation of Akt and more importantly of Erk1/2 compared to control siRNA

conditions. This shows that insulin receptor is a mitogenic receptor, but under normal conditions its mitogenic ability is impaired due to presence of IGF-IR.

4.7 Migratory and invasive potency of Insulin Glargine

IGF-I via activation of IGF-IR has been shown to stimulate not only proliferative but also motile cell responses (Leventhal and Feldman, 1997; Bredin *et al*., 1999; Meyer *et al*., 2001). In MCF7 cells contrasting data has been reported regarding the role of IGF-I in cell migration. Guvakova *et al*. (2002) showed that the release of cell-cell adhesion (cell separation) was enhanced in MCF7 cells overexpressing the IGF-IR and blocked in cells expressing a kinase-dead mutant of this receptor. Furthermore, IGF-IR mediated cell separation was demonstrated to be due to the activation of α-actinin through the PI3K pathway which results in the rapid organization of actin into microspikes at the cell-cell junctions. On the contrary, Mauro *et al*. (2001) showed that IGF-I treatment of MCF7 cells results in increase in cell-cell adhesion via activation of E-cadherin protein.

We studied MCF7 cell migration in the presence of regular insulin, Insulin Glargine or IGF-I by using wound healing assay. Our results show that there was no significant difference in the wound healing rate between the cells treated with insulin, Insulin Glargine and IGF-I. As IGF-I treatment of MCF7 cells did not induce significant migration under our experimental conditions, it may be suggsted that IGF-I has weak potency in stimulating cell migration in our system. In addition to the wound healing experiment, we also studied the effect of Insulin Glargine on MCF7 cell invasion by matrigel assay (data not shown). Control experiments showed increased migration through the matrigel layer by treatment of the cells with 10%DCC-FCS as compared with 2%DCC-FCS, but there was no effect of IGF-I, Insulin Glargine or regular insulin on invasion. In summary, insulin, Insulin Glargine and IGF-I demonstrate poor migratory and invasive potency in MCF7 cells. It is important to point out that the validity of these data is limited in the terms of time-points and concentrations of the compounds studied. Further investigations are required to make a conclusive statement.

4.8 Outlook

Our findings show that at concentrations ≥ 150pM, Insulin Glargine exerted significantly higher proliferation relative to regular insulin in MCF7 cells. At lower concentrations (1.5pM and 15pM) a stronger proliferative effect of Insulin Glargine compared to regular insulin was clearly visible although not statistically significant. The question is whether this stronger

proliferative effect of Insulin Glargine is of relevance to diabetic patients undergoing Insulin Glargine therapy. Only few data are available on serum concentrations of Insulin Glargine. Free serum insulin levels of healthy volunteers and of patients with type I diabetes who received Insulin Glargine injections for 1 to 11 days varied between 70pM and90pM, with peak levels of about 220pM after injection (Gerich *et al.,* 2006; Heise *et al.,* 2002; Heise *et al.,* 2004; Becker *et al.,* 2008). Considering these studies in the light of our results, we postulate that at these Insulin Glargine serum levels, cell proliferation may be significantly stimulated in diabetic patients. We found that the difference in proliferation between Insulin Glargine and Actrapid treated MCF7 cells was approximately 1.5-fold. Although this difference seems not to be very high, it must be considered that insulin therapy is prescribed lifelong. The higher proliferative ability of Insulin Glargine must therefore be considered a matter of concern. Nevertheless, proliferation assays must be performed at Insulin Glargine concentrations between 15pM and 150pM to achieve precise data for concentrations that are found in serum of patients treated with Insulin Glargine.

Normal breast epithelial cells do not exhibit very high IGF-IR/IR ratio, whereas, importantly, a high IGF-IR/IR ratio is observed in many breast tumours (Frasca *et al*., 2003). In the present study, we observed that Insulin Glargine induced stronger proliferation than regular insulin only in MCF7 cells which are characterized by a high IGF-IR/IR ratio. The mammary epithelial cells exhibiting lower IGF-IR/IR ratio responded to Insulin Glargine and regular insulin to a similar extent. These observations may explain why the two-year carcinogenicity experiment on normal mice and rats reported by Stammberger *et al.* (2002) did not show a significant difference in the incidence of mammary tumors between groups treated with Insulin Glargine or regular insulin. As high IGF-IR/IR ratio is observed mostly in tumour cells, we assume that Insulin Glargine may act as a tumour promoter rather than a tumour initiator. We also hypothesize that Insulin Glargine may be of risk for diabetic patients with breast cancer or as yet undetected (pre-) cancerous lesions. In the future, animal studies must be carried out to gain more information on the potential tumour promoting effects of Insulin Glargine in the mammary gland. Attempts can be made to initiate breast cancer in rats or mice by chemical carcinogens and then compare the effect of Insulin Glargine and regular insulin on tumour progression. In addition, transgenic mouse models of breast cancer should be analysed.

We reported preliminary experiments to study the migratory and invasive potency of Insulin Glargine in comparison to regular insulin. Our results show slightly higher migratory potency

of Insulin Glargine relative to regular insulin. As a diabetic patient is on insulin therapy for lifelong even a slight increase can compromise the health of the diabetic patient. A detailed and comprehensive study of migratory and invasive potency of Insulin Glargine must be performed.

Our study reveals that Insulin Lispro and Insulin Detemir where equipotent to regular insulin regarding mitogenicity. However, in comparison to regular insulin, Insulin Aspart had slightly increased proliferative effect at all concentrations in MCF7 cells. Nowadays, doctors prescribe one short acting insulin analogue together with one long acting insulin analogue for better control of glycaemia and there are reports that Insulin Glargine and Insulin Aspart are prescribed together (Garber, 2006). Our results suggest that this particular combination (Insulin Glargine + Insulin Aspart) may pose a higher safety risk compared to any of them administered alone. There is no information about the proliferative potency of such combinations. Thus, there is urgent need to compare the proliferative ability of regular insulin with the combination of Insulin Aspart and Insulin Glargine.

Last but not least, type II diabetes, which is characterized by hyperinsulinemia has been reported to be positively associated with the risk to develop not only breast cancer but also cancer in other organs such as colon, pancreas and prostate (Key, 2001; Silverman, 2001; Giovannucci, 2007; Venkateswaran *et al*., 2007). A similar study as presented in this thesis must also be carried out with respect to the proliferative potency of insulin analogues on colon, pancreas and prostate cell lines.

5 MATERIALS

5.1. Equipment

Name of the Equipment	Name of the Company
General Equipments	
Analytical balance 2002 MP1	Sartorius, Göttingen, Germany
Blotting chamber for wet blotting	Sigma, Deisenhofen, Germany
Centrifuge GPK	Beckman Instruments, München , Germany
Centrifuge for Eppendorf tubes Biofuge	Heraeus, Hanau, Germany
CHROMO4 System for Real-Time PCR Detection	MJ Research, Miami, USA
Heating block Dri Block DB3	Thermo-Dux, Wertheim, Germany
Luminometer Biolumat LB9505	Berthold, Bad Wildbad, Germany
Magnetic stirrer MR 2002	Heidolph, Kelheim, Germany
Microscope camera DFC 480	Leica Microsystems, Solms, Germany
Microwave Micromat 135	AEG, Nürnberg, Germany
Multiplate ® PCR plates	Bio-Rad, Hercules, CA, USA
Multiskan Ex (Plate Reader)	Thermo Fisher Scientific, Dreieich, Germany
Nanodrop	Peqlab, Erlangen, Germany
PCR Primus96 Plus machine	MWG Biotech, Martinsried, Germany
PTC-200 Peltier Thermal Cycler	MJ Research, Miami, USA
pH electrode InLab 410	Mettler Toledo, Steinbach, Germany
pH meter 535 Multical	WTW, Weilheim, Germany
Photometer Ultrospec III	Amersham Pharmacia Biotech, Freiburg, Germany
Pipetboy acu	Integra, Hamburg, Germany
Powersupply EPS 300	Amersham Pharmacia Biotech, Freiburg, Germany

Refrigerated centrifuge “fresco”	Heraeus, Hanau, Germany
SDS-PAGE Mini Protean II and III	Bio-Rad, München, Germany
Spin-over rotator Bioblock	Novodirect, Kehl, Germany
Thermomixer compact	Eppendorf, Hamburg, Germany
Water-purification system Milli-Q	Millipore, Eschwege, Germany
Vortex mixer	Bender u. Hobein AG, Zürich, Switzerland
X-ray film developer Hyperprocessor	Amersham Pharmacia Biotech, Freiburg, Germany
Cell Culture Equipments	
Humidified CO_2 incubator	Forma Scientific, Labotech Göttingen, Germany
Microscope (phase contrast)	Leica Microsystems, Solms, Germany
Neubauer hemacytometer	Schott, Hofheim, Germany
Sterile bench	Heraeus, Hanau, Germany

5.2. Chemicals

If not otherwise mentioned, all chemicals used in the present study were of analytical grade and sourced from following companies: Merck (Darmstadt, Germany), Roche (Mannheim, Germany), and Sigma (Deisenhofen, Germany). All consumables for cell culture work were of cell culture grade and sourced mainly from Becton and Dickenson Labware (Heidelberg, Germany), Eppendorf (Hamburg, Germany) and Greiner Bio-One (Frickenhausen, Germany).

Name of the chemical	Name of the company
General Chemicals	
Acrylamide/bis-acrylamide, 30% solution	Sigma, Deisenhofen, Germany
Ammonium persulfate	Merck, Darmstadt, Germany
Bovine serum albumin, 30% solution	Bio Rad, München, Germany
Bromophenol blue	Serva, Heidelberg, Germany
Complete mini EDTA-free protease inhibitor cocktail tablet	Roche, Mannheim, Germany

DC protein assay kit	Bio Rad, München, Germany
Dimethyl sulphoxide (DMSO)	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Sigma, Deisenhofen, Germany
dNTPs mix	Stratagene, Amsterdam, The Netherlands
ECL-plus reagent	Amersham Pharmacia Biotech, Freiburg, Germany
Luciferase assay reagent (LAR)	Promega, Mannheim, Germany
Luciferase cell culture lysis 5 x reagent	Promega, Mannheim, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany
Milk powder	Fluka, Buchs, Switzerland
Oligonucleotide PCR primers	Operon, Köln, Germany
Paraformaldehyde (PFA)	Merck, Darmstadt, Germany
Protein A agarose beads	Roche Diagnostics, Mannheim, Germany
PVDF-membrane Immobilon-P	Millipore, Eschwege, Germany
SYBR green supermix	Bio Rad, München, Germany
N,N,N',N'-tetramethyl ethylenediamine (TEMED)	Serva, Heidelberg, Germany
Tris-base	Sigma, Deisenhofen, Germany
Tris-HCl	Roth, Karlsruhe, Germany
Triton X-100	Serva, Heidelberg, Germany
Tween-20	GERBU, Gaiberg, Germany
X-ray film	Konica, Tokyo, Japan

Cell Culture Chemicals

Charcoal (Norrit A)	Serva, Heidelberg, Germany
Dextran 60	Serva, Heidelberg, Germany
EDTA 1 % (w/v) in PBS	Biochrom, Berlin, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
L-glutamine, 200 mM	Biochrom, Berlin, Germany

Matrigel	R & D Systems, Minneapolis, USA
Oligofectamine	Invitrogen, Karlsruhe, Germany
Penicillin/streptomycin (10.000 U/10.000 µg/ml)	Biochrom, Berlin, Germany
Transwell plates	Corning, NY, USA
Trypsin/EDTA, 0,05 %/0,02 % (w/v) in PBS	Biochrom, Berlin, Germany
U0126	Calbiochem, Darmstadt, Germany
Wortmannin	Calbiochem, Darmstadt, Germany

5.3. Hormones/Growth factors used

Name of the compound	Name of the company
17β-estradiol (E2)	Sigma, Deisenhofen, Germany
Actrapid®	Novo Nordisk, Bagsvard, Denmark
Bovine insulin	Sigma, München, Germany
IGF-I	R & D Systems, Minneapolis, USA
Humalog®	Lilly Deutschland GmbH, Bad Homburg, Germany
Lantus®	Sanofi Aventis
Levemir®	Novo Nordisk, Bagsvard, Denmark
Novorapid®	Novo Nordisk, Bagsvard, Denmark

5.4. Disposable materials

All disposable laboratory plastic and reaction tubes were sourced from Becton Dickinson (Heidelberg, Germany), Bio-Rad (München, Germany), Eppendorf AG (Hamburg, Germany), Greiner (Frickenhausen, Germany) and Sarstedt (Nümbrecht, Germany).

5.5. Growth Medium

Name of the medium	Name of the company
Dulbecco's MEM, 3.7 g/l NaHCO3, 4.5 g/l D-glucose, phenol-red free, glutamine-free, pyruvate-free (DMEM)	Invitrogen, Karlsruhe, Germany
Mammary Epithelial Growth Medium (MEGM)	Provitro, Berlin, Germany
RPMI 1640, phenol-red free, glutamine-free, pyruvate-free (RPMI)	PAA, Cölbe, Germany

5.6. Cell lines studied

Cell line	Growth Medium	Source-Description
MCF7	DMEM	Human (ER+) breast epithelial adenocarcinoma cell line derived from metastatic pleural effusion (DSMZ, Germany)
MELN	DMEM	MCF-7 cells (ER+) stably transfected with ERE-controlled luciferase reporter plasmid (Balaguer *et al.* 2001)
MDA-MB231	DMEM	Human (ER-) breast epithelial adenocarcinoma cell line derived from pleural effusion (ATCC, Wesel)
HCC1937	DMEM	A near-tetraploid human (ER-) breast epithelial adenocarcinoma cell line (ATCC, Wesel)
BT474	RPMI-1640	Human (ER+) breast epithelial ductal carcinoma cells isolated from solid invasive tumour (ATCC, Wesel)
T47D	RPMI-1640	Human (ER+) breast epithelial ductal carcinoma cells derived from metastatic pleural effusion (ATCC, Wesel)
ZR75-1	RPMI-1640	Human (ER+) breast epithelial ductal carcinoma cells derived from ascites (ATCC, Wesel).
MCF10A	MEGM	Human non-tumorigenic epithelial cell line (ER-) produced by long term culture in serum free medium with low Ca++ concentration (ATCC, Wesel).

5.7. Solutions and media for cell culture work

M2

phenol-red free DMEM or RPMI	
Penicillin/Streptomycin	100 U/ml resp. 100 µg/ml
FCS	10 %
sodium pyruvate	1 mM
L-glutamine	2 mM

DCC solution

Tris-HCl (pH 8.0)	0.01 M
Charcoal Norrit A	0.25 % (w/v)
Dextran 60	0.0025 % (w/v) in distilled H_2O

M3

phenol-red free DMEM or RPMI	
Penicillin/Streptomycin	100 U/ml resp. 100 µg/ml
dextran-coated charcoal-treated FCS (DCC-FCS)	10 %
sodium pyruvate	1 mM
L-glutamine	2 mM

M4

phenol-red free DMEM or RPMI	
Penicillin/Streptomycin	100 U/ml resp. 100 µg/ml
sodium pyruvate	1 mM
L-glutamine	2 mM

MEGM

serum-free MEGM	
Human recombinant EGF	10 ng
Hydrocortisone	0.5 µg

Insulin	5.0 µg
BPE 26 mg Protein/2 ml	4.0 µl
Gentamicin	50.0 µg
Amphotericin B	50.0 ng

5.8. Description of kits used

Kit	Description	Company
Bio-Rad DC Protein Assay kit	For determining protein concentration in cell lysates	Bio Rad, München, Germany
FITC BrdU Flow Kit	For BrdU incorporation in proliferating cells	BD Biosciences, HD, Germany
QIAquick gel extraction	Extraction of nucleotide fragments from agarose gels	Qiagen,Hilden, Germany
QIAquick PCR purification and nucleotide removal	To purify DNA fragments from enzymatic reactions	Qiagen, Hilden, Germany
RevertAid^{TM}H Minus First Strand cDNA Synthesis	For the synthesis of cDNA from total RNA	Fermentas, St.Leon-Rot, Germany
RNeasy	Extraction of total RNA from cells or tissues	Qiagen, Hilden, Germany

5.9. siRNA sequences

Name	siRNA references and/or sequences	Company
CT2	Non-targeting siRNA sequence (ON-TARGETplus Non-targeting siRNA #2; Cat # D-001810-02-05)	Dharmacon, Lafayette, CO, USA
IGF-IR	IGF-IR targeting sequence (ON-TARGET plus SMART pool; Cat # L-003012-00-0005)	Dharmacon, Lafayette, CO, USA

IR	IR targeting sequence (ON-TARGET plus SMART pool; Cat # L-003014-00-0005)	Dharmacon, Lafayette, CO, USA
ERα	ERα targeting sequence (Cat # N2010)	New England Biolabs, Frankfurt, Germany

5.10. Quantitative real time PCR primers

Name	Specificity	Organism	Length (N)	Sequence (5' → 3')	Tm (°C)
SYBR GAPDHfwd	GAPDH	H.Sapiens	18	AGCCACATCGCTCAGACA	59.9
SYBR GAPDHrev	GAPDH	H.Sapiens	19	GCCCAATACGACCAAATCC	60.1
SYBR CCND1fwd	Cyclin D1	H.Sapiens	20	CCTGTCCTACTACCGCCTCA	64.5
SYBR CCND1rev	Cyclin D1	H.Sapiens	18	TGGGGTCCATGTTCTGCT	59.9

5.11. Buffers and Solutions

Specification	Composition
Acrylamide solution	
acrylamide/bisacrylamide	30:0.8 in distilled H_2O
APS	
Ammonium Persulfate	10 % (w/v)
Cell lysis buffer (made in distilled H_2O)	
HEPES (pH 7.6)	50 mM
NaCl	150 mM
$MgCl_2$	1.5 mM
$Na_4P_2O_7$ x 10 H_2O	10 mM
EDTA	2 mM
Glycerol	10 % (v/v)
Triton X-100	1.5 % (v/v)

Na-Fluoride	100 mM
Na-Orthovanadate (Na_3VO_4)	2.7 mM
Protease Inhibitor Cocktail Tablet	1 (/10 ml)

LiCl buffer (made in distilled H_2O)

LiCl	1M
Tris-HCl, pH 8.0	100mM
Na-Azide	0.1%

PBS (10 x)

NaCl	0.86M
Na_2HPO_4	0.58M
NaH_2PO_4xddH_2O	0.17M

Sample buffer (5 x)

Tris-HCl (pH 6.7)	50 mM
DTT	200 mM
SDS	2 %
Glycerol	2.5 %
Bromophenol blue	0.1 %
β-Mercaptoethanol	1.75 M

SDS

10 %	made in distilled H_2O

TBS (10 x)

Tris-HCl (pH 7.6)	0.1 M
NaCl	1.5 M

TBS-T

TBS	1 x
Tween	0.1 %

TE buffer (pH 8.0)

Tris-HCl	10mM
EDTA	1M

Tris-HCl

Tris solution which pH is adjusted with HCl

SDS-PAGE gel (10 %, 2 mini gels)

H_2O	4.02 ml
30 % acrylamide solution	3.3 ml
1.5 M Tris (pH 8.8)	2.5 ml
10 % SDS	100 µl
10 % Ammonium Persulfat	50 µl
Temed	30 µl

Stacking gel (5 %, 2 mini gels)

H_2O	3 ml
30 % acrylamide solution	0.66 ml
1 M Tris (pH 6.8)	1.26 ml
10 % SDS	50 µl
10 % Ammonium Persulfat	38 µl
Temed	30 µl

Running buffer (1 x)

Tris-HCl (pH 8.3)	25 mM
Glycine	250 mM
SDS	0.1 %

Protein Standard

Protein All Blue precision plus standard (Bio- Rad)

Transfer buffer

Tris-base	50 mM
Glycine	380 mM
Methanol	20 %

Blocking buffer

Non-fat dry milk in TBS/T	5 %

Stripping buffer

Tris-HCl (pH 6.7)	62 mM
β-Mercaptoethanol	7.18 ml
10 % SDS	200 ml

5.12. Antibodies

Primary antibodies

Name	Species	Dilution	Company
α IGF-IRβ	rabbit (P)	1:1000	Santa Cruz, Heidelberg, Germany
α IGF- IRβ	mouse (M)	1:1000	Upstate, Millipore, Eschwege, Germany
α IR-β	rabbit (P)	1:1000	Santa Cruz, Heidelberg, Germany
α IR-β	rabbit (M)	1:1000	Cell Signaling, NEB, Frankfurt, Germany
α phospho Akt (Ser 473)	mouse (M)	1:1000	Cell Signaling, NEB, Frankfurt, Germany
α phospho GSK3α/β (Ser 21/9)	rabbit (P)	1:800	Cell Signaling, NEB, Frankfurt, Germany
α phospho Erk1/2	mouse (M)	1:1000	Cell Signaling, NEB, Frankfurt, Germany
α phospho p38	mouse (M)	1:1000	Cell Signaling, NEB, Frankfurt, Germany
α Akt-1/2	rabbit (P)	1:1000	Santa Cruz, Heidelberg, Germany

α GSK3α/β	mouse (M)	1:50,000	BioSource, Solingen, Germany
α Erk1/2	rabbit(P)	1:1000	Cell Signaling, NEB, Frankfurt, Germany
α p38	rabbit (P)	1:1000	Cell Signaling, NEB, Frankfurt, Germany
α phospho Tyr 4G10	mouse (M)	1:1000	Upstate, Millipore, Eschwege, Germany
IgG	rabbit (P)	1:1000	Upstate, Millipore, Eschwege, Germany
α β-actin	mouse (M)	1:200,000	Abcam, Cambridge, UK
α ERα	rabbit (P)	1:1000	Santa Cruz, Heidelberg, Germany
α ERα	mouse (M)	1:1000	Novocastra, Newcastle, UK
α phospho ERα (Ser 118)	mouse (M)	1:5000	Cell Signaling, NEB, Frankfurt, Germany

P denotes polyclonal and **M** denotes monoclonal in the above list.

Secondary antibodies

The secondary antibodies used were tagged with HRP.

Specificity	Species	Dilution	Company
α mouse	goat	1:20,000	Dianova, Hamburg, Germany
α rabbit	goat	1:1000	Dianova, Hamburg, Germany

6 METHODS

6.1 Cell culture

6.1.1 Maintenance of various cell lines

MCF7, MELN (a sub cell line of MCF7 cell line [Balaguer *et al.,* 2001]), MDA-MB231 and HCC1937 cells were all maintained in phenol red-free DMEM (4.5 g/liter glucose) containing 10% FBS and penicillin/streptomycin (100 U/ml / 100 µg/ml, respectively). BT474, T47D and ZR75-1 were maintained in RPMI-1640. Three days before the experiment, cells were grown in medium supplemented with 10% dextran-coated charcoal-treated FBS (DCC-FBS) (prepared as described by Migliaccio *et al.* (1993)). MCF10A cells were maintained in mammary epithelial growth medium (MEGM) supplemented with growth factor cocktail and antibiotics. Three days before the experiment, MCF10A cells were cultured in MEGM without insulin.

6.1.2 Cell harvesting

The growth medium was aspirated from the culture dish. Cells were washed with 3 ml of EDTA for 3-4 minutes. Thereafter, cells were incubated with 3-4 ml of Trypsin/EDTA solution for 5 minutes except MCF10A cells which detached only after 10 min of trypsinisation. Reaction was stopped by adding equal volume of M3 medium into the dish. The cells were collected in 15ml/50ml Falcon tubes and then spun at 1000 rpm for 5 minutes. The medium was aspirated and cell pellet was resuspended in growth medium. The cell number in the cell suspension was determined by counting with Neubauer hemacytometer. Cells were plated as per the requirement of the experiment.

6.1.3 Proliferation assays

a. Colorimetric method using crystal violet dye

1 x10^4 cells/well were plated in a 96-well plate in 10% DCC-FBS medium (M3). After 24 h, cells were starved for next 24 h in medium containing 2% DCC-FBS except MCF10A cells which were starved in MEGM without insulin. Thereafter, cells were stimulated with insulin or insulin analogues every 24 h for total of 72 h, MCF10A cells were stimulated for 48 h. Controls remained untreated. At the end of incubation time, cells were washed with 100µl of PBS, fixed for 5 min with 100µl of 3% paraformaldehyde in PBS and stained for 10 min with 100µl of 1% crystal violet dye dissolved in 10% ethanol. Excess crystal violet dye was

removed and plates were extensively washed with water to remove traces of unbound crystal violet dye. After air drying, the bound dye was dissolved in 100µl of 10% acetic acid. Optical density was read at 595 nm using a plate reader (Multiscan MX, Thermo, Dreieich, Germany).

b. Colorimetric method using XTT

T47D cells were plated and treated with insulin or insulin analogue as described above. After 68 h of treatment, tetrazolium salt XTT was added to the cells. Four hours after the XTT addition, the optical density of the plate was read at 450nm using a plate reader.

c. FACS analysis of BrdU incorporation

MCF7 cells were starved in medium containing 2% DCC-FBS for 24 h and then were stimulated with regular insulins and insulin analogues for 16 h. BrdU incorporation was allowed by incubating cells with 10µM BrdU for 1 h and the whole process was carried out as suggested in the Manual of the Becton Dickinson kit (FITC BrdU Flow Kit). Thereafter, cells were submitted to FACS analysis and BrdU incorporation was measured using FACS Calibur equipment from Becton Dickinson. BrdU incorporation of insulin treated cells was compared with BrdU incorporation in untreated cells. Fold of control was determined by normalizing data from treated cells over untreated cells.

6.1.4 Migration assays

a. Wound healing assay

MCF7 cells were plated in a 6-well plate containing M3 medium and allowed to grow till they reach confluency. Then, cells were treated for 1 h with 2µg/ml mitomycin C to inhibit cell proliferation. The monolayers were scratched using a 200 µl pipette tip, washed with PBS and incubated with different concentrations of Actrapid or Lantus (15nM, 150nM or 1.5µM). IGF-I (15nM) was used as a positive control. Wound was photographed first at the beginning of the experiment and then after 3 h, 6 h, 12 h and 21 h. Healing was determined at every timepoint by measuring distance between two wound margins. Migration was calculated by comparing the healing at the particular timepoint with the initial wound.

b. Transwell assay

MCF7 cells were harvested from the culture dish and washed with PBS twice to get rid of the serum traces. Cell pellet was resuspended in medium containing 2%DCC-FBS. 1 x 10^5 cells were seeded in the upper chamber of the transwell plate (diameter 6.4mm, pore size 8µm) which was inserted in the 24-well plate. Medium containing 2%DCC-FBS (control) or

2%DCC-FBS in combination with 15nM Actrapid, 15nM Lantus or 15nM IGF-I was added to the lower chamber. Compounds were added freshly every 24 h for 2 days. M4 medium or M2 medium in the lower chamber acted as negative and positive control, respectively, in this experiment. After 48 h, cells from the upper surface of the membrane were removed by cotton swab. Cells which had migrated to the lower surface of the membrane were washed with PBS twice, fixed with 3% PFA and stained with haematoxylin dye. Membrane was cut out and mounted with moviol on microscopic slides. Thereafter, cells were counted by light microscopy.

c. Invasion assay using matrigel

Basement Membrane Extract (BME) was aliquoted and stored at -80 ^{o}C. Before starting the experiment, BME was thawed at 4^{o}C and reconstituted with medium containing 2%DCC-FBS to prepare a working dilution of 0.8µg/µl. Care was taken not to introduce any bubbles during reconstitution. 66µl of this dilution (200 µl/cm^2) was added to the transwell insert and left for 30 minutes at 37^{o}C to gel. The gel was rehydrated by adding 100µl of medium containing 2%DCC-FBS to the upper chamber and 600µl of medium containing 2%DCC-FBS to the lower chamber for at least one hour. Thereafter, medium was removed from the upper chamber and 1 x 10^5 MCF7 cells, resuspended in medium containing 2%DCC-FBS, were seeded on it. The lower chamber contained either medium containing 2%DCC-FBS (control) or medium containing 2%DCC-FBS in combination with 15nM Actrapid, 15nM Lantus or 15nM IGF-I. Compounds were added freshly every 24 h for 3 days. M4 medium or M2 medium in the lower chamber acted as negative and positive control, respectively, in this experiment. The transwell plates were incubated for 72 h at 37^{o}C.

At the end of the experiment, cells were removed from the upper chamber by cotton swab. Invaded cells at the lower surface of the membrane were washed with PBS twice, fixed with 3% PFA and stained with haematoxylin dye. Membrane was cut out and mounted with moviol on microscopic slides. Thereafter, cells were observed and counted by light microscopy.

6.1.5 Silencing of IGF-IR or IR by transient transfection of MELN cells with siRNA

a. Effect on signalling pathways

3 x 10^5 MELN cells were plated in M3 in each well of a 6-well plate. After 24 h, cells were transfected with 25nM siRNA either targeting IGF-IR or IR using oligofectamine (10µl/well). For control, cells were transfected with 25nM non-targeting siRNA. After 48 h of

transfection, cells were starved in M4 for 24 h. At the end of the starvation period, cells were left unstimulated or stimulated with 15nM Actrapid, 15nM Lantus or 15nM IGF-I for 10 min. To stop the experiment, cells were washed with ice-cold PBS and cell lysate was made by standard lysis method.

b. Effect on cell proliferation

1 x 10^4 MELN cells were plated in M3 in each well of a 96-well plate. After 24 h, cells were transfected with 25nM siRNA targeting IGF-IR using oligofectamine (2µl/well). For control, cells were transfected with 25nM non-targeting siRNA. After 48 h of transfection, cells were starved in medium containing 2% DCC-FBS for 24 h. Cells were then treated for next 72 h with fresh medium containing 2% DCC-FBS (control) or medium containing 2% DCC-FBS supplemented with 150nM Actrapid or 150nM Lantus or 150nM IGF-I. At the end of the experiment, medium was aspirated, cells were washed, fixed and stained with crystal violet as described in section 6.1.3.a.

6.2 Biochemistry

6.2.1 Cell lysis

a. Standard lysis protocol

Medium was aspirated and cells were washed with PBS. 500µl – 750µl of ice-cold PBS was added to the cells and cells were scraped with a disposable cell scraper. Cell suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged at 4°C for 5 min at 1000 rpm. The supernatant was discarded and the cell pellet was suspended in ice-cold lysis buffer, incubated on ice for 1 h and centrifuged at 4°C for 10 min at 13,000 rpm. The supernatant represented the lysate and was used for protein estimation. For long term storage, the lysate was stored at - 80^{o}C.

b. Direct lysis protocol

In short-term experiments with identical cell numbers per well, lysis was performed directly in the plate. For this, medium was aspirated and cells were washed with ice-cold PBS. 100µl of 1 x SDS-loading dye was added to each well and plates were left for 15 min on ice with intermittent agitation. After 15 min, lysate was collected and boiled and processed as a normal SDS sample.

6.2.2 Western blotting

The protein concentration of cell lysate was determined by Bio-Rad DC Protein Assay kit, a colorimetric assay based on Lowry assay. The standard curve was generated by measuring absorbance values (750nm) of samples with known BSA concentrations (0.25 to 2 mg/ml). Each cell lysate was read in duplicates and protein concentration was determined by using the BSA standard curve. Protein samples were prepared by adding 5 x SDS buffer to the cell lysates and boiling at 95°C for 5 min. The samples were loaded and resolved on a 10% SDS-polyacrylamide gel. The separated proteins were then electro-blotted on an activated Immobilon-P membrane (Millipore, Eschwege, Germany) by the wet transfer method. Thereafter, the membrane was incubated for 1 h in blocking buffer at RT, washed three times with TBS-T and then incubated overnight at 4°C with specific primary antibody. Excess primary antibody on the membrane was removed by washing three times with TBS-T. The membrane was incubated with a peroxidase-labelled secondary antibody for 1 h at RT and then was washed three times with TBS-T. Immunoreactive protein bands were detected with ECL-plus system from Amersham (GE Healthcare Biotech, Freiburg, Germany). For studying the phosphorylation status of proteins of signalling pathways, the membrane was first probed for the phosphorylated form and then for the total protein. To probe the membrane a second time, the membrane was first stripped by incubating in stripping buffer for 20 min at 60°C and then the whole process from blocking step onward was repeated with another primary antibody.

6.2.3 Immunoprecipitation

50µl of protein-A agarose beads were processed by washing first twice in PBS and then twice with lysis buffer. 750µg of protein (cell lysate) together with 2µg of primary antibody was added to the washed beads (final volume was 750µl) and left overnight for incubation at 4°C on a rotating shaker. Next morning, tubes were centrifuged and supernatant was discarded. To remove proteins bound unspecifically, beads were washed thoroughly twice with lysis buffer, then two times with LiCl buffer and again twice with lysis buffer (to remove the excess salt). Thereafter, beads were suspended in 30µl of 2.5 x SDS buffer, boiled at 95°C for 5 min to separate the protein complex from beads. The beads were subjected to centrifugation at 10,000 rpm for 10 min and supernatant was loaded and resolved on 10% SDS-polyacrylamide gel.

6.2.4 Firefly luciferase reporter gene assay

MELN cells are MCF7 cells stably transfected with ERE controlled firefly luciferase reporter gene (Balaguer *et al.,* 2001). 3 x 10^5 MELN cells per well were plated in M3 in a 6-well plate. After 10 h treatment with compounds, cells were washed with PBS and were directly lysed in the plate by incubating with 150 µl/well of luciferase lysis reagent for 30 min on ice. Cell lysate was collected and centrifugation (10,000 × g, 10 min) was performed to separate cell debris from supernatant. 20 µl of the supernatant was mixed with 100µl of luciferase assay reagent (Promega, Mannheim, Germany) and luminescence was measured for 1 min at 560 nm in a luminometer. The luciferase activity (cpm/mg protein) was calculated by normalizing luminescence (expressed in counts per minute) with the protein concentration of the lysate.

6.3 Molecular biology

6.3.1 RNA extraction from cultured cells

3 x 10^5 MCF7 cells were seeded per well of a 6-well plate in M3. After 12 h cells were starved for 24 h in M4. Cells were stimulated with required compounds for 1 h, 3 h or 6 h. After treatment, cells were washed with PBS and incubated for 10 min with the lysis buffer from RNeasy kit (Qiagen, Hilden, Germany). For shearing genomic DNA, cell lysate was passed 5 times through a fine needle syringe (20 Gauge) and then transferred to Eppendorf tubes. From here on RNA purification was performed according to the instructions provided in the Manual of the RNeasy kit. RNA bound to the matrix of the column (provided in the kit) was eluted by adding 20 µl of nuclease-free water to the column. For maximum yield, elution was repeated and thus the total volume of RNA eluted was 40µl. RNA concentration was measured by determining absorbance at 260nm in a Nanodrop device (Peqlab, Erlangen, Germany). RNA or DNA is considered pure if absorbance ratio (260nm/280nm) is between 1.5 and 2.0. Therefore, only those RNA samples which had absorbance ratio between 1.5 and 2.0 were considered for further processing. For long term storage, RNA samples were kept at -80°C.

6.3.2 cDNA synthesis by reverse transcription

500ng of total RNA was reverse transcribed to generate cDNA. oligo-dT primers were used for producing cDNA and the whole process was performed as suggested in the Manual of 'RevertAid^{TM}H Minus First Strand cDNA Synthesis' kit (Fermentas, St. Leon-Rot, Germany).

cDNA, thus synthesised, was purified with the QIAquick PCR purification kit (Qiagen). After elution from the matrix, cDNA concentration was measured by determining absorbance at 260nm in Nanodrop device. Only cDNA samples which showed absorbance ratio (260nm/280nm) between 1.5 and 2.0 were considered for quantitative PCR. For long term storage, cDNA samples were kept at -20°C.

6.3.3 Quantitative polymerase chain reaction

Primers for quantitative PCR were designed using 'Universal Probe Library' website of 'Roche Diagnostics'. 50ng of cDNA was incubated with 500nM of SYBR Fwd primer and SYBR Rev primer. $MgCl_2$ was added to obtain a final concentration of 25mM Mg^{2+} in the reaction. Reaction was started by adding 10 µl of iQ SYBR Green supermix (Bio-Rad, München, Germany). PCR amplification was performed following the manufacturer's instructions on PTC-200 Peltier Thermal Cycler (MJ Research, Miami, FL, USA) and the MJ OpticonMonitor analysis software, version 3.1 (Bio Rad).

6.4 Statistical analysis

Immunoblots were quantitatively evaluated using ImageJ software (NIH, USA). Signal intensities of phospho-proteins were normalized to the corresponding protein signals. Data are presented as mean ± SD of at least three independent experiments. Significance of differences between groups was calculated by *t*-test. Proliferation assays (dose-response curves) were evaluated using a four-parametric log-logistic model (Ritz and Streibig, 2005) and t-tests in a multiple contrast testing approach (Hothorn *et al.,* 2008). P-values below 0.05 were considered statistically significant.

7 REFERENCES

7.1 Websites

ATCC website	http://www.lgcstandards-atcc.org/
Beta Cell Biology Consortium-	http://www.betacell.org/
Davidson College website	http://www.bio.davidson.edu/
European Union Public Health Information System	http://www.euphix.org
Labor Lademannbogen	http://www.labor-lademannbogen.de
Labor Limbach	http://www.labor-limbach.de
National Cancer Institute	http://www.cancer.gov/
University of California, Irvine website	http://www.bio.uci.edu
World Health Organization	http://www.who.int/en/

7.2 Research Articles

Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI & Westphal H 1996 Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet* **12** 106-109.

Adams TE, Epa VC, Garrett TP & Ward CW 2000 Structure and function of the type 1 insulin-like growth factor receptor. *Cell Mol Life Sci* **57** 1050-1093.

Ahn NG, Seger R & Krebs EG 1992 The mitogen-activated protein kinase activator. *Curr Opin Cell Biol* **4** 992-999.

Ali S & Coombes RC 2002 Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer* **2** 101-112.

Allen NE, Roddam AW, Allen DS, Fentiman IS, Dos Santos Silva I, Peto J, Holly JM & Key TJ 2005 A prospective study of serum insulin-like growth factor-I (IGF-I), IGF-II, IGF-binding protein-3 and breast cancer risk. *Br J Cancer* **92** 1283-1287.

Allred DC, Brown P & Medina D 2004 The origins of estrogen receptor alpha-positive and estrogen receptor alpha-negative human breast cancer. *Breast Cancer Res* **6** 240-245.

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq

JM & Hemmings BA 1997 Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272** 31515-31524.
Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, 3rd, Johnson RS & Kahn CR 1994 Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* **372** 186-190.

Bailyes EM, Bennett DL & Hutton JC 1991 Proprotein-processing endopeptidases of the insulin secretory granule. *Enzyme* **45** 301-313.

Balaguer P, Boussioux AM, Demirpence E & Nicolas JC 2001 Reporter cell lines are useful tools for monitoring biological activity of nuclear receptor ligands. *Luminescence* **16** 153-158.
Baserga R 2000 The contradictions of the insulin-like growth factor 1 receptor. *Oncogene* **19** 5574-5581.

Bates P, Fisher R, Ward A, Richardson L, Hill DJ & Graham CF 1995 Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II). *Br J Cancer* **72** 1189-1193.

Bates P, Fisher R, Ward A, Richardson L, Hill DJ & Graham CF 1995 Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II). *Br J Cancer* **72** 1189-1193.
Becker AB & Roth RA 1990 Insulin receptor structure and function in normal and pathological conditions. *Annu Rev Med* **41** 99-115.

Becker RH, Frick AD, Teichert L, Nosek L, Heinemann L, Heise T & Rave K 2008 Fluctuation and reproducibility of exposure and effect of insulin glargine in healthy subjects. *Diabetes Obes Metab*.

Belfiore A 2007 The role of insulin receptor isoforms and hybrid insulin/IGF-I receptors in human cancer. *Curr Pharm Des* **13** 671-686.

Belfiore A, Frittitta L, Costantino A, Frasca F, Pandini G, Sciacca L, Goldfine ID & Vigneri R 1996 Insulin receptors in breast cancer. *Ann N Y Acad Sci* **784** 173-188.

Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J & Tsichlis P 1998 Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* **17** 313-325.

Binoux M, Roghani M, Hossenlopp P, Hardouin S & Gourmelen M 1991 Molecular forms of human IGF binding proteins: physiological implications. *Acta Endocrinol (Copenh)* **124 Suppl 2** 41-47.

Bjornholm M, He AR, Attersand A, Lake S, Liu SC, Lienhard GE, Taylor S, Arner P & Zierath JR 2002 Absence of functional insulin receptor substrate-3 (IRS-3) gene in humans. *Diabetologia* **45** 1697-1702.

Bluher M, Kahn BB & Kahn CR 2003 Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* **299** 572-574.

Bluher M, Michael MD, Peroni OD, Ueki K, Carter N, Kahn BB & Kahn CR 2002 Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Dev Cell* **3** 25-38.

Bray F, McCarron P & Parkin DM 2004 The changing global patterns of female breast cancer incidence and mortality. *Breast Cancer Res* **6** 229-239.

Bredin CG, Liu Z, Hauzenberger D & Klominek J 1999 Growth-factor-dependent migration of human lung-cancer cells. *Int J Cancer* **82** 338-345.

Bruning PF, Bonfrer JM, van Noord PA, Hart AA, de Jong-Bakker M & Nooijen WJ 1992 Insulin resistance and breast-cancer risk. *Int J Cancer* **52** 511-516.

Bruning PF, Van Doorn J, Bonfrer JM, Van Noord PA, Korse CM, Linders TC & Hart AA 1995 Insulin-like growth-factor-binding protein 3 is decreased in early-stage operable pre-menopausal breast cancer. *Int J Cancer* **62** 266-270.

Byrd JC, Devi GR, de Souza AT, Jirtle RL & MacDonald RG 1999 Disruption of ligand binding to the insulin-like growth factor II/mannose 6-phosphate receptor by cancer-associated missense mutations. *J Biol Chem* **274** 24408-24416.

Calle EE & Kaaks R 2004 Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* **4** 579-591.

Chance RE, Kroeff EP, Hoffmann JA & Frank BH 1981 Chemical, physical, and biologic properties of biosynthetic human insulin. *Diabetes Care* **4** 147-154.

Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE & Saltiel AR 2001 Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature* **410** 944-948.

Chiang SH, Hwang J, Legendre M, Zhang M, Kimura A & Saltiel AR 2003 TCGAP, a multidomain Rho GTPase-activating protein involved in insulin-stimulated glucose transport. *EMBO J* **22** 2679-2691.

Clemons M & Goss P 2001 Estrogen and the risk of breast cancer. *N Engl J Med* **344** 276-285.
Couse JF & Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* **20** 358-417.

Collaborative Group on Hormonal Factors in Breast Cancer 1996 Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. *Lancet* **347** 1713-1727.

Da Silva Xavier G, Qian Q, Cullen PJ & Rutter GA 2004 Distinct roles for insulin and insulin-

like growth factor-1 receptors in pancreatic beta-cell glucose sensing revealed by RNA silencing. *Biochem J* **377** 149-158.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y & Greenberg ME 1997 Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91** 231-241.

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.

Del Giudice ME, Fantus IG, Ezzat S, McKeown-Eyssen G, Page D & Goodwin PJ 1998 Insulin and related factors in premenopausal breast cancer risk. *Breast Cancer Res Treat* **47** 111-120.

Diaz B, Serna J, De Pablo F & de la Rosa EJ 2000 In vivo regulation of cell death by embryonic (pro)insulin and the insulin receptor during early retinal neurogenesis. *Development* **127** 1641-1649.

Dideriksen LH, Jorgensen LN, Drejer K 1992 Carcinogenic effect on female rats after 12 months administration of the insulin B10ASP. Abstract. *Diabetes* **41** 143A.

Dodson G & Steiner D 1998 The role of assembly in insulin's biosynthesis. *Curr Opin Struct Biol* **8** 189-194.

Dombrowski F, Mathieu C & Evert M 2006 Hepatocellular neoplasms induced by low-number pancreatic islet transplants in autoimmune diabetic BB/Pfd rats. *Cancer Res* **66** 1833-1843.

Drake PG & Posner BI 1998 Insulin receptor-associated protein tyrosine phosphatase(s): role in insulin action. *Mol Cell Biochem* **182** 79-89.

Dufourny B, Alblas J, van Teeffelen HA, van Schaik FM, van der Burg B, Steenbergh PH & Sussenbach JS 1997 Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *J Biol Chem* **272** 31163-31171.

Dumitrescu RG & Cotarla I 2005 Understanding breast cancer risk -- where do we stand in 2005? *J Cell Mol Med* **9** 208-221.

Dunn MF 2005 Zinc-ligand interactions modulate assembly and stability of the insulin hexamer -- a review. *Biometals* **18** 295-303.

Dupont J & Le Roith D 2001 Insulin-like growth factor 1 and oestradiol promote cell proliferation of MCF-7 breast cancer cells: new insights into their synergistic effects. *Mol Pathol* **54** 149-154.

Dutta J, Fan Y, Gupta N, Fan G & Gelinas C 2006 Current insights into the regulation of programmed cell death by NF-kappaB. *Oncogene* **25** 6800-6816.

Eckardt K, May C, Koenen M & Eckel J 2007 IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts. *Diabetologia* **50** 2534-2543.

Fantin VR, Wang Q, Lienhard GE & Keller SR 2000 Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis. *Am J Physiol Endocrinol Metab* **278** E127-133.

Filardo EJ, Quinn JA & Sabo E 2008 Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. *Steroids* **73** 870-873.

Fowlkes JL, Suzuki K, Nagase H & Thrailkill KM 1994 Proteolysis of insulin-like growth factor binding protein-3 during rat pregnancy: a role for matrix metalloproteinases. *Endocrinology* **135** 2810-2813.

Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A & Vigneri R 1999 Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol Cell Biol* **19** 3278-3288.

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.

Frasca F, Pandini G, Vigneri R & Goldfine ID 2003 Insulin and hybrid insulin/IGF receptors are major regulators of breast cancer cells. *Breast Dis* **17** 73-89.

Froesch ER, Schmid C, Schwander J & Zapf J 1985 Actions of insulin-like growth factors. *Annu Rev Physiol* **47** 443-467.

Furlanetto RW, Harwell SE & Frick KK 1994 Insulin-like growth factor-I induces cyclin-D1 expression in MG63 human osteosarcoma cells in vitro. *Mol Endocrinol* **8** 510-517.

Gamayunova VB, Bobrov Yu F, Tsyrlina EV, Evtushenko TP & Berstein LM 1997 Comparative study of blood insulin levels in breast and endometrial cancer patients. *Neoplasma* **44** 123-126.

Garber AJ 2006 Premixed insulin analogues for the treatment of diabetes mellitus. *Drugs* **66** 31-49.

Gerich J, Becker RH, Zhu R & Bolli GB 2006 Fluctuation of serum basal insulin levels following single and multiple dosing of insulin glargine. *Diabetes Technol Ther* **8** 237-243.

Giani C, Cullen KJ, Campani D & Rasmussen A 1996 IGF-II mRNA and protein are expressed in the stroma of invasive breast cancers: an in situ hybridization and immunohistochemistry study. *Breast Cancer Res Treat* **41** 43-50.

Giani C, Cullen KJ, Campani D & Rasmussen A 1996 IGF-II mRNA and protein are expressed in the stroma of invasive breast cancers: an in situ hybridization and immunohistochemistry study. *Breast Cancer Res Treat* **41** 43-50.

Giani C, Pinchera A, Rasmussen A, Fierabracci P, Bonacci R, Campini D, Bevilacqua G, Trock B, Lippman ME & Cullen KJ 1998 Stromal IGF-II messenger RNA in breast cancer: relationship with progesterone receptor expressed by malignant epithelial cells. *J Endocrinol Invest* **21** 160-165.

Giani C, Pinchera A, Rasmussen A, Fierabracci P, Bonacci R, Campini D, Bevilacqua G, Trock B, Lippman ME & Cullen KJ 1998 Stromal IGF-II messenger RNA in breast cancer: relationship with progesterone receptor expressed by malignant epithelial cells. *J Endocrinol Invest* **21** 160-165.

Giovannucci E 2007 Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. *Am J Clin Nutr* **86** s836-842.

Greger JG, Fursov N, Cooch N, McLarney S, Freedman LP, Edwards DP & Cheskis BJ 2007 Phosphorylation of MNAR promotes estrogen activation of phosphatidylinositol 3-kinase. *Mol Cell Biol* **27** 1904-1913.

Grisouard J, Medunjanin S, Hermani A, Shukla A & Mayer D 2007 Glycogen synthase kinase-3 protects estrogen receptor alpha from proteasomal degradation and is required for full transcriptional activity of the receptor. *Mol Endocrinol* **21** 2427-2439.

Gucev ZS, Oh Y, Kelley KM & Rosenfeld RG 1996 Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta2-induced growth inhibition in human breast cancer cells. *Cancer Res* **56** 1545-1550.

Guiochon-Mantel A, Delabre K, Lescop P & Milgrom E 1996 The Ernst Schering Poster Award. Intracellular traffic of steroid hormone receptors. *J Steroid Biochem Mol Biol* **56** 3-9.

Haber EP, Ximenes HM, Procopio J, Carvalho CR, Curi R & Carpinelli AR 2003 Pleiotropic effects of fatty acids on pancreatic beta-cells. *J Cell Physiol* **194** 1-12.

Hancock JF & Parton RG 2005 Ras plasma membrane signalling platforms. *Biochem J* **389** 1-11.
Hankins GR, De Souza AT, Bentley RC, Patel MR, Marks JR, Iglehart JD & Jirtle RL 1996 M6P/IGF2 receptor: a candidate breast tumor suppressor gene. *Oncogene* **12** 2003-2009.

Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, Rosner B, Speizer FE & Pollak M 1998 Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* **351** 1393-1396.

Hansen BF, Danielsen GM, Drejer K, Sorensen AR, Wiberg FC, Klein HH & Lundemose AG 1996 Sustained signalling from the insulin receptor after stimulation with insulin analogues

exhibiting increased mitogenic potency. *Biochem J* **315 (Pt 1)** 271-279.

Hebert E 2006 Mannose-6-phosphate/insulin-like growth factor II receptor expression and tumor development. *Biosci Rep* **26** 7-17.

Heise T, Bott S, Rave K, Dressler A, Rosskamp R & Heinemann L 2002 No evidence for accumulation of insulin glargine (LANTUS): a multiple injection study in patients with Type 1 diabetes. *Diabet Med* **19** 490-495.

Heise T, Nosek L, Ronn BB, Endahl L, Heinemann L, Kapitza C & Draeger E 2004 Lower within-subject variability of insulin detemir in comparison to NPH insulin and insulin glargine in people with type 1 diabetes. *Diabetes* **53** 1614-1620.

Heise T & Pieber TR 2007 Towards peakless, reproducible and long-acting insulins. An assessment of the basal analogues based on isoglycaemic clamp studies. *Diabetes Obes Metab* **9** 648-659.

Hellman B 1975 Modifying actions of calcium ionophores on insulin release. *Biochim Biophys Acta* **399** 157-169.

Henquin JC 2000 Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49** 1751-1760.

Hernandez-Sanchez C, Bartulos O & de Pablo F 2005 Proinsulin: much more than a hormone precursor in development. *Rev Endocr Metab Disord* **6** 211-216.

Heuson JC & Legros N 1972 Influence of insulin deprivation on growth of the 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction. *Cancer Res* **32** 226-232.

Hothorn T, Bretz F & Westfall P 2008 Simultaneous inference in general parametric models. *Biom J* **50** 346-363.

James DE & Piper RC 1994 Insulin resistance, diabetes, and the insulin-regulated trafficking of GLUT-4. *J Cell Biol* **126** 1123-1126.

Jhun BH, Meinkoth JL, Leitner JW, Draznin B & Olefsky JM 1994 Insulin and insulin-like growth factor-I signal transduction requires p21ras. *J Biol Chem* **269** 5699-5704.

Jimenez-Feltstrom J, Lundquist I, Obermuller S & Salehi A 2004 Insulin feedback actions: complex effects involving isoforms of islet nitric oxide synthase. *Regul Pept* **122** 109-118.

Joshi RL, Lamothe B, Cordonnier N, Mesbah K, Monthioux E, Jami J & Bucchini D 1996 Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. *EMBO J* **15** 1542-1547.

Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R & Grohe C 2000 Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. *J Biol Chem* **275** 18447-18453.

Karasik A, Rothenberg PL, Yamada K, White MF & Kahn CR 1990 Increased protein kinase C activity is linked to reduced insulin receptor autophosphorylation in liver of starved rats. *J Biol Chem* **265** 10226-10231.

Kato S, Masuhiro Y, Watanabe M, Kobayashi Y, Takeyama KI, Endoh H & Yanagisawa J 2000 Molecular mechanism of a cross-talk between oestrogen and growth factor signalling pathways. *Genes Cells* **5** 593-601.

Key TJ 2001 Glycemic index, hyperinsulinemia, and breast cancer risk. *Ann Oncol* **12** 1507-1509.

Khan MN, Baquiran G, Brule C, Burgess J, Foster B, Bergeron JJ & Posner BI 1989 Internalization and activation of the rat liver insulin receptor kinase in vivo. *J Biol Chem* **264** 12931-12940.

Kidd PM 2008 Alzheimer's disease, amnestic mild cognitive impairment, and age-associated memory impairment: current understanding and progress toward integrative prevention. *Altern Med Rev* **13** 85-115.

Klinge CM 2000 Estrogen receptor interaction with co-activators and co-repressors. *Steroids* **65** 227-251.

Klip A & Paquet MR 1990 Glucose transport and glucose transporters in muscle and their metabolic regulation. *Diabetes Care* **13** 228-243.

Kuliawat R, Prabakaran D & Arvan P 2000 Proinsulin endoproteolysis confers enhanced targeting of processed insulin to the regulated secretory pathway. *Mol Biol Cell* **11** 1959-1972.

Kupriyanova TA & Kandror KV 1999 Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin. *J Biol Chem* **274** 1458-1464.

Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C & Trub T 2000 Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* **49** 999-1005.

Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR & Avruch J 1992 Raf-1 activates MAP kinase-kinase. *Nature* **358** 417-421.

Lannigan DA 2003 Estrogen receptor phosphorylation. *Steroids* **68** 1-9.

Larsson O, Girnita A & Girnita L 2005 Role of insulin-like growth factor 1 receptor signalling in cancer. *Br J Cancer* **92** 2097-2101.

Lee J, O'Hare T, Pilch PF & Shoelson SE 1993 Insulin receptor autophosphorylation occurs asymmetrically. *J Biol Chem* **268** 4092-4098.

Lee JS, Weiss J, Martin JL & Scott CD 2003 Increased expression of the mannose 6-phosphate/insulin-like growth factor-II receptor in breast cancer cells alters tumorigenic properties in vitro and in vivo. *Int J Cancer* **107** 564-570.

Leibiger IB & Berggren PO 2008 Insulin signaling in the pancreatic beta-cell. *Annu Rev Nutr* **28** 233-251.

Leventhal PS & Feldman EL 1997 Insulin-like Growth Factors as Regulators of Cell Motility Signaling Mechanisms. *Trends Endocrinol Metab* **8** 1-6.

Levin ER & Pietras RJ 2008 Estrogen receptors outside the nucleus in breast cancer. *Breast Cancer Res Treat* **108** 351-361.

Linos E & Willett WC 2007 Diet and breast cancer risk reduction. *J Natl Compr Canc Netw* **5** 711-718.

Liu SC, Wang Q, Lienhard GE & Keller SR 1999 Insulin receptor substrate 3 is not essential for growth or glucose homeostasis. *J Biol Chem* **274** 18093-18099.

Longo N, Shuster RC, Griffin LD, Langley SD & Elsas LJ 1992 Activation of insulin receptor signaling by a single amino acid substitution in the transmembrane domain. *J Biol Chem* **267** 12416-12419.

Lowy DR & Willumsen BM 1993 Function and regulation of ras. *Annu Rev Biochem* **62** 851-891.

Lu K & Campisi J 1992 Ras proteins are essential and selective for the action of insulin-like growth factor 1 late in the G1 phase of the cell cycle in BALB/c murine fibroblasts. *Proc Natl Acad Sci U S A* **89** 3889-3893.

Lupulescu AP 1985 Effect of prolonged insulin treatment on carcinoma formation in mice. *Cancer Res* **45** 3288-3295.

Maor S, Mayer D, Yarden RI, Lee AV, Sarfstein R, Werner H & Papa MZ 2006 Estrogen receptor regulates insulin-like growth factor-I receptor gene expression in breast tumor cells: involvement of transcription factor Sp1. *J Endocrinol* **191** 605-612.

Marshall S 1985 Dual pathways for the intracellular processing of insulin. Relationship between retroendocytosis of intact hormone and the recycling of insulin receptors. *J Biol Chem* **260** 13524-13531.

Martensson UE, Salehi SA, Windahl S, Gomez MF, Sward K, Daszkiewicz-Nilsson J, Wendt A, Andersson N, Hellstrand P, Grande PO, et al. 2008 Deletion of the G protein-coupled Receptor

GPR30 Impairs Glucose Tolerance, Reduces Bone Growth, Increases Blood Pressure, and Eliminates Estradiol-stimulated Insulin Release in Female Mice. *Endocrinology*.

Mathieu MC, Clark GM, Allred DC, Goldfine ID & Vigneri R 1997 Insulin receptor expression and clinical outcome in node-negative breast cancer. *Proc Assoc Am Physicians* **109** 565-571.

Mauro L, Salerno M, Panno ML, Bellizzi D, Sisci D, Miglietta A, Surmacz E & Ando S 2001 Estradiol increases IRS-1 gene expression and insulin signaling in breast cancer cells. *Biochem Biophys Res Commun* **288** 685-689.

McClain DA 1991 Different ligand affinities of the two human insulin receptor splice variants are reflected in parallel changes in sensitivity for insulin action. *Mol Endocrinol* **5** 734-739.

McClenaghan NH, Yoon TW, Barnett CR, Wilson AM, Abdel-Wahab YH, Swanston-Flatt S, Ah-Singh E & Flatt PR 1994 Effect of glucose and amino acids on insulin-secretion from a novel pancreatic B-cell line produced by electrofusion. *Biochem Soc Trans* **22** 237S.

McPherson K, Steel CM & Dixon JM 2000 ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ* **321** 624-628.

Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Barone MV, Ametrano D, Zannini MS, Abbondanza C, et al. 2000 Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *EMBO J* **19** 5406-5417.

Migliaccio A, Pagano M & Auricchio F 1993 Immediate and transient stimulation of protein tyrosine phosphorylation by estradiol in MCF-7 cells. *Oncogene* **8** 2183-2191.

Milazzo G, Giorgino F, Damante G, Sung C, Stampfer MR, Vigneri R, Goldfine ID & Belfiore A 1992 Insulin receptor expression and function in human breast cancer cell lines. *Cancer Res* **52** 3924-3930.

Milazzo G, Sciacca L, Papa V, Goldfine ID & Vigneri R 1997 ASPB10 insulin induction of increased mitogenic responses and phenotypic changes in human breast epithelial cells: evidence for enhanced interactions with the insulin-like growth factor-I receptor. *Mol Carcinog* **18** 19-25.

Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A & McClain DA 1990 Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO J* **9** 2409-2413.

Mueckler M 1994 Facilitative glucose transporters. *Eur J Biochem* **219** 713-725.

Nandi S, Guzman RC & Yang J 1995 Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proc Natl Acad Sci U S A* **92** 3650-3657.

Nave BT, Ouwens M, Withers DJ, Alessi DR & Shepherd PR 1999 Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for

opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* **344 Pt 2** 427-431.

Oesterreich S & Fuqua SA 1999 Tumor suppressor genes in breast cancer. *Endocr Relat Cancer* **6** 405-419.

Oesterreich S, Zhang P, Guler RL, Sun X, Curran EM, Welshons WV, Osborne CK & Lee AV 2001 Re-expression of estrogen receptor alpha in estrogen receptor alpha-negative MCF-7 cells restores both estrogen and insulin-like growth factor-mediated signaling and growth. *Cancer Res* **61** 5771-5777.
Ogawa W, Matozaki T & Kasuga M 1998 Role of binding proteins to IRS-1 in insulin signalling. *Mol Cell Biochem* **182** 13-22.

Pang DT & Shafer JA 1984 Evidence that insulin receptor from human placenta has a high affinity for only one molecule of insulin. *J Biol Chem* **259** 8589-8596.

Papa V & Belfiore A 1996 Insulin receptors in breast cancer: biological and clinical role. *J Endocrinol Invest* **19** 324-333.

Papa V, Milazzo G, Goldfine ID, Waldman FM & Vigneri R 1997 Sporadic amplification of the insulin receptor gene in human breast cancer. *J Endocrinol Invest* **20** 531-536.

Papa V, Pezzino V, Costantino A, Belfiore A, Giuffrida D, Frittitta L, Vannelli GB, Brand R, Goldfine ID & Vigneri R 1990 Elevated insulin receptor content in human breast cancer. *J Clin Invest* **86** 1503-1510.

Perez-Villamil B, de la Rosa EJ, Morales AV & de Pablo F 1994 Developmentally regulated expression of the preproinsulin gene in the chicken embryo during gastrulation and neurulation. *Endocrinology* **135** 2342-2350.

Peyrat JP & Bonneterre J 1992 Type 1 IGF receptor in human breast diseases. *Breast Cancer Res Treat* **22** 59-67.

Pezzino V, Papa V, Milazzo G, Gliozzo B, Russo P & Scalia PL 1996 Insulin-like growth factor-I (IGF-I) receptors in breast cancer. *Ann N Y Acad Sci* **784** 189-201.

Pollak MN 1998 Endocrine effects of IGF-I on normal and transformed breast epithelial cells: potential relevance to strategies for breast cancer treatment and prevention. *Breast Cancer Res Treat* **47** 209-217.

Pons S, Asano T, Glasheen E, Miralpeix M, Zhang Y, Fisher TL, Myers MG, Jr., Sun XJ & White MF 1995 The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol* **15** 4453-4465.

Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA & Hathaway HJ 2008 Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* **70**

165-190.

Ramakrishnan R, Khan SA & Badve S 2002 Morphological changes in breast tissue with menstrual cycle. *Mod Pathol* **15** 1348-1356.

Resnik JL, Reichart DB, Huey K, Webster NJ & Seely BL 1998 Elevated insulin-like growth factor I receptor autophosphorylation and kinase activity in human breast cancer. *Cancer Res* **58** 1159-1164.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB & Prossnitz ER 2005 A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307** 1625-1630.

Rinderknecht E & Humbel RE 1978 The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem* **253** 2769-2776.

Ritz C & Streibig JC 2005 Bioassay analysis using R. *J Stat Soft* **12** 1-22.

Rolla A 2008 Pharmacokinetic and pharmacodynamic advantages of insulin analogues and premixed insulin analogues over human insulins: impact on efficacy and safety. *Am J Med* **121** S9-S19.

Sachdev D & Yee D 2001 The IGF system and breast cancer. *Endocr Relat Cancer* **8** 197-209.
Saltiel AR & Kahn CR 2001 Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414** 799-806.

Saltiel AR & Pessin JE 2002 Insulin signaling pathways in time and space. *Trends Cell Biol* **12** 65-71.

Sasaki H, Jones PA, Chaillet JR, Ferguson-Smith AC, Barton SC, Reik W & Surani MA 1992 Parental imprinting: potentially active chromatin of the repressed maternal allele of the mouse insulin-like growth factor II (Igf2) gene. *Genes Dev* **6** 1843-1856.

Schernhammer ES, Holly JM, Pollak MN & Hankinson SE 2005 Circulating levels of insulin-like growth factors, their binding proteins, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **14** 699-704.

Schmid C, Rutishauser J, Schlapfer I, Froesch ER & Zapf J 1991 Intact but not truncated insulin-like growth factor binding protein-3 (IGFBP-3) blocks IGF I-induced stimulation of osteoblasts: control of IGF signalling to bone cells by IGFBP-3-specific proteolysis? *Biochem Biophys Res Commun* **179** 579-585.

Schnarr B, Strunz K, Ohsam J, Benner A, Wacker J & Mayer D 2000 Down-regulation of insulin-like growth factor-I receptor and insulin receptor substrate-1 expression in advanced human breast cancer. *Int J Cancer* **89** 506-513.

Sesti G, Federici M, Hribal ML, Lauro D, Sbraccia P & Lauro R 2001 Defects of the insulin

receptor substrate (IRS) system in human metabolic disorders. *FASEB J* **15** 2099-2111.
Silverman DT 2001 Risk factors for pancreatic cancer: a case-control study based on direct interviews. *Teratog Carcinog Mutagen* **21** 7-25.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW & Liao JK 2000 Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* **407** 538-541.

Simpson ER 2003 Sources of estrogen and their importance. *J Steroid Biochem Mol Biol* **86** 225-230.

Singer CF, Mogg M, Koestler W, Pacher M, Marton E, Kubista E & Schreiber M 2004 Insulin-like growth factor (IGF)-I and IGF-II serum concentrations in patients with benign and malignant breast lesions: free IGF-II is correlated with breast cancer size. *Clin Cancer Res* **10** 4003-4009.

Sjogren K, Liu JL, Blad K, Skrtic S, Vidal O, Wallenius V, LeRoith D, Tornell J, Isaksson OG, Jansson JO, et al. 1999 Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *Proc Natl Acad Sci U S A* **96** 7088-7092.

Slieker LJ, Brooke GS, DiMarchi RD, Flora DB, Green LK, Hoffmann JA, Long HB, Fan L, Shields JE, Sundell KL, et al. 1997 Modifications in the B10 and B26-30 regions of the B chain of human insulin alter affinity for the human IGF-I receptor more than for the insulin receptor. *Diabetologia* **40 Suppl 2** S54-61.

Smith CL 1998 Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol Reprod* **58** 627-632.

Sommer S & Fuqua SA 2001 Estrogen receptor and breast cancer. *Semin Cancer Biol* **11** 339-352.

Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R & Santen RJ 2004 The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. *Proc Natl Acad Sci U S A* **101** 2076-2081.

Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R & Santen RJ 2002 Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. *Mol Endocrinol* **16** 116-127.

Spiegelman BM & Flier JS 1996 Adipogenesis and obesity: rounding out the big picture. *Cell* **87** 377-389.

Stacey DW 2003 Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. *Curr Opin Cell Biol* **15** 158-163.

Stammberger I, Bube A, Durchfeld-Meyer B, Donaubauer H & Troschau G 2002 Evaluation of the carcinogenic potential of insulin glargine (LANTUS) in rats and mice. *Int J Toxicol* **21** 171-179.

Sternlicht MD 2006 Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. *Breast Cancer Res* **8** 201.

Sun M, Paciga JE, Feldman RI, Yuan Z, Coppola D, Lu YY, Shelley SA, Nicosia SV & Cheng JQ 2001 Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. *Cancer Res* **61** 5985-5991.

Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, et al. 1994 Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* **372** 182-186.

Taniguchi CM, Emanuelli B & Kahn CR 2006 Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* **7** 85-96.

Tanti JF, Gremeaux T, Rochet N, Van Obberghen E & Le Marchand-Brustel Y 1987 Effect of cyclic AMP-dependent protein kinase on insulin receptor tyrosine kinase activity. *Biochem J* **245** 19-26.

Taylor R 1991 Insulin action 1991. *Clin Endocrinol (Oxf)* **34** 159-171.

Taylor SD & Hill B 2004 Recent advances in protein tyrosine phosphatase 1B inhibitors. *Expert Opin Investig Drugs* **13** 199-214.

Tran H, Brunet A, Griffith EC & Greenberg ME 2003 The many forks in FOXO's road. *Sci STKE* **2003** RE5.

Turner BC, Haffty BG, Narayanan L, Yuan J, Havre PA, Gumbs AA, Kaplan L, Burgaud JL, Carter D, Baserga R, et al. 1997 Insulin-like growth factor-I receptor overexpression mediates cellular radioresistance and local breast cancer recurrence after lumpectomy and radiation. *Cancer Res* **57** 3079-3083.

Venkateswaran V, Haddad AQ, Fleshner NE, Fan R, Sugar LM, Nam R, Klotz LH & Pollak M 2007 Association of diet-induced hyperinsulinemia with accelerated growth of prostate cancer (LNCaP) xenografts. *J Natl Cancer Inst* **99** 1793-1800.

Watson CS, Alyea RA, Jeng YJ & Kochukov MY 2007 Nongenomic actions of low concentration estrogens and xenoestrogens on multiple tissues. *Mol Cell Endocrinol* **274** 1-7.
White MF & Kahn CR 1994 The insulin signaling system. *J Biol Chem* **269** 1-4.

White MF, Shoelson SE, Keutmann H & Kahn CR 1988 A cascade of tyrosine autophosphorylation in the beta-subunit activates the phosphotransferase of the insulin receptor.

J Biol Chem **263** 2969-2980.

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.

Yam D, Fink A, Mashiah A & Ben-Hur E 1996 Hyperinsulinemia in colon, stomach and breast cancer patients. *Cancer Lett* **104** 129-132.

Yee D 1994 The insulin-like growth factor system as a target in breast cancer. *Breast Cancer Res Treat* **32** 85-95.

Yee D, Paik S, Lebovic GS, Marcus RR, Favoni RE, Cullen KJ, Lippman ME & Rosen N 1989 Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Mol Endocrinol* **3** 509-517.

Yee D, Paik S, Lebovic GS, Marcus RR, Favoni RE, Cullen KJ, Lippman ME & Rosen N 1989 Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. *Mol Endocrinol* **3** 509-517.

Zhang W & Liu HT 2002 MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* **12** 9-18.

Zhao WQ & Alkon DL 2001 Role of insulin and insulin receptor in learning and memory. *Mol Cell Endocrinol* **177** 125-134.

ABBREVIATIONS

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
AF1	Transcriptional Activation Function1
AF2	Transcriptional Activation Function2
APS	adaptor protein containing PH and SH2 domains
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumine
cAMP	cyclic AMP
Cdk	Cyclindependent kinase
Cdk2	Cyclin dependent kinase 2
cDNA	complementary or copy DNA
CNS	Central Nervous System
CO_2	Carbon Dioxide
COX2	Cyclooxygenase2
cpm	counts per minute
CREB	cAMP Response Element Binding protein
cSrc	Cellular homologue of vSrc
DBD	DNABinding Domain
DCC	DextranCoated Charcoal
DCIS	Ductal Carcinoma In Situ
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dsDNA	doublestranded DNA
dNTPs	deoxynucleotides Triphosphate
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylen diaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eIF2B	eukaryotic Initiation Factor 2B
ER	Estrogen Receptor
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
ERE	Estrogen Response Element
ERK	Extracellular signalRelated Kinase
ER	ERα negative
E2	17βEstradiol
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FGFR	Fibroblast Growth Factor Receptor
Fos	nuclear DNA binding protein (product of the cFos protooncogene)
Fwd primer	Forward primer
GAPDH	Glyceraldehyde3Phosphate Dehydrogenase
Gab1	Grb2-associated binder-1
GDP	Guanosine diphosphate
GF	Growth Factor
GFP	Green Fluorescence Protein
GLUT	glucose transporter
GMCC	Protein kinases group including GSK, MAPK, Cdk and CTD kinases.
GMP	Guanosine Monophosphate
Grb2	Growth factor receptor bound protein 2
GSK3α/β	Glycogen Synthase Kinase3alpha/beta
h	hour
H & E	Hematoxylin and Eosin
HCl	Hydrochloric acid

HEPES	4(2Hydroxyethyl)1Piperazineethanesulfonic acid
HEPES/KOH	HEPES/Potassium hydroxide
HER2	Human epidermal growth factor receptor 2 or ErbB2
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
IDC	Invasive Ductal Carcinoma
IGF-I	Insulin Like Growth Factor-I
IGF-II	Insulin Like Growth Factor-II
IGF-IR	Insulin Growth Factor –I Receptor
IGFBP	Insulin Growth Factor Binding Protein
IgG	Immunoglobulin G
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
IL6	Interleukin6
IR	Insulin Receptor
IR-A	Insulin Receptor isoform lacking exon 11
IR-B	Insulin Receptor isoform with full transcript
IRS	Insulin Receptor Substrate
JNK	cJun Nterminal kinase
Jun	nuclear DNA binding protein (product of the cJun protooncogene)
KCl	Potassium Chloride
kDa	kilo Dalton
l	litre
LAR	Luciferase Assay Reagent
LBD	Ligand Binding Domain
LCIS	Lobular Carcinoma In Situ
m	milli
M	Molar
MAPKAPK1	MAPKActivated Protein Kinase 1 or p90Rsk
MAPK	MitogenActivated Protein Kinase
MEK	MAPK/ERK kinase
µ	micro
min	minutes
M6PR	Mannose 6-Phosphate Receptor or IGF-II receptor
MMTV	Mouse Mammary Tumor Virus
MNAR	Modulator of Nongenomic Action of estrogen Receptor
mRNA	messenger RNA
mTOR	mammalian Target Of Rapamycin
Myc	protein inducing cell growth and encoded by the cMyc protooncogene
MW	molecular weight
NaAc	Sodium Acetate
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
NaF	Sodium Fluoride
Na_2HPO_4	Disodiumhydrogenphosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
Na_3VO_4	Sodium orthovanadate
NCAM	Neural CellAdhesion Protein
NCI	National Cancer Institute (USA)
NFAT	Nuclear Factor of Activating T cells
NFκB	Nuclear Factor kappa B
NGF	Nerve Growth Factor
NIH	National Institutes of Health (USA)
NLS	Nuclear Localization Signal
NPH	Neutral Protamine Hagedorn
nm	nanometres
O.D.	Optical Density
O/N	Overnight
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PDK1/2	3PhosphoinositideDependent Protein Kinase1/2
PFA	Paraformaldehyde
PH domain	Pleckstrin-homology domain
PKA	Protein Kinase A
PKB	Protein Kinase B or Akt
PKC	Protein Kinase C
PI3K	Phosphatidylinositol 3Kinase
PIK3CA	Gene encoding the PI3K Catalytic subunit (p110 Alpha)
PTB domain	Phosphotyrosine-binding domain
PTK	Protein Tyrosine Kinase
PVDF	Polyvinylidenfluoride
p38 MAPK	Mitogen Activated Protein Kinase p38
Rev primer	Reverse Primer
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute cell culture medium
Rsk	Ribosomal S6 Kinase or MAPKAPK1
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
RTPCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
sec	seconds
SEM	Standard Error of the Mean
Ser (S)	Serine, amino acid
Shc	Src-homology-2-containing protein
SH2 domain	Src-homology-2 domain
siRNA	small interfering RNA
SNPs	SingleNucleotide Polymorphisms
SYBR	SYBR green (DNA intercalating dye)
TAE	Tris Acetate EDTA buffer
TBS	Trisbuffered saline
TBS-T	TBS-Tween 20
TE	TrisEDTA buffer
TEMED	N, N, N', N'Tetramethyl ethylenediamine
Tm	melting Temperature
Tris	trishydroxymethylaminomethane
U	Unit of the enzyme activity
UV	Ultraviolet
vSrc	Oncoprotein encoded by Rous sarcoma virus
v/v	volume per volume
w/v	weight per volume

ERKLÄRUNG

Hiermit erkläre ich, Ashish Shukla, daß ich die vorliegende Dissertation selbst angefertigt habe und keine anderen als die aufgeführten Hilfsmittel und angegebenen Quellen benutzt habe. Ich habe die vorliegende Arbeit an keiner anderen in- oder ausländischen Fakultät eingereicht.

Heidelberg, 27. November 2008

Ashish Shukla

ACKNOWLEDGEMENTS

I thank Prof. Dr. Doris Mayer for this interesting project. Her guidance and knowledge has allowed me to enhance my scientific expertise and awareness. Due to her observational skills, I have become more careful scientist.

I also thank my other PhD supervisors, namely Prof. Dr. Lutz Gissmann and Dr. Stephan Herzig for their interesting discussion regarding my project and its aims. Moreover, I thank Prof. Dr. Bernhard Dobberstein and Prof. Dr. Bernd Bukau to take part in my examination committee.

I thank Dr. Milen Kirilov for his help in designing primers for quantitative PCR. Along with him, I thank Dr. Jan Rodriguez for valuable help in analysing quantitative PCR data. I thank Nitin Patil for guiding me whenever I was stuck in carrying out molecular biology.

I thank Alexander Hermani and Jean Grisouard for their help in troubleshooting, analysis of data and excellent scientific discussions. Regarding scientific discussions, I will also like to thank Anke Petzold, Katrin Hahn and Nibedita Gupta.

I thank Raphael Bleiler and Katharina Becke for their valuable technical assistance. In this regard, special thanks to Gabriele Rincke. I thank Anke Petzold for her help with microscope. I thank Nibedita Gupta for her help with luciferase assay and immunoprecipitation.

I thank all my lab colleagues, Biswajit Mukerjee, Bogdan Munteanu, Elif Cholak, Milan, Zlatko Radev for the pleasant working atmosphere. Special thanks to Gabriele Rincke for always correcting my Indian-German.

I thank Kabeer, Rashmi, Nibedita and Shazia for helping me with thesis writing.

PhD in DKFZ has been a nice opportunity to meet students from other fields. In this regard, I will like to thank Kiran Maß for helpful discussions and her valuable advices.

During the PhD tenure, the Indian Students Association (ISA) of Heidelberg has often been my refuge, my platform to avoid nostalgic feelings and through it I had opportunity to meet

highly talented students. I will like to specially acknowledge Alok and Vijayan as the skits directed by them allowed me to realize my own creative self. I thank all my skit-colleagues (Alok, Arun, Deepitha, Kabeer, Kannabiran, Jonnah, Lucas, Meeta, Ram, Sadanand Sarvanan, Sashidhar, Shipa,and Vijayan) for the funny and artistic break from the monotony of daily life.

My special thanks to Vanitha and Kabeer, two *Homo sapiens* who buffeted me from most of the emotional disturbances. I will also like to thank Nitin, Pooja and Hritvik, who have been a family to me in Heidelberg. I thank my roommates – Bhaskar, Prasad and Lakshmi (Mr. President) – for the great food and equally great discussions. I extend special thanks to Lakshmi for taking care of me whenever I was ill.

Last but not least, I will like to thank other ISA members (Irfan, Kalai, Mohini, Nitin Patil, Prachiti, Ranjeet, Obul, Sowjanya, Suresh, Sharada) for making my stay lively and *peaceful*.

PEER-REVIEWED PUBLICATIONS

Shukla A, Grisouard J, Ehemann V, Hermani A, Enzmann H & Mayer D Analysis of signaling pathways related to cell proliferation stimulated by insulin analogs in human mammary epithelial cell lines. *Endocr Relat Cancer* (*Manuscript under revision*).

Hermani A, **Shukla A**, Rincke G, Medunjanin S, Werner H & Mayer D Insulin-like growth factor binding protein-4 and -5 modulate ligand dependent estrogen receptor-α activation in breast cancer cells in an IGF independent manner. *Endocrinology* (*Manuscript under revision*).

Mayer D, **Shukla A** & Enzmann H 2008 Proliferative effects of insulin analogues on mammary epithelial cells. *Arch Physiol Biochem* **114** 38-44.

Grisouard J, Medunjanin S, Hermani A, **Shukla A** & Mayer D 2007 Glycogen synthase kinase-3 protects estrogen receptor alpha from proteasomal degradation and is required for full transcriptional activity of the receptor. *Mol Endocrinol* **21** 2427-2439.

POSTERS PRESENTED

Shukla A, Bleiler R, Becker K, Enzmann H & Mayer D 2008 Mitogenic potency of insulin analogues. *33rd FEBS-IUBMB Meeting*, Athens, Greece.

Grisouard J, Medunjanin S, Hermani A, **Shukla A** & Mayer D 2007 Glycogen synthase kinase-3 protects estrogen receptor alpha from proteasomal degradation and is required for full transcriptional activity of the receptor. *ZMBH-FSPA Retreat*, Altleiningen, Germany.

Shukla A, Bleiler R, Becker K, Enzmann H & Mayer D 2008 Mitogenic potency of insulin analogues. *3rd DKFZ Alumni Meeting*, Heidelberg, Germany.

Shukla A, Bleiler R, Becker K, Enzmann H & Mayer D 2007 Effect of insulin analogues on human mammary cell lines. *Deutsch-Indischer Workshop*, Heidelberg, Germany.

Shukla A, Bleiler R, Becker K & Mayer D 2007 Effect of insulin analogues on human mammary cell lines. *Graduate Students Forum*, Heidelberg, Germany

Shukla A, Bleiler R & Mayer D 2006 Effect of insulin analogues on breast cancer cell lines. *DKFZ PhD Retreat*, Weil der Stadt, Germany.

CURRICULUM VITAE

PERSONAL INFORMATION

First Name	Ashish
Last Name	Shukla
Birth Date	26th February 1980
E-mail	*ashishshukla@email.com*
Nationality	Indian

EDUCATION

2005-2008	PhD	German Cancer Research Centre (DKFZ) Heidelberg, Germany
2003-2005	JRF	Tata Institute of Fundamental Research Mumbai, India
2001-2003	M.Sc.	Jawaharlal Nehru University New Delhi, India
1997-2001	B.Sc.	Chhatrapati Shahu Ji Maharaj University, Kanpur, India

RESEARCH EXPERIENCE

Oct/2005 – Present **Project title:**

Insulin analogues: Analysis of proliferative potency and characterization of receptors and signalling pathways activated in human mammary epithelial cells.

Publications:

Mayer D, **Shukla A** & Enzmann H 2008 Proliferative effects of insulin analogues on mammary epithelial cells. *Arch Physiol Biochem* **114** 38-44.

Grisouard J, Medunjanin S, Hermani A, **Shukla A** & Mayer D 2007 Glycogen synthase kinase-3 protects estrogen receptor alpha from proteasomal degradation and is required for full transcriptional activity of the receptor. *Mol Endocrinol* **21** 2427-2439.

Sept/2003 – Sep/2004 **Project title:**

To study the possible interaction of hRad51 and hRad52 with MCM complex.

Publications:

Shukla A, Navadgi VM, Mallikarjuna K & Rao BJ 2005 a cross-talk between recombination and replication proteins. *Biochem Biophys Res Commun* **329** 1240-1245.

Project title:

To characterization binding property of hRad51 and hRad52 to DNA by *in vitro* approach.

Publications:

Navadgi VM, **Shukla A**, Vempati RK & Rao BJ 2006 DNA mediated disassembly of hRad51 and hRad52 proteins and recruitment of hRad51 to ssDNA by hRad52. *FEBS J* **273** 199-207.

Navadgi VM, **Shukla A** & Rao BJ 2005 Effect of DNA sequence and nucleotide cofactors on hRad51 binding to ssDNA: role of hRad52 in recruitment. *Biochem Biophys Res Commun* **334** 696-701.

ACHIEVEMENTS

Cleared twice (**Dec. 2003 and Dec. 2002**) **All-India** Junior Research Fellowship (**JRF**) exam held by Council of Scientific and Industrial Research (CSIR).

GRE Score: 600/800 in vocabulary skills, 730/800 in quantitative skills and 3.5/6.0 in essay writing.

TOEFL Score: 283/300.

INTERESTS

Sports: Chess, computer games especially Age of Empires, gilli-danda and kabbadi.

Others: Cooking, reading novels,

REFERENCES

Personal references available upon request.
