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The Role of Tumour Suppressor Tyrosine Kinase SYK in Glioblastoma and Breast Cancer

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Erklärung:

Ich erkläre an Eides statt, dass ich die der Fakultät für Chemie der Technischen Universität München vorgelegte Dissertationsarbeit mit dem Titel:

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München, den

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Sushil Kumar

Dedicated to my Parents

For Nessy

1 Introduction

The ability of mammalian cells to respond to a wide variety of extracellular signals is essential for multicellular organisms during embryonic development and adult life. These responses are co-ordinated through different signalling pathways that transduce and exchange the information between different cells or inside the cell between different compartments. Apart from direct cell-cell contact i.e., communication between neighbouring cells, signalling also occurs between distant cells by growth factors and hormones via the bloodstream. These molecules bind to their cognate receptors on the cell surface and initiate a signalling cascade to finally stimulate physiological responses such as proliferation, differentiation, migration, and apoptosis. Regulation of the signal transmission is critical for the definition for physiological outcome as is the tight control of its generation. Therefore, irregularities in the activation and processing of signal transduction pathways can result in severe disorders e.g., cardiovascular diseases, diabetes, obesity, immunological disorders and cancer (Cohen, 2000; Hanahan and Weinberg, 2000).

Signals are translated in the cell in the form of post translational modifications of macromolecules e.g., ligation of phosphoryl, alkyl, lipid or sugar moieties. Among all these modifications, reversible protein phosphorylation is a key mechanism in the signal transduction process. The importance of phosphorylation in cellular signalling is reflected by the fact that about 30% of the cellular proteins are phosphorylated (Cohen, 2000). Regulation of the protein phosphorylation state by the combined activity of protein kinases and phosphatases can modify protein function in various ways e.g., by increasing or decreasing its enzymatic activity, by targeting it to degradation, by changing its subcellular localisation, or by initiating or disrupting its interaction with other molecules.

The cloning and sequencing of the human genome identified 518 putative kinase genes and 130 phosphatase encoding genes (Blume-jensen and Hunter, 2001; Manning, et al., 2002). Both protein kinases and phosphatases can be subdivided in membrane spanning or cytoplasmic enzymes based on their localization or as tyrosine, serine-threonine or dual specificity kinases based on the prefered amino acid for their kinase activity. However, it is the tyrosine kinase signalling which is found the most crucial in the oncogenesis and involves the largest fraction of over 100 known oncogenic protein kinases (Blume-jensen and Hunter, 2001; Cohen, 2000).

Deregulation of phosphorylation patterns by abberant expression or enzymatic activity of kinases and phosphatases is a key feature of malignant diseases (Lim, 2005).

1.1 Receptor Tyrosine Kinases

A large family of cell surface receptors contains intrinsic protein tyrosine kinase activity. These receptor tyrosine kinases (RTKs) consist of a glycosylated extracellular domain, a single transmembrane helix and an intracellular domain containing a protein kinase core which catalyses the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosine residue on target proteins (Hunter, 1998). The activation of RTKs occurs through ligand-induced dimerisation, which triggers transphosphorylation of specific tyrosine residue in the cytoplasmic domain, generating docking sites for various intracellular signalling molecules containing phosphotyrosine interaction domains (Hunter, 2002; Schlessinger, 2003). Ligand binding also triggers vesicle mediated internalisation of the activated receptor which eventually regulate the receptor population on cell surface and continuous signal generation. Once internalised, the RTKs are either degraded or recycled back to the membrane, a process called desensitisation (Kim et al., 2007; Orth and McNiven, 2006).

RTKs play a critical role in the regulation of various cellular processes such as proliferation, differentiation, metabolism, and survival. RTKs are catogorised in 20 different subfamilies depending on the domain composition of their extracellular ligand binding domain such as cystein rich domains, EGF-like (epidermal-growth factor-like) domains, immunoglobuline-like domains, cadherin-like domains and kringle-like domains among others (Fig.1).

1.1.1 The Epidermal Growth Factor Receptor (EGFR) Family and their Cognate Ligands

The epidermal growth factor receptor (EGFR) is one of the most prominent RTKs and was the first cell surface signalling protein to be identified by molecular genetic methods (Ullrich et.al., 1984). The cloning of EGFR cDNA and subsequent elucidation of its priamary structure has been a major landmark in the investigation of epithelial cancers due to its domainant role in cell proliferation, migration and escape from apoptosis (Ullrich et al., 1984; Downward et al., 1984).

The EGFR family constitutes of four members namely, the prototype member EGFR/ErbB1, HER-2/ErbB2/neu, HER-3/ErbB3, and HER-4/ErbB4.

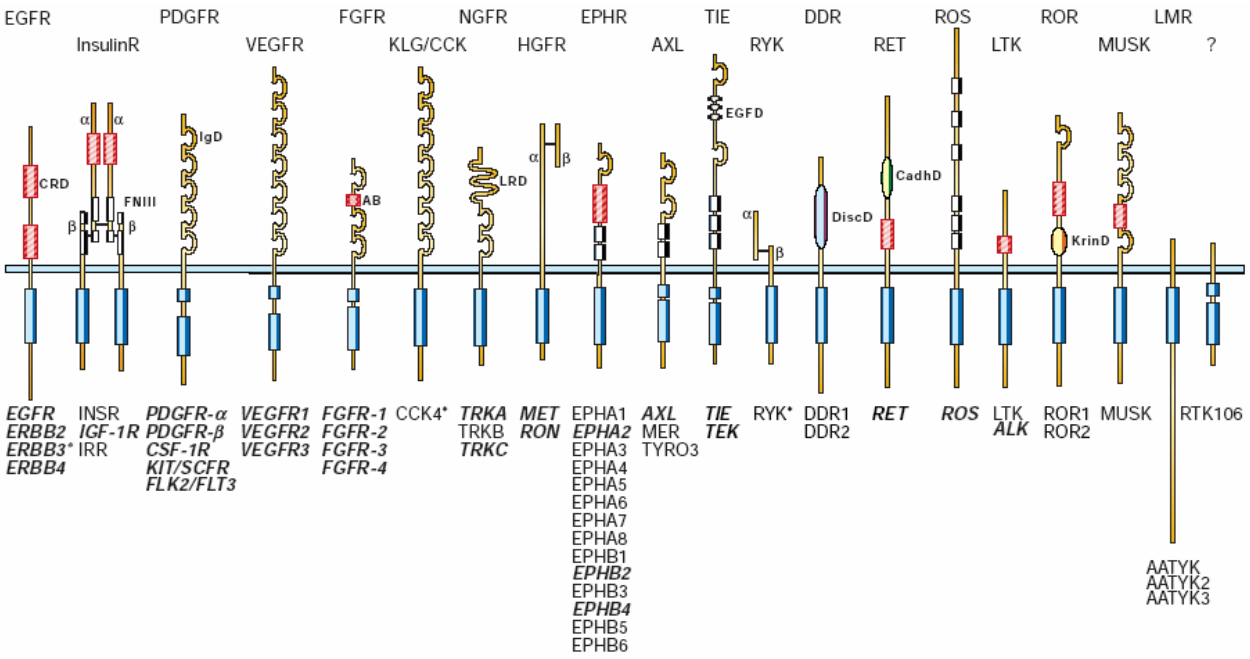


Figure 1 The subfamilies of receptor tyrosine kinases: The receptor tyrosine kinases are grouped based on their specificity for the ligands. The specificity in turn differs based on the extra cellular domains represented in the diagramme. Moreover, the tyrosine kinases also differ in the kinase domain. Various subfamilies express a split kinase domain. The specificity of certain anti-angiogenic compounds might rely on such structural aspects of kinase domains.
Abbreviations: AB Acidic Box, CadhD Cadherin like domain, CRD Cysteine rich domain, DiscD Discoidin like domain, EGFD EGF like domain, FNIII Fibronectin type III like domain, IgD Immunoglobulin like domain, KrinD Kringle like domain, LRD leucine rich domain, (**Blume-Jensen and Hunter; 2000**).

While EGFR is a classical example of the RTKs, its other family members show irregularities in their structure and functions. HER-2/ErbB2 is considered to be an orphan receptor since its ligand binding domain is missing (Citri et al., 2004). As its heterodimerization with other family members does not appear to require a ligand, HER-2/ErbB2 is highly oncogenic and the gene encoding this RTK is found amplified in more than 25% of all breast cancers (Slamon et.al, 1987). In fact, the first therapeutic monoclonal antibody Herceptin® targets HER-2/ErbB2 and is prescribed for HER-2/ErbB2 overexpressing breast cancer (Baselga et al., 1998; Pegram et al., 1998; Pegram et al., 1999). HER-3/ErbB3 is an atypical kinase with an unusual kinase function due to specific sequence features in its kinase domain. HER-3/ErbB3 is known to serve as a

platform for PI3-K regulatory subunits and propagates the anti-apoptotic or migratory signals in various cancer cells (Van Der Horst et al., 2005; Van Der Horst et al, 2003; Schulz et al., 2005).

Multiple ligands and various combinations of homo or heterodimerisation within the EGFR family couple to a complex and diverse set of biochemical pathways. The EGFR family members are activated upon dimerisation and therefore, deregulated co-expression of any of these receptors leads to an impaired cellular signalling. In concurrence, such deregulated co-expression of these receptors is commonly observed in a variety of cancer types e.g., lung cancer, breast cancer, kidney cancer, neuro-endocrine cancers etc. (Bianchi et al., 2006; Lee et al., 2005; Buck et al., 2006; Mendelsohn and Baselga, 2000).

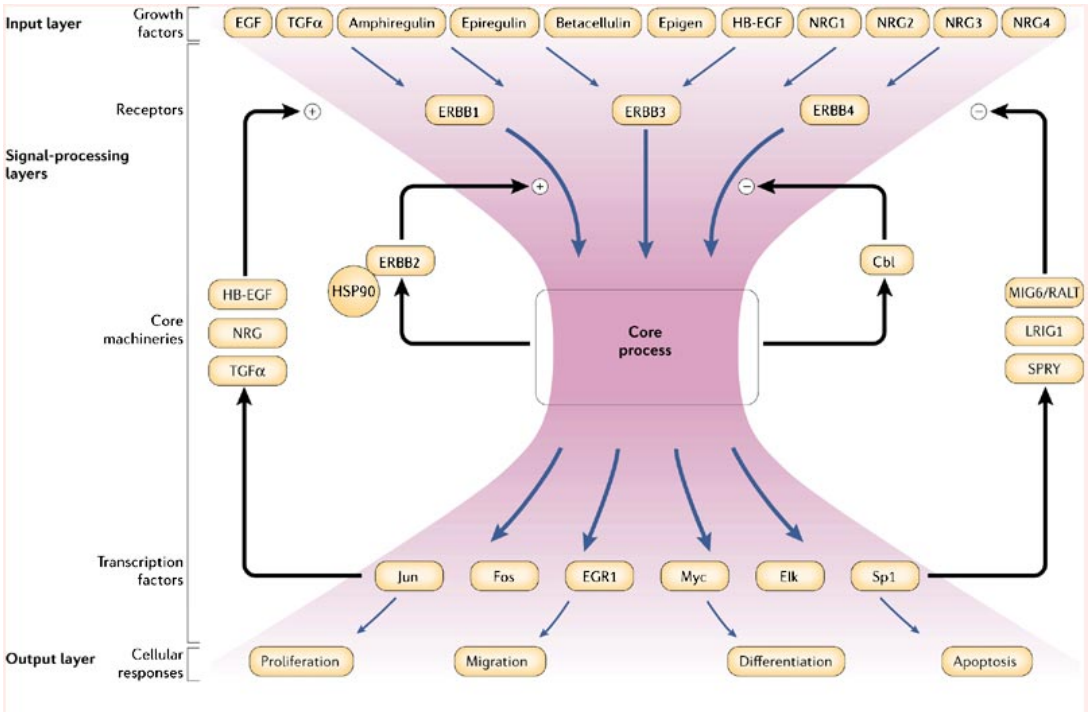


Figure 2 EGFR family and their ligands: EGFR family consist of four members of which only three members are activated by ligands (ErbB1, ErbB3, ErB4) whereas ErbB2 is an orphan receptor and heterodimerise with the other family members without ligand binding. Upon binding of their preferred ligands, the ErB receptor transmits signals to the cytoplasmic kinases which relay the signal further to the nucleus by modulating the activity of various transcription factors. In this way these differential signalling pathway regulate distinct physiological processes e.g., Proliferation, Migration, Apoptosis and Differentiation **(Citri and Yarden, 2006)**.

There are 8 known ligands which act as direct agonists for EGFR: epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor alpha

(TGFα), amphiregulin, betacellulin, epigen, epiregulin and cripto which, with the exception of cripto, are synthesized as transmembrane precursors and need to be proteolytically cleaved by metalloproteases to release the mature growth factors (Massague and Pandiella, 1993; Riese and Stern, 1998; Salomon et al., 1999; Strachan et al., 2001).

The Neuregulins act as ligands for HER-3/ErbB-3 and HER-4/ErbB-4 (NRG-1 and NRG-2) and HER-4/ErbB-4 (NRG-3 & NRG-4).

1.2 Cytoplasmic Tyrosine Kinases and their Modular Domains

Cytoplasmic tyrosine kinases, also known as non-receptor tyrosine Kinases (NRTKs), are key molecules in signalling which transmit the signal generated on the cell surface to various intracellular compartments within the cell. Moreover, the receptors lacking intrinsic enzymatic activity are truly dependent on these kinases to relay signals to their respective effector molecules. There are 32 NRTKs which are subdivided into 10 subfamilies namely, ABL, ACK, CSK, FAK, FES, FRK, JAK, SRC, SYK, and TEK kinase (Blume-jensen & Hunter 2001).

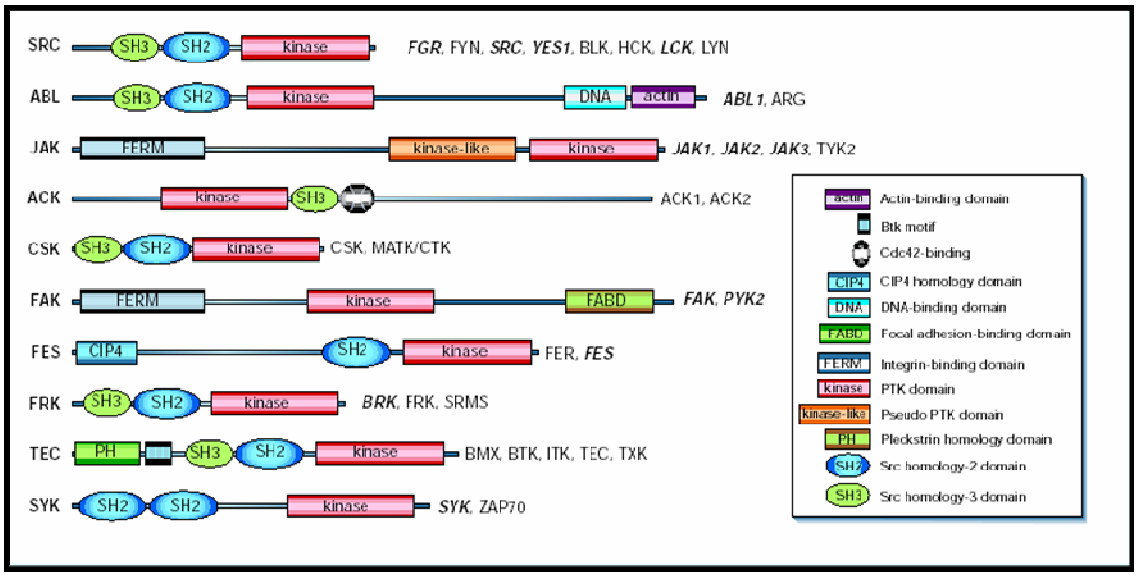


Figure 3 Domain based representation of cytoplasmic tyrosine kinases.
Cytoplasmic kinases are structurally diverse class of molecules and classified based on the sequence homology and domain similarities **(Blume-Jensen and Hunter, 2000)**.

Apart from their interactions with the membrane receptors, NRTKs are localized at several subcellular sites including the nucleolus, mitochondria, the endoplasmic reticulum and the inner face of cell membrane through amino-terminal modifications, such as myristylation or palmitoylation (Hantschel and Superti-Furga, 2004).

The NRTKs bind to their receptors either by the phosphotyrosine interacting domains e.g., PTB or the SH-2 domains or by docking molecules which are inserted in membrane by the PH domains to recruit the cytoplasmic kinases (Seet et al., 2006). The latter mode of interaction is generally observed in the receptor signalling where intrinsic enzymatic activity of the receptor is absent and the signalling is dependent on the recruitment of cytoplasmic tyrosine kinase. Whereas SH-2 domains are highly specific to pTyr residues, the PTB domains could bind to the non-phosphorylated peptides as well and therefore, serve as protein interaction modules to gather different molecules in a signalling zone (Pawson and Nash, 2003).

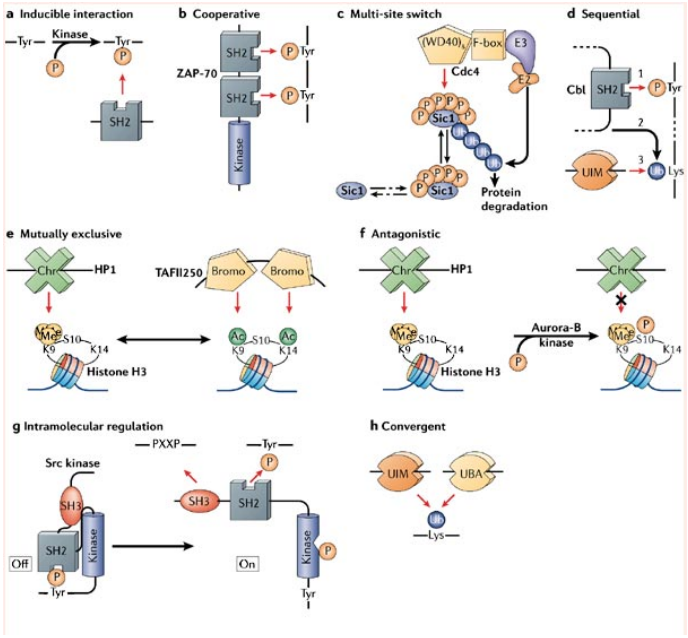


Figure 4 Different modes of molecular interaction: Several themes of molecular interaction can be observed in the regulatory processes e.g., **inducible interaction** where a modification of the molecule induces its interaction, **Cooperative interaction** in which modifications cooperate to bring another level of specificity (Syk binds to doubly phosphorylated ITAM via its tandemSH2 domains), multiple site switch induces interaction via multiple residues modified, **Sequential** mode displays how one interaction can create the opportunity for a series of interactions by employing the otherwise unrelated enzymatic activity to the molecule, **Mutually exclusive** model shows that a residue can have exclusive interactions based on the kind of modification it displays, **Antagonistic** mode is relatively opposite to the inducible as modification of a residue can be inhibitory on occasions, **Intramolecular interaction** are regulatory events and alters the self activity of the enzymes, **Convergent** mode exploits the leaky nature of specificity and shows that similar modifications can be recognised by different domains **(Seet et al., 2006)**.

Another class of molecules possesses interaction domains as well as enzymatic activity, such as cytoplasmic tyrosine kinase Src that contains a SH-2 domain and tyrosine kinase activity; and PLC-γ containing a SH-2 domain and phospholipase activity. In addition, signalling proteins consisting entirely of the interaction domains have been reported. Their examples include Grb2, Crk, or Shc that contain SH-2 and SH-3 domains to link activated RTKs to downstream signaling pathways like mitogen activated protein kinases (MAPKs) pathway.

1.2.1 Spleen Tyrosine Kinase (Syk)

Syk was first discovered as a proteolytic product of nearly 40kDa derived from a 72kDa tyrosine kinase in the spleen, thymus and lung. Since the cloning of its cDNA, Syk has been addressed as an essential component of the haematopoietic signalling system (Taniguchi et al., 1991). Syk is recruited to the tyrosine phosphorylated receptors in the haematopoietic cells by two tandem SH-2 domains to the Immune receptor Tyrosine based Activation Motif (ITAM) of B-cell receptors, T-cell receptors, or Fc receptors. Once phosphorylated by proximal kinases e.g., Src family kinases (SFKs), Syk gets activated and binds to its substrate to transmit the signal (Yaghini et al., 2007; Underhill and Goodbridge, 2007). Syk is a key signalling molecule in the haematopoietic cells and its abrogation in mice leads to lack in B-cell and T-cell development which causes perinatal lethality (Cheng et al., 1996; Turner et al., 1996). Though early efforts to understand the role of Syk in signalling were devoted in haematopoietic cells, its importance in the epithelial cells remained elusive until it was found that the metastatic cells are Syk deficient and they lose their invasive phenotype upon Syk expression (Coopman et.al, 2000). Since then Syk has been reported as a tumor suppressor in many malignancies enlisted in the table 1.

Although it was evident from the phenotypical profiling that loss of Syk is a key event during tumor progression, its functional significance has largely been unknown in these tumours. Several lines of evidence suggest that Syk might not only act as signal transducer in the cytoplasm but it could also modulate gene expression owing to its probable nuclear localisation (Wang et al., 2005). However, there are conflicting observations that have been reported regarding Syk translocation based on a predicted Nuclear Localization Signal in its protein sequence (NLS) (Fig. 5). Alternative splicing is a common post-transcriptional modification which alters the enzymatic activity as well as localisation of proteins (Garcia-Blanco, 2006;

Novoyatleva et al., 2006). Since Syk is known to be alternatively spliced, it is tempting to hypothesise the role of splicing in its regulation (Rowley et al., 1995). In fact, it has been shown that a NLS is located in the spliced sequence which impairs the nuclear localisation of the spliced variant and sequesters it in the cytoplasm (Wang et al., 2003). Furthermore, it was shown in the same report that the alternatively spliced form of Syk increases the aggressiveness in tumours whereas the unspliced form suppresses the invasive phenotype.

Fig. 3A

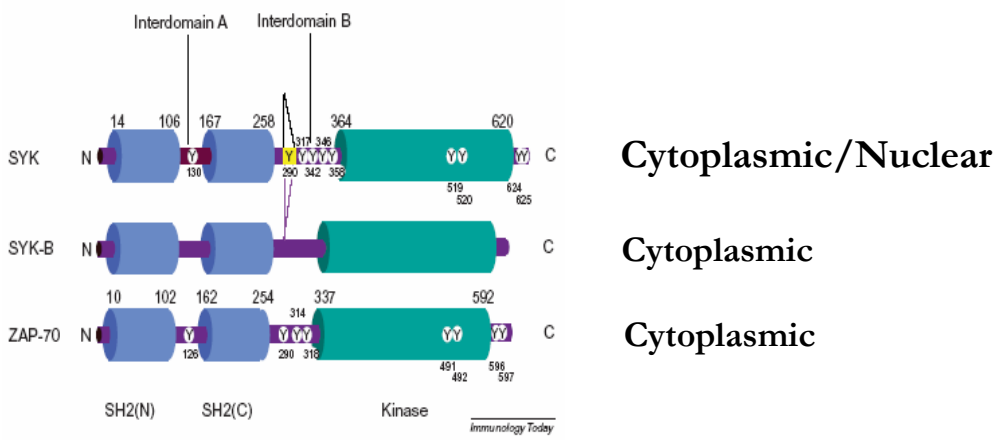


Fig. 3B

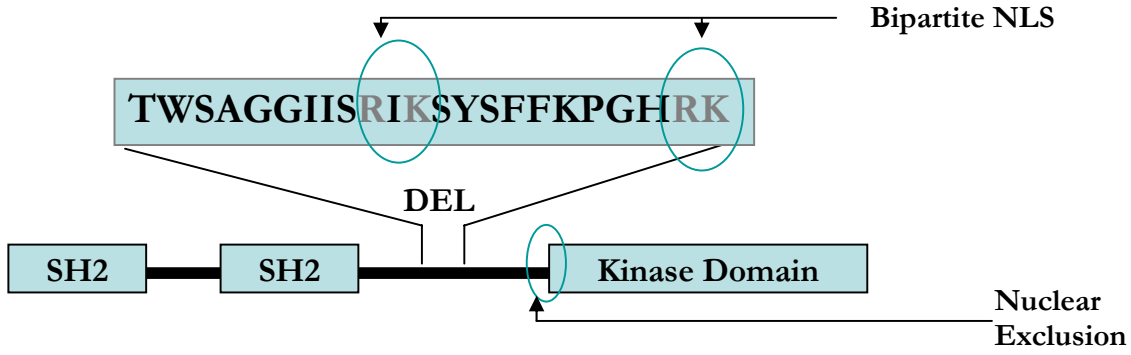


Figure 5 Diagrammatic representation of Syk and molecular determinant of its nuclear localisation. Syk family of tyrosine kinases consists of Syk isoforms and ZAP70. Syk isoforms differs by 23 amino acids which undergoes splicing and therefore, creates Syk B from Syk A transcripts (Fig. 3A) **(Turner et al., 2000)**. The nuclear localisation characterised as a regulated phenomenon determined by two putative sequences shown in the Fig.3B. The bipartite NLS and the N-terminal residues of kinse domain are shown affecting the nuclear localisation in two independent and contrasting reports (Adapted from **Wang et al., 2003** and **Zhou et al., 2006**).

Though the role of Syk as a transcriptional modulator is not yet confirmed, its localization in the nucleus is observed as a crucial event for its tumour suppressive nature (Coopman et al., 2000). It

has been shown that Syk co-localises to the centrosomes and aberrant mitosis is observed in the cells with ectopic Syk expression (Zyss et al., 2005). This phenomenon is supported by the observation that Syk co-localises with the microtubules in the cell and interacts with the γ-tubulin although no regulation is observed in tubulin polymerization or de-polymerization (Faruki et al., 2000). Moreover, tumour samples from breast cancer patients tend to loose Syk expression which correlates with high proliferation index (Moroni et al., 2004; Dong et al., 2001; Repana et al., 2006).

Tumour	Technical approaches	Observations	Referances
Breast	Western Blot analysis RT-PC (after LCM) *in situ* hybridization tumourogenicity in athymic mice	Loss of Syk in invasive breast carcinoma tissue and cell lines. Suppression of metastasis growth after Syk transfection in Syk (-) cells.	**Coopman et al., Nature; 2000**
Breast	qRT-PCR and LCM	Reduced Syk expression in primary Breast cancer (*n=90)*; Correlation of Reduced Syk expression with increased risk of metastasis and poor prognosis.	**Toyoma, et al., 2000; Cancer Lett.**
Breast	*In situ* hybridization	Progressive loss of Syk mRNA during progression from normal to hyperplasia to DCIS to metastasis. (*n=113)*	**Moroni, et al., 2004; Cancer Res.**
Breast	Immunohistochemistry & *in situ* hybridization	Decreased Syk protein levels in invasive carcinoma (*n=24)* and preserved Syk expression predicts favourable outcome.	**Dejmek et al., 2004; Cli. Cancer Res.**
Breast	Western Blot, RT-PCR	Expression of full length Syk and shorter alternatively spliced variant in breast cancer cell lines and primary tumours. Aberrant expression of shorter form of Syk in breast cancer but not in normal tissue types.	**Wang, et al., 2003; Cancer Res.**
Stomach	RT-PCR	Lower expression of mRNA in lymph node positive patients than in lymph node negative patients.	**Wang, et al., 2004; World J. Gastroent.**

Stomach	Immunohistochemistry	Nuclear Syk expression significantly associated with T1 tumours, absence of venous and lymphatic Invasion (*n=250*).	**Nakashima, et al., 2005; Cancer Lett.**
Squamous cell carcinoma	Differential display and cDNA arrays	Decreased Syk expression during tumour progression	**Dong, et al., 2001; Cancer Res.**
Melanoma	Western Blot qRT-PCR, tumorogenicity in nude mice.	Syk express in melanocytes but not in melanoma. Decreased tumour growth after re-infection of Syk.	**Hoeller, et. al., 2005; J. Clin. Invest.**
Pro B cell acute lymphobl -astic leukemia	Kinase Assay, Western Blot, RT-PCR, Nucleotide Sequencing	Markedly reduced Syk activity and Syk levels, mRNA species encodes abnormal protein with missing or truncated domain.	**Goodman, et. al., 2005; Oncogene**
Chronic Lymphocytic Leukemia	qRT-PCR	Downregulation of Syk expression in the genomic abberation 17p subgroup (n=82)	**Kienle, et. al., 2005; J. Clin. Oncol.**
Classical Hodgkin disease	Immunohistochemistry	Reed-steenberg cells are consistently Syk Negative.	**Marafioti, et. al., 2004; Blood**
Anaplastic Large cell Lymphoma	cDNA microarray analysis	Anaplastic Lymphoma kinase (ALK) poitive group express more Syk than ALK negative group.	**Thompson, et. al., 2005; Hum. Pathol.**

Table 1 The expression analysis of Syk carried in different tumour types. The table shows the results from different studies conducted to analyse the expression of different genes in various tumours. Syk expression is lost in majority of tumour types and aberrantly expressed in the haematopoietic malignancies. Abbreviations: LCM: Laser Capture Microtome DCIS: Ductal Carcinoma *in situ.* (Adapted from **Coopman et al., 2005**)

An interesting and important aspect of Syk, especially for the present study, is its involvement in the regulation of EGFR activity. It has been shown that Syk expression in breast epithelium modulates EGFR phosphorylation (Ruschel and Ullrich, 2004). Furthermore, it was reported that Syk knockdown by siRNA results in higher phosphorylation of EGFR whereas the over expression of Syk causes the dephosphorylation of EGFR. Moreover, Syk knockdown or its overexpression in breast epithelium led to an increase or decrease in apoptosis by reactive oxygen species respectively. Therefore, it warrants more analysis to identify the molecular mechanism involved in Syk mediated EGFR regulation.

1.3 G Protein Coupled Receptors

G protein coupled receptors (GPCRs) represent the largest family membrane receptors, comprising more than 800 members that are encoded by more than 2 % of the total genes in the human genome (Dorsam and Gutkind, 2007). GPCRs regulate diverse physiological functions such as neurotransmission, hormone release, immune response, cardiac and blood pressure regulation etc. Their malfunctioning causes a variety of diseases prevalent in humans and more than 60% of the targets of currently approved drugs are GPCRs. (Pierce et al., 2002). Though GPCRs are activated by a large variety of agonists, they share a common core structure. The characteristic structural features of GPCRs, which possess no intrinsic enzymatic activity, are seven transmembrane helices of 20-27 amino acids each. While the C terminus, the three extracellular, the three intracellular loop and the N-terminal extracellular portion vary in their length, and a weak correlation between ligand size and the length of the N-terminal portion has been observed, suggesting a role of this extracellular domain in ligand binding (Marinissen and Gutkind, 2001).

Binding of an agonist to the GPCRs activate the heterotrimeric G proteins (alpha, beta, and gamma) by conformational changes that leads to exchange of the bound GDP from G-α subunit with the GTP. Furthermore, the GTP bound G-α subunit dissociates from the G-$\beta\gamma$ complex and both complexes initiate their specific signalling cascades. In addition, both C-terminus and intracellular loops interact with other signalling molecules containing SH3, PTB or PDZ domains to add the complexity in the system. The effectors of the activated G proteins are rather limited and many G proteins are coupled with the same intracellular effectors. These effectors act through varying enzymatic activities and include adenylyl and guanylyl cyclase, Protein kinases e.g., PKA, PKC and Src, GTPase activating proteins (GAPs), Guanine nucleotide exchange factors (GEFs), Phosphodiestrases, and Phospholipases.

1.3.1 GPCRs in cancer

GPCRs control various crucial physiological functions in a cell and abnormalities in their signalling can lead to carcinogenesis and its progression. Various GPCR ligands have been shown to be potent mitogens e.g., acetylcholine, angiotensin, bombesin, bradykinin, endothelin-I, isoproterenol, lysophosphatidic acid (LPA), neurotensin, prostaglandin, and thrombin etc. and are

able to induce mitogenic responses in tissue culture systems (Daaka et al., 2004). Characterization of the MAS1 oncogene isolated from a human epidermoid carcinoma cell line, revealed the presence of multiple transmembrane domain, a structure similar to GPCRs (Young, et al. 1986). Persistent GPCR activation or activating mutations are shown to contribute to malignant transformations and cancer (Julius et al. 1989; Allen et al. 1991). Moreover, various transforming viruses e.g. Kaposi's sarcoma associated herpesvirus, contain sequences encoding constitutively active GPCRs, are shown to induce cancer in animal models (Montaner et al. 2003; Sodhi et al., 2004).

The oncogenic potential of mutated G protein subunits has also been shown. GTPase deficient mutants of $G\alpha_i$, $G\alpha_q$, $G\alpha_o$, $G\alpha_{12}$, and $G\alpha_{13}$ are found to be oncogenic in several cellular systems. In addition, naturally occurring activating mutations have been identified in various disease states, including cancer (Dhanasekaran et al. 1995). This led to the designation of several activated Gα mutants as oncogenes, including $G\alpha_s$, $G\alpha_{i2}$, and $G\alpha_{12}$, referred to as the gsp, gip2, and gep oncogenes, respectively (Landis et al. 1989; Lyons et al. 1990; Xu et al. 1993).

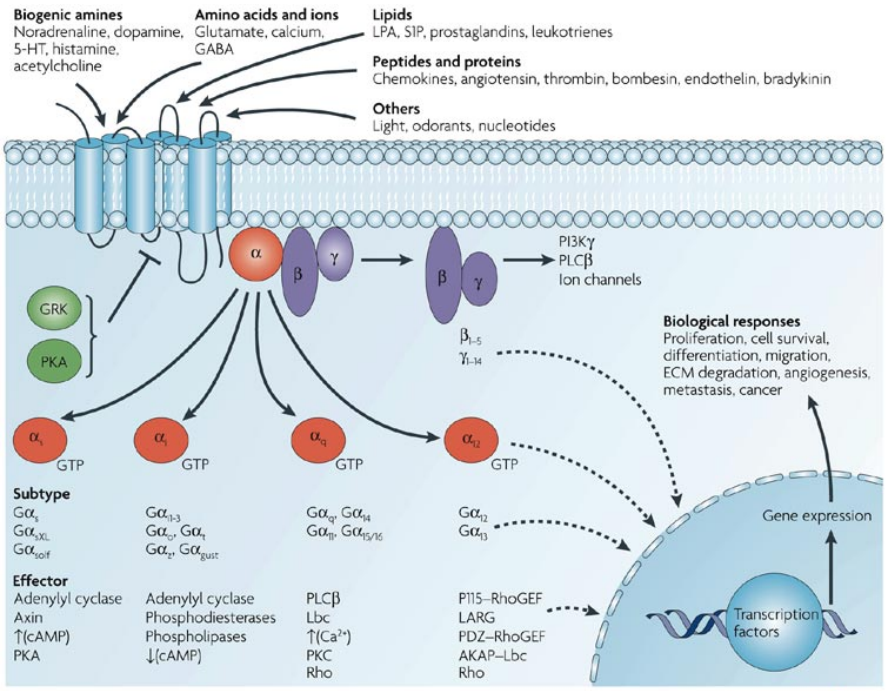


Figure 6 GPCR signalling and its physiological significance. GPCRs, upon binding of their ligands, activate heterotrimeric G proteins which transmit the signal by various effectors e.g., PLC, PKC, PKA etc. The signal can also be translated into the structural changes in the cell. These changes then regulate biological processes such as proliferation, migration, angiogenesis, metastasis and cancer **(Dorsam and Gutkind, 2007)**.

1.3.2 Lysophosphatidic acid (LPA) in cancer development

LPA is arguably one of the simplest lipids with the most diverse biological functions. It is a water soluble lipid with a single fatty acid chain, a glycerol backbone and a phosphate group. It is present abundantly (> 1μM) in the body fluids such as serum, saliva, follicular fluid, and malignant effusions (Mills and Moolenaar, 2003). The receptors of LPA on the cell surface are the members of EDG (Endothelial Differentiation Gene) family which are specific for LPA (EDG1, EDG2, and EDG3) or structurally related bioactive lipid sphingosine-1-phosphate (S1P) (EDG1, EDG3, EDG5, and EDG7) (Saba, 2004; Mills and Moolenaar, 2003). The biological responses of LPA are remarkably diverse and rapid that occurs independent of the protein synthesis. These responses include the cell morphological changes, gap junction closure and tight junction opening, motility, invasiveness, and chemotaxis. A rather unusual effect of LPA at cellular level is the activation of EGFR by GPCR mediated activation of Matrix Metalloproteases (MMPs) which act upon the membrane bound proligands of EGFR to release them in extracellular milieu. This seemingly complicated signalling mechanism is known as EGFR transactivation and is prevalent in many cell types (Daub et al., 1996; Prenzel et al. 1999; Fischer et al., 2006; Hart et al., 2004; Gschwind et al., 2001). Apart from the immediate LPA responses there are many responses which depend on protein synthesis e.g., cell cycle progression, wound healing, increased cell viability, production of angiogenic factors (endothelin-1, VEGF etc.) or matrix degrading enzymes (MMPs, uPA etc.) (Mills and Moolenaar, 2003). One of the developments in signifying the role of LPA in cancer, however, is the finding that a previously understudied enzyme autotaxin (ATX) involved in cancer invasiveness, acts by producing LPA (Hoelzinger et al., 2005; Kishi et al., 2006).

1.4 EGFR Transactivation

EGFR transactivation was first described by Daub et al (Daub et al., 1996). In this report activation of EGFR was shown in rat fibroblasts upon treatment with GPCR agonists. Subsequently, this phenomenon was shown in a variety of cell types with different GPCR agonists (Gschwind et al. 2002; Schäfer et al. 2004). Initially, EGFR activation by GPCRs was believed to be occurring via intracellular signaling. However, Prenzel and colleagues showed for the first time the metalloprotease mediated processing of the EGF like ligand HB-EGF and therefore a ligand dependent mechanism in EGFR transactivation (Prenzel et al. 1999). Blocking

both proHB-EGF and metalloprotease function abrogated GPCR stimulated EGFR, Shc, and MAPK phosphorylation, revealing the involvement of metalloproteases and the EGF like ligand HB-EGF in the transactivation pathway. Since then all of the 7 ligands binding to EGFR have been shown to be cleaved to release a soluble form and six of them are shown to be cleaved by metalloproteases (Sahin et al. 2004; Kochupurakkal et al. 2005). Various metalloproteases

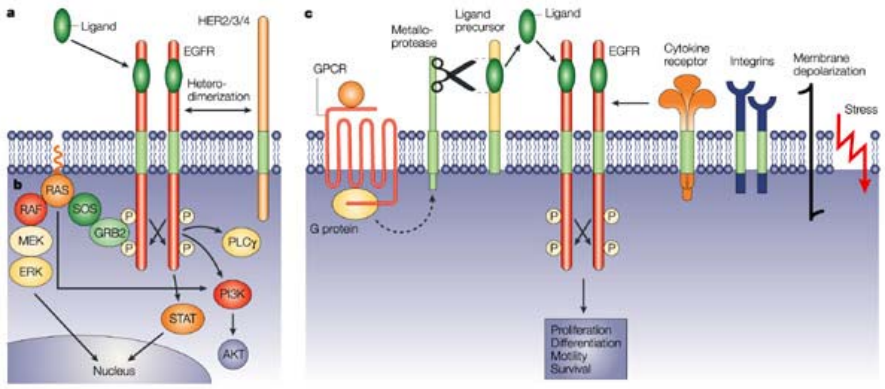


Figure 7 EGFR transactivation by GPCRs: The GPCRs or a physical stimulus (e.g., UV) activates the signaling via secondary messangers (e.g., Ca2+) or kinases (e.g., Src) which leads to the activation of metalloproteinases. Metalloproteinases act on a membrane bound proligand (e.g., HB-EGF) and cleaves it from the mebrane which then bind to its receptor. The activation of the receptor by ligand binding induces various signalling pathways which finally stimulates a cellular response e.g., proliferation, migration, survival etc (**Gschwind A et al., 2004**).

including various ADAMs and matrix metalloproteases (MMPs) have also been shown to cleave EGF family ligands (Gschwind et al. 2002; Gschwind et al. 2003).

In addition to this pathway Src family kinases have been suggested as both upstream and downstream mediators of the GPCR induced EGFR transactivation. Besides Src kinases, the serine/threonine kinase PKC has been frequently suggested to be involved in EGFR transactivation (Izumi et al., 1998; Carpenter, 2000). Moreover, in different cellular systems the intracellular Ca^{2+} concentration and the Ca^{2+} regulated tyrosine kinase Pyk2 have been discussed as a mediator of EGFR signal transactivation (Zwick et al. 1997; Eguchi et al. 1998; Keely et al. 2000). These pathways could also activate EGFR in addition to the metalloprotease induced proligand shedding pathway. Furthermore, inhibition of pathways negatively regulating RTK activity could also indirectly prolong the RTK signaling. Typical examples are inactivation of protein tyrosine phosphatases which dephosphorylate RTKs, reducing their activity and subsequent signaling (Knebel et al. 1996).

1.4.1 Matrix Metalloproteinases

The role of matrix metalloproteinases (MMPs) in the development of an organism is so significant that the first matrix metalloproteinase collagenase was found in the tail of a tadpol undergoing metamorphosis (Gross & Lapiere 1962). Research focusing on MMPs increased in the late 1960s and 1970s following observations that MMPs are upregulated in the disease states e.g., rheumatoid arthritis and cancer. However, the relationship between MMPs and the diseases is becoming more complicated since both increased and decreased expressions of MMPs have been associated with the clinical symptoms. Moreover, essential role of MMPs can not be directly comprehended by the clinical correlations coming but by their physiological role in the organisms. Many MMPs have been knocked out in mice with no lethality which suggests that MMPs might be redundant in their functions (Egeblad and Werb, 2002).

Biochemically, MMPs are members of Metzincin group (derived from Methionine and the Zinc ion present in their active site). Mammalian MMPs are conserved in their structure and consist of a catalytic domain and an autoinhibitory domain. The functions of MMPs are diverse ranging from the release of cytokines and pro-ligands via shedding (e.g., HB-EGF) or releasing them from sequestering matrix (e.g., VEGF), creating space for cells to migrate, activating receptors (e.g., Protease activated receptors) (Boire A et al., 2005), modulation of tissue architecture, and modifying the biological activity of the proteins. These observations combined with the correlation of MMPs expression with bad prognosis in various cancers hints at a strong link between MMPs and cancer.

1.5 Regulation of the Cell Cycle and its Implication in Cancer

The Cell cycle is a critical process for cells since any aberration (either in pace or genomic segregation) in its progression can cause serious anomalies. Therefore, cell cycle has to proceed in a unidirectional mode with temporal regulation of the processes involving DNA replication and protein turnover. Based on granularity of the cells, DNA content and, morphological feature, the cell cycle is divided into different phases. These are G1 and S phase which are marked with the duplication of the chromosomes; G2 and M phase which undertake the proper segregation of the chromosomes and cytokinesis to give rise to new daughter cells. These different phases are

further regulated by different cyclins which associate and activate their partners termed as CDKs (cyclin dependant kinases). Cyclins and their associated molecules are regulated by the phosphorylation and their turnover during cell cycle. Moreover, cyclins are also regulated by the inhibitors of the cyclin-CDK interactions. Therefore, it is not surprising to note that abnormalities in the expression of cyclins, CDKs, and their inhibitors are seen in many tumour types (Nakayama and Nakayam, 2006; Shapiro, 2006).

Since genomic maintenance is crucial for cell survival or its homeostasis, it is checked at different phases of the cell cycle and any abnormality in the genomic DNA or chromosomes induces a cell cycle arrest. The cell cycle arrest is regulated by several cell cycle checkpoints. These checkpoints are named after the stages they regulate e.g., G1, S, G2 or spindle checkpoints.

CDK1 is the master regulator of mitosis progression and its deregulation causes improper chromosomal movements as well as their segregation. CDK1 is positively regulated by phosphorylation at Threonine residues by MAPKs and negatively regulated by phosphorylation at Thr14 and Tyr15 by dual specificity kinases Wee-1 and Myt (Ohi and Gould, 1999; Berry and Gould, 1996; Nigg, 2001). Timely activation and inactivation of the CDK1 is crucial for proper M phase progression. Last but not least, the structural components such as the actomyosin complex also play a crucial role in the cytokinesis. The Actomyosin complex in particular generates the force (contractile ring) to cleave the cell into two halves. As cell cycle regulation is critically involved with the proper genomic distribution into the daughter cells, the disregulation of these events cause fatal diseases in humans e.g., congenital diseases, cancer etc. Major defects which are observed in the defective mitotic daughter cells are aneuploidy, multinucleation, or deletions and recombinations in chromosomes. Majority of aggressive tumours show unequal distribution of chromosomes and are aneuploid (van Deursen, 2007; Jefford and Irminger-Finger, 2006). Therefore, the concerted expression and activity of mitotic checkpoints are highly significant. In fact, many mitotic as well as the spindle checkpoints are defective or abberantly expressed in advanced tumours which is again suggests that these molecules are of high importance in understanding the tumour progression and finding a mean of intercepting them could be useful in cancer treatment.

1.6 Mechanisms of Metastasis in cancer

Tumours are continuously growing tissues which then disseminate into the blood stream to colonise new sites favourable for their growth. This process was explained with a great understanding by an English clinician Stephen Paget (1889) describing cancer cells as seed and the metastatic niche as the soil. Briefly, the metastasis can be explained as a series of events where a cancer cell invade to the neighbouring tissue (invasion), enter in the blood stream (intravasation) and colonise new tissues (Extravasation and proliferation)(Fig. 8).

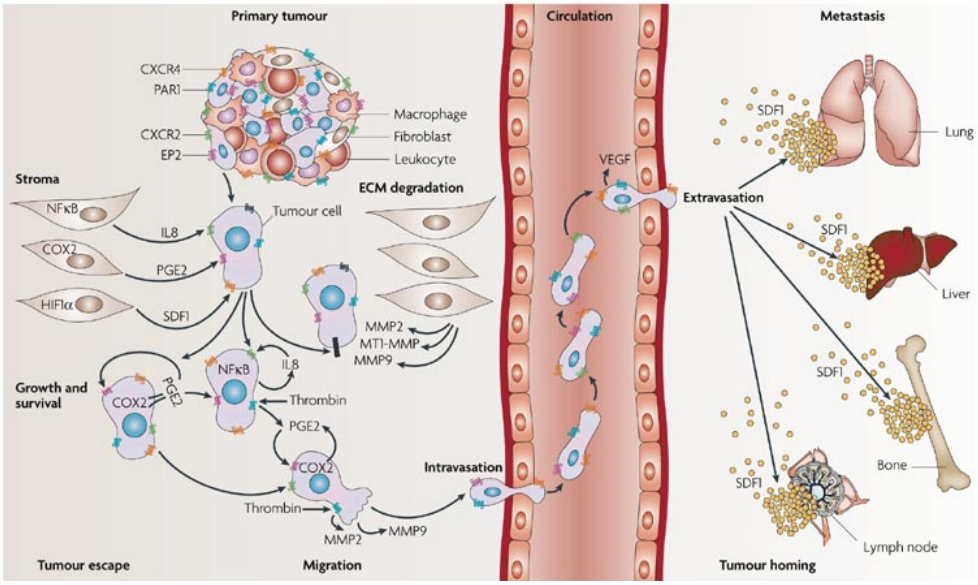


Figure 8 Metastatis from priamary tumour site to a distant organ: The tumour growth at the primary site causes several changes in the surrounding environment (e.g., infiltration of inflammatory cells, pH, hypoxia etc.). These changes modulate gene expression (e.g., MMPs to degrade the ECM) which enables the tumour to invade the surrounding tissue. The next step in metastasis is intravsataion which is marked as the entry point of escaped cancer cells in the main blood circulation. In the blood circulation the cells which are able to form aggregates or complexes with platelets, survives. These cells then, depending on largely unknown cues, extravasate to the new metastatic site (e.g., lung, liver, bone or lymph nodes) **(Dorsam RT and Gutkind JS, 2007)**.

Recently the concept of "Soil and Seed" has been re-envisioned and the properties of both cancer cells and metastatic niche are scrutinised to the molecular details (Minn et al., 2005; Kang et al., 2003; Weigelt et al., 2005). It has been shown in these studies that cancer cells are differential in there capabilities to colonise a new tissue type. The cell population in a growing tissue mass is heterogeneous based on their proteome constitution. These changes come into effect due to the spontaneous (epigenetic changes as well as mutations) as well as acquired genetic changes (e.g., Mutations caused by carcinogens). These metastatic cells are selected in the testing conditions of

the blood streams and the new tissue site. There are variety of changes that have been observed in the cells which metastasise to a new site in the body e.g., up regulation of MMPs, cytokines (e.g., SDF-1, CTGF), anti apoptotic proteins (Minn et al., 2005; Kang et al., 2003) & down regulation of caspases (Stupack et al., 2006). Original tumour site also undergoes modulation by the growing cancer into a hypoxic, acidic, inflammatory environment which is partially responsible for the invasive phenomenon. All these changes around the growing tumour have been shown to stimulate the production of cytokines (migratory) and matrix degrading enzymes (migratory and invasive).

1.7 Aim of the study

The aim of the present study is to investigate the role of Syk tyrosine kinase in pathophysiology of human cancers. Syk is apparently a tumor suppressor but little information exists about its molecular mechanism of action. Therefore, this work is aimed at understanding the molecular mechanisms underlying Syk mediated tumor suppression.

To address these problems, different tumour cell lines of breast cancer and glioblastoma origin were used. Various biochemical stimuli as well as genetic methods to regulate the expression or activity of Syk tyrosine kinase were employed to explore Syk dependent molecular mechanisms. Further, the physiological relevance of these molecular changes was sought by performing cell biological assays (e.g., proliferation, apoptosis, migration & invasion assays) in comparable settings to the previously performed biochemical experiments.

A special interest of this study was to identify the molecules involved in Syk regulated EGFR attenuation. The crucial molecules involved in Syk dependent EGFR regulation were scrutinize by employing the genetic as well as biochemical methods.

There is growing interest in identifying the Syk dependent signalling network in epithelial cells as well as epithelial tumours. The present work addresses this matter by utilising the power of mass spectrometry to identify and analyse the Syk interacting molecules in different physiological states.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory Chemicals and Biochemicals

Acrylamide	Serva, Heidelberg
Agar	Difco, Detroit, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine 3´-triphosphate)	Pharmacia, Freiburg
Batimastat	British Biotech, Oxford, UK
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Collagenase (Type III)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Heparin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid))	Serva, Heidelberg
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine	Gibco, Eggenstein
Leupeptin	Sigma, Taufkirchen
Lipofectamine®	Invitogen, Karlruhe

Lysozyme	Sigma, Taufkirchen
LY-294,002	Alexis, Grünberg
MBP (Myelin basic protein)	Sigma, Taufkirchen
Mineral oil	Sigma, Taufkirchen
MOPS (3-Morpholinopropanesulfonic acid)	Biomol, Haub
N,N-Dimethylsphingosine	Sigma, Taufkirchen
Oligofectamine®	Invitrogen, Karlruhe
Piceatannol	Sigma, Taufkirchen
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
pNPP (p-Nitrophenyl phosphate)	Sigma, Taufkirchen
Polybrene (Hexadimethrine bromide)	Sigma, Taufkirchen
PD98059	Alexis, Grünberg
PEG (Polyethylene glycol) 4000, 6000	Serva, Heidelberg
Ponceau S	Sigma, Taufkirchen
PP2	Calbiochem, Bad Soden
PTX (Pertussis toxin) List,	Campbell, USA
Ro 31-8220	Upstate Biotech, USA
Salmon sperm DNA	Sigma, Taufkirchen
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sphingosine-1-phosphat, D-erythro	Biomol, PA, USA
SKI-606	Vichem
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Aldrich, Steinheim
Scintillation cocktail (Rotiszint®ecoplus)	Roth, Karlsruhe
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg

All other chemicals were purchased from Merck (Darmstadt).

2.1.2 Enzymes

Alkaline Phosphatase	Roche, Mannheim
Restriction Endonucleases	Pharmacia, Freiburg
	Roche, Mannheim
	NEB, Frankfurt/ Main
	MBI Fermentas, St. Leon-Rot
T4-DNA Ligase	Roche, Mannheim
T7-DNA Polymerase	Pharmacia, Freiburg
Taq-DNA Polymerase	Roche, Mannheim
	Takara, Japan
Trypsin	Gibco, Eggenstein

2.1.3 Radiochemicals

[γ-^{32}P] ATP >5000 Ci/mmol

[α-^{33}P] dATP 2500 Ci/mmol

All radiochemicals were obtained from PerkinElmer Life Sciences, Köln.

2.1.4 “Kits" and other Materials

Cell culture materials	Greiner, Solingen
	Nunclon, Dänemark
	Falcon, U.K.
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
Dowex AG1-X8	Bio-Rad, München
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-Sepharose	Pharmacia, Freiburg

Protein G-Sepharose	Pharmacia, Freiburg
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Random-Primed DNA Labeling Kit	Pharmacia, Freiburg
Sephadex G-50 (DNA Quality)	Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Transwells	Corning, New York, USA
Whatman 3MM	Whatman, USA

2.1.5 Growth Factors and Ligands

Amphiregulin	R&D Systems
HB-EGF	R&D Systems
Bradykinin	Calbiochem
EGF (murine)	Toyoba, Japan
FGFb	
Lysophosphatidic Acid (LPA)	Sigma
TGFb	

All other growth factors and ligands were purchased from Sigma.

2.1.6 Media and Buffers

Medium for E.coli

LB-Medium	1.0 % Tryptone
	0.5 % Yeast Extract
	1.0 % NaCl
	pH 7.2
2xYT-Medium	1.6 % Tryptone
	1.0 % Yeast Extract
	1.0 % NaCl
	pH 7.2

When necessary the following antibiotics were added to the media after autoclavation:
Ampicillin 100 µg/mL
Kanamycin 100 µg/mL
Chloramphenicol 30 µg/mL
LB-plates additionally contained 1.5% Agar.

2.1.7 Cell Culture Media

All cell culture media and additives were from Gibco (Eggenstein), fetal calf serum (FCS) was purchased from Sigma. Dulbecco's modified eagle medium (DMEM) with 4.5 mg/mL glucose, 2 mM L-glutamine, 1mM sodium-pyruvate. Eagle´s minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. RPMI 1640 medium supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate. Complete medium for primary epithelial cells: Dulbecco's modified eagle medium (DMEM) with 4.5 mg/mL glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x nonessential amino acids, 5µg/mL insulin, 10ng/mL EGF, 10% heat-inactivated FCS (All are Gibco reagents, except insulin and EGF are from Sigma) Freeze medium: 90% heat-inactivated FCS, 10% DMSO.

2.1.8 Stock Solutions for Buffers

BBS (2x)	50 mM BES
	280 mM NaCl
	1.5 mM Na2HPO4
	pH 6.96 (NaOH)
HBS (2x)	46 mM HEPES pH 7.5
	274 mM NaCl
	1.5 mM Na2HPO4
	pH 7.0
Denhardt (100x)	2.0 % Polyvinylpyrollidon
	2.0 % Ficoll
	2.0 % BSA
DNA loading buffer (6x)	0.25 % Bromphenol blue

	0.25 % Xylencyanol
	30.0 % Glycerol
	100.0 mM EDTA pH 8.0
Laemmli buffer (2x)	187.5 mM Tris/HCl pH 6.8
	6.0 % SDS
	30.0 % Glycerol
	0.01 % Bromphenol blue
	5.0 % ß-Mercaptoethanol
NET (1x)	150.0 mM NaCl
	5 mM EDTA
	50 mM Tris
	0.05 % Triton X-100
	pH 7.4 (HCl)
PBS	13.7 mM NaCl
	2.7 mM KCl
	80.9 mM Na2HPO4
1.5 mM KH2PO4, pH 7.4 (HCl)	
SD-Transblot	50.0 mM Tris/HCl pH 7.5
	40.0 mM Glycine
	20.0 % Methanol
	0.004 % SDS
“Strip” buffer	62.5 mM Tris/HCl pH 6.8
	2.0 % SDS
	100 mM ß-Mercaptoethanol
	SSC (20x) 3.0 M NaCl
	0.3 M Sodium citrate
TAE (10x)	400 mM Tris/Acetate
	10 mM EDTA pH 8.0 (Acetic acid)
TE10/0.1	10.0 mM Tris/HCl pH 8.0
	0.1 mM EDTA pH 8.0
Tris-Glycine-SDS (10x)	248.0 mM Tris/HCl pH 7.5
	1918.0 mM Glycine

1.0 % SDS

2.1.9 Bacterial Strains, Cell Lines and Antibodies

E. coli Description	**Origin/ Reference**	
DH5α	F' F'/endA1 hsd17 (rk-mk-),supE44, recA1, gyrA (Nal), thi-1, (lacZYA-argF)	Genentech, San Francisco, USA
CJ236	dut-, ung-, thi-, relA-	(Kunkel, 1985)

Cell Line	**Description**	**Source/Referance**
Cos-7	African Green Monkey, SV-40 transformed kidney fibroblast.	Genentech
HEK-293	T Human Embryonic Kidney fibroblasts transformed with adenovirus Typ V DNA.	ATCC CRL-1573
Phoenix E, A	Retrovirus producer cell lines for the generation of helper free ectropic and amphotropic retroviruses, based on HEK-293.	Nolan, Stanford
MDA-MB-231	Human mammary carcinoma	ATCC HTB-26
BT-20	Human Breast cancer	ATCC
MCF7	Human breast adenocarcinoma metastatic to brain	ATCC HTB-22
MCF-10A	Human mammary epithelium	ATCC
MDA-MB-435s	Human Mammary carcinoma, metastatic to Lung	ATCC
SF-767	Human Glioblastoma	SUGEN
U118	Human Glioblastoma	SUGEN

U373-MG	Human glioblastoma	DKFZ

ATCC, American Type Culture Collection, Manassas, USA

DKFZ, Deutsches Krebsforschungszentrum, Heidelberg

2.1.10 List of Antibodies

P-Tyr (4G10)	Mouse, monoclonal; recognizes phospho-(3)-tyrosine residues	UBI, Lake Placid
EGFR	Sheep, polyclonal/part of cytoplasmic domain of the human EGFR	UBI, Lake Placid
EGFR (108.1)	Mouse, monoclonal/ectodomain of the human (Daub et al., 1997) EGFR	(Daub et al., 1997)
HER2/neu	Rabbit, polyclonal/C-terminal peptide of human (Daub et al., 1996) HER2/neu	(Daub et al., 1996)
Akt1/2	Rabbit, polyclonal/AA 345-480 of human Akt1	Santa Cruz, USA
SHC	Mouse, monoclonal	Santa Cruz, USA
P-ERK	Rabbit, polyclonal; recognizes phospho-p44/p42 (Thr-202/Tyr-204) MAPK	NEB, Frankurt/M.
P-p38	Rabbit, polyclonal; recognizes phospho-p38 (Thr-180/Tyr-182) MAPK	NEB
P-Akt/PKB	Rabbit, polyclonal; recognizes phospho-Akt (Ser-473)	NEB
ERK2 (C-14)	Rabbit, polyclonal/peptide at C-terminus of rat ERK2	Santa Cruz, USA
ERK2 (K-23)	Rabbit, polyclonal/peptide from sub-domain XI of rat ERK2	Santa Cruz, USA
Pan-ERK	Mouse monoclonal/AA 219-358 of human ERK2	Transduction Lab.
HA	Mouse, monoclonal; recognizes the influenza	Babco, California,

	hemagglutinin epitope USA	
VSV (P5D4)	Mouse, monoclonal; recognizes an epitope of eleven AA derived from the vesicular stomatits virus glycoprotein VSV-G	Roche, Mannheim
Syk	Mouse, monoclonal antibody	Santa Cruz, USA
FAK	Rabbit, polyclonal antibody	Santa Cruz, USA
c-Src	Rabbit, polyclonal antibody	Santa Cruz, USA
Hck	Rabbit, polyclonal antibody	Santa Cruz, USA
Yes	Rabbit, polyclonal antibody	Santa Cruz, USA
Lyn	Mouse, monoclonal antibody	Santa Cruz, USA
EGFR-1008	Rabbit, polyclonal antibody	Santa Cruz, USA
CDK1	Mouse, monoclonal antibody	Santa Cruz, USA
pCDK1	Rabbit, polyclonal antibody	Abcam
cdc27	Mouse, monoclonal antibody	Upstate biotech
Tubulin	Mouse, monoclonal antibody	Sigma
Actin	Rabbit, polyclonal antibody	Sigma

2.2 Plasmids and Oligonucleotides

2.2.1 Plasmid Preparation for Analytical Purpose

Small amounts of plasmid DNA were prepared as described previously (Lee and Rasheed, 1990).

2.2.2 Plasmid Preparation in Preparative Scale

For transfection experiments of mammalian cells DNA of high quality was prepared using Qiagen Maxi-Kits (Qiagen, Hilden) according to the manufacturers´ recommendations.

2.1.3 Plasmid and Oligonucleotides

Primary Vectors

Vector	Description	Origin/ Reference

pcDNA3	Mammalian expression vector, Amp_r, CMV promotor, BGH poly A, high copy number plasmid	Invitrogen, USA
pLXSN	Expression vector for retroviral gene transfer, Ampr, Neor, ori from pBR322, 5'-LTR and 3'-LTR from MoMuLV, SV40 promotor	Clontech, Palo- , Alto USA
pLXSN-ESK	Modified pLXSN vector with multiple cloning site from pBluescript	J. Ruhe
pRK5	Expression vector, Ampr, CMV Promoter, SV 40 poly A, high copy number plasmid	Genentech
pRETRO-SUPER	shRNA expression vector	OligoEngine

2.2.4 Constructs

pcDNA3-Syk: Syk subcloned from pLXSN-Syk in pcDNA3 (Ruschel A and Ullrich A, 2004)

pcDNA3-Syk K/R: Syk K/R subcloned from pLXSN-Syk K/R in pcDNA3 (Ruschel A and Ullrich A, 2004)

pcDNA3-Syk AP: Syk autophosphorylation mutant (Tyr525+Tyr526) generated from pcDNA3-Syk

pcDNA3-SykB: Spliced form of Syk generated by deleting the 69bp sequence coding the Interdomain B from pcDNA3-Syk (Dai Jl et al., 2003).

2.2.5 Important Primers

pcDNA3-Syk(Fw) 5’-gcgggatccgccaccatggccagcagcggcatggc-3’

pcDNA3-Syk (Rev) 5’-gcgggatccttagttcaccacgtcatag-3’

pcDNA3-Syk AP (525F) 5'-ctgcgtgctgatgaaaac**gca**tacaaggcccagaccc-3'

pcDNA3-Syk AP(525R) 5'-gggtctgggccttgta**tgc**gttttcatcagcacgcag-3'

pcDNA3-Syk AP (525+526F) 5'-cgtgctgatgaaaacgcagcaaaggcccagacccatg-3'

pcDNA3-SykAP (525+526R) 5’-catgggtctgggcctttgctgcgttttcatcagcacg- 3’

pcDNA3-SykB (Fw) 5’-gggct**gcaagg**cccaaggaggtttacctggaccgaa-3’

pcDNA3-SykB (Fw1) 5’-gggct**gcaagg**cccaaggaagtttacctggaccgaa-3’

pcDNA3-SykB (R) 5’-cttgggccttgcagcccagggtgcaagttctg-3’

pRetro-Super constructs were generated as per suppliers instructions (OligoEngine). The 64mer oligonucleotide used for the generation of constructs are as follows:

pRS-Syk1(F): gatcccc**gtcgagcattattcttata**ttcaagaga**tataagaataatgctcgac**tttttggaaa

pRS-Syk1(R): agcttttccaaaaa**gtcgagcattattcttata**tctcttgaa**tataagaataatgctcgac**ggg

pRS-Syk2 (F): gatcccc**ggatgctggttatggagat**ttcaagaga**atctccataaccagcatcc**tttttggaaa

pRS-Syk2(R): agcttttccaaaaa**ggatgctggttatggagat**tctcttgaa**tctccataaccagcatcc**ggg

pRS-Scramble(F): gatcccc**gtacctcttaccaatccaa**ttcaagag**ttggattggtaagaggtac**tttttggaaa

pRS-Scramble(R): agcttttccaaaaa**gtacctcttaccaatccaa**tctcttgaa**ttggattggtaagaggtac**ggg

2.2.6 Primers for RT-PCR

MMP1 (Fw.): 5'-CGACTCTAGAAACACAAGAGCAAGA-3'

MMP1 (Rev.): 5'-AAGGTTAGCTTACTGTCACACGCTT-3'

GAPDH (Fw.): 5’-ACCACAGTCCATGCCATCAC-3’

GAPDH (Rev.): 5’-TCCACCACCCTGTTGCTGTA-3’

MMP14 (Fw.): 5'-CGCTACGCCATCCAGGGTCTCAAA-3'

MMP14 (Rev.): 5'-CGGTCATCATCGGGCAGCACAAAA-3'

2.2.7 Primers for Methylation specific PCR:

uSyk (Fw): 5'-ATTTTGTGGGTTTTGTTTGGTG-3'

uSyk (Rev): 5'-ACTTCCTTAACACACCCAAAC-3'

mSyk (Fw): 5'-CGATTTCGCGGGTTTCGTTC-3'

mSyk (Rev): 5'-AAAACGAACGCAACGCGAAAC-3'

2.3 Enzymatic Manipulation of DNA

2.3.1 Digestion of DNA Samples with Restriction Endonucleases

Restriction endonuclease cleavage was accomplished by incubating the enzyme(s) with the DNA in appropriate reaction conditions. The amounts of enzyme and DNA, the buffer and ionic concentrations, and the temperature and duration of the reaction were adjusted to the specific application according to the manufacturers´recommendations.

2.3.1.2 Dephosphorylation of DNA 5'-Termini with Calf Intestine Alkaline Phosphatase (CIAP)

Dephosphorylation of 5´-termini of vector DNA in order to prevent self-ligation of vector termini. CIP catalyzes the hydrolysis of 5´-phosphate residues from DNA, RNA, and ribo and deoxyribonucleoside triphosphates. The dephosphorylated products possess 5´-hydroxyl termini. For dephosphorylation 1-20 picomoles of DNA termini were dissolved in 44 µL deionized water, 5 µL 10x reaction buffer (500 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.5) and 1 µL CIP (1 U/µL). The reaction was incubated 30 min at 37°C and stopped by heating at 85°C for 15 minutes.

2.3.1.3 DNA Insert Ligation into Vector DNA

T4 DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA. T4 DNA Ligase thereby joins doublestranded DNA with cohesive or blunt termini.

In a total volume of 10 µL the digested, dephosphorylated and purified vector DNA (200 ng), the foreign DNA to be inserted, 1 µL 10x T4 DNA Ligase buffer (0.66 M Tris/HCl pH 7,5, 50 mM MgCl2, 50 mM DTT, 10 mM ATP) and 1 µL T4 DNA Ligase (2 U for sticky ends and 4 U for blunt ends) were mixed. The reaction was incubated at 15°C overnight. T4 DNA Ligase was inactivated by heating the reaction mixture at 65°C for 10 minutes. The resulting ligation reaction mixture was directly used for bacterial transformation.

2.3.1.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying 0.5- to 25 kb DNA fragments. 0.6-2%, horizontal agarose gel with 1x TAE electrophoresis buffer was used for separation. The voltage was set typically to 1-10 V/cm of gel. Gels were stained by covering the gel in a dilute solution of ethidium bromide (0.5 µg/mL in water) and gently agitating for 30 min and destained by shaking in water for an additional 30 min.

2.3.1.5 Isolation of DNA Fragments Using Low Melting Temperature Agarose Gels

Following preparative gel electrophoresis using low melting temperature agarose, the gel slice containing the band of interest was removed from the gel. This agarose slice was then melted and subjected to isolation using the QIAquick Gel Extraction Kit (Qiagen).

2.3.2 Introduction of Plasmid DNA into E.coli

2.3.2.1 Preparation of Competent Cells

Competent cells were made according to the procedure described before (Chung and Miller, 1988). For long-term storage competent cells were directly frozen at –70°C. Transformation frequency ranged between 10^6 and 10^7 colonies/µg DNA.

2.3.2.2 Transformation of Competent Cells

100 µL competent cells were added to 10 µL ligation mix and 20 µL 5x KCM (500 mM KCl, 150 mM CaCl2, 250 mM MgCl2) in 70 µL H2O and incubated on ice for 20 min. Upon incubation at room temperature for 10 min 1 mL LB medium was added and incubated 45 min at 37°C with mild shaking to allow expression of the antibiotic resistance gene. Transformants were selected on appropriate plates.

2.3.3 Oligonucleotide-Directed Mutagenesis

A DNA sequence can be specifically altered by synthesizing the desired sequence change within an oligonucleotide, and then converting this into a biologically active circular DNA strand by using the oligonucleotide to prime in vitro synthesis on a single-stranded circular template. This protocol (Kunkel, 1985; Messing, 1983) uses a DNA template containing a small number of uracil residues in place of thymine. Use of the uracil-containing template allows rapid and efficient recovery of mutants.

2.3.3.1 Preparation of Uracil-Containing, Single-Stranded DNA Template

CJ236 bacteria were transformed with the DNA of interest (typically pcDNA3 constructs). 2 mL 2xYT-medium was inoculated with several colonies of transformed CJ236 at 37°C until the early log-phase was reached. Cultures were infected with 2x107 M13K07 phages/mL (Amersham) and incubated for further 1.5 h. Next, kanamycin was added (70 µg/mL final concentration) and the culture was incubated with vigorous shaking at 37 °C overnight. Cells were pelletet twice by centrifugation (13000 rpm, 5 min) to clear the supernatant. Phage was then precipitated by adding 200 µL 2.5 M NaCl/ 20% PEG 6000 and incubation for 15 min at room temperature. Precipitated phage was collected by centrifugation. The phage sediment was resuspended in 100 µL TE10/0.1 buffer and subjected to phenol extraction/ ethanol precipitation in order to purify the single-stranded phage DNA. Quality and concentration of DNA was determined spectrophotometrically at 260 nm. For visual examination and documentation an aliquot of the single-stranded DNA was run on a 1% agarose gel.

2.3.3.2 Primer Extension

The uracil-containing DNA was used as a template in oligonucleotide-directed mutagenesis experiments: 200 ng single-stranded template DNA, 2-3 pmol phosphorylated oligonucleotide, 1 µL 10x hybridization buffer (20 mM Tris/HCl pH 7,4, 2 mM MgCl2, 50 mM NaCl) in a total volume of 10 µL were incubated for 2 min at 90°C and allowed to cool to room temperature. To the hybridization mixture 1 µL 10x synthesis buffer (5 mM dNTPmix, 100 mM Tris/HCl pH 7.5, 50 mM MgCl2, 20 mM DTT), 5 U T4-DNA Ligase (1 µL), 1 µg T4-Gen 32 Protein (0.5 µL) and 3 U T4-DNA Polymerase (1 µL) were added. The reaction was incubated for 5 min on ice, 5 min at 25 °C and finally for 90 min at 37°C. The reaction was stopped by adding 66 µL TE. 100 ng of double-stranded DNA product were used for transformation of E. coli. Resulting clones were chosen randomly for isolation of plasmid DNA which was analysed by sequencing.

2.3.4 Enzymatic Amplification of DNA by Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA (Mullis and Faloona, 1987). A multitude of applications have been developed including direct cloning from cDNA, in vitro mutagenesis and engineering of DNA, genetic fingerprinting of forensic samples, assays for the presence of infectious agents and analysis of allelic sequence variations. For long and accurate cDNA amplification LATaq ™ polymerase (TaKaRa) was used:

0.5 µL template cDNA
2 µL "sense" oligonucleotide, 10 pmol/µL
2 µL "antisense" oligonucleotide, 10 pmol/µL
5 µL 10x LA PCR buffer II (w/o MgCl2)
5 µL MgCl2, 25 mM
8 µL dNTP-Mix, 2.5 mM each
0.5 µL LA-Taq™ (5 U/µL)
ad 50 µL H_2O

PCR reactions were performed in an automated thermal cycler („Progene“, Techne). The following standard protocol was adjusted to the specific application:

first denaturation:	3 min 94°C
amplification 25-30 cycles:	1 min 94°C (denaturation)
	1 min 58°C (hybridization)
	1 min/ kb product 72°C (extension)
last extension:	7 min 72°C

10 µL from each reaction were electrophoresed on an agarose gel appropriate for the PCR product size expected. PCR products were subjected to isolation using the PCR purification kit (Qiagen).

2.3.5 DNA Sequencing

DNA sequencing was performed according to the "Big Dye Terminator Cycle Sequencing Protocol" (ABI). The following mix was subjected to a sequencing-PCR run:
0.5 µg DNA of interest
10 pmol oligonucleotide
4 µL Terminator Ready Reaction Mix
ad 20 µL H2O
25cycles: 30sec 94°C
15 sec 45-60°C
4 min 60°C
The sequencing products were purified by sodium acetate/ EtOH precipitation, dissolved in 20 µL template suppression reagent, denatured for 2 min at 90°C and analysed on a 310-Genetic Analyzer (ABI Prism).

2.3.6 cDNA Array Hybridization

Filters spotted with genes of interest (cloned into pBluescript SKII+) were a gernerous gift from J. Ruhe, cDNA probes of the various cell lines were from T. Knyazeva and generated according to standard molecular biology methods. Labeling of 3– 5 µL of cDNA was performed with the Megaprime kit (Amersham) in the presence of 50 µCi of [α-^{33}P] dATP. The prehybridization solution was replaced from filters by the hybridization solution containing 5x SSC, 0.5% (v/v)

SDS, 100 µg/mL baker yeast tRNA (Roche), and the labeled cDNA probe (2–5 x 10^6 cpm/mL) and incubated at 68°C for 16 h. Filters were washed under stringent conditions. A phosphorimager system (Fuji BAS 1000; Fuji) was used to quantify the hybridization signals. Average values for each slot were calculated using the formula: A = (AB - B) x 100/B; [A, final volume; AB, intensity of each slot signal (pixel/mm2); B, background (pixel/mm2)].

2.4 Methods in Mammalian Cell Culture

2.4.1 General Cell Culture Techniques

Cell lines were grown in a humidified 93% air, 7% CO_2 incubator (Heraeus, B5060 Ek/CO2) at 37°C and routinely assayed for mycoplasma contamination using a bisbenzimidestaining kit (Sigma). Before seeding cells were counted with a Coulter Counter (Coulter Electronics). SCC9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM): Ham´s F12 medium (1:1) containing 400 ng/mL hydrocortisone and 10% FCS. U373MG and NCI-H292 were cultured in Eagle´s Minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and 10% FCS. MDA-MB-231, MCF7, U373MG were cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FCS. HEK-293, Cos-7, Rat-1, 1321N1, T98G were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate and 10% FCS

2.4.2 Transfection of Cultured Cell Lines

2.4.2.1 Transfection of Cells with Calcium Phosphate

SCC9 cells or HEK-293 cells in six-well dishes were transfected transiently at about 70% confluency with a total of 2 µg DNA by using a modified calcium phosphate precipitation method as described previously (Chen and Okayama, 1987). In this protocol, a calcium phosphate-DNA complex is formed gradually in the medium during incubation with cells. The transfection mix of DNA and CaCl2 in water was prepared as follows:

Dish	6 Well	6 cm	10 cm
Area	10 cm^2	21 cm^2	57 cm^2
Volume of medium	1 mL	2 mL	4 mL
DNA in H_2O	2 µg in 90 µL	5 µg in 180 µL	10 µg in 360 µL
2.5 M $CaCl_2$	10 µL	20 µL	40 µL
2 x BBS (pH 6.96)	100 µL	200 µL	400 µL
Total volume	200 µL	400 µL	800 µL

To initiate the precipitation reaction the adequate volume of 2x BBS was added and mixed by vortexing. The reaction was incubated for 10 min at room temperature before being added to each well. Plates were placed in a humidified container at 3% CO2 overnight. One day following transfection, cells were serum-starved for 24 hours in standard cell culture medium without FCS. Transfection efficiency of SCC9 cells was typically about 50% as determined by LacZ staining after transfection of a LacZ-containing expression plasmid. For transfection of Phoenix cells HBS was used instead of BBS.

2.4.2.2 Transfection of Cos-7 Cells Using Lipofectamine®

Cos-7 cells were transiently transfected using Lipofectamine® (Gibco-BRL) essentially as described (Daub et al., 1997). For transfections in 6-well dishes, 1.0 mL of serum-free medium containing 10 µL of Lipofectamine and 1.5 µg of total plasmid DNA per well were used. After 4 h the transfection mixture was supplemented with an equal volume of medium containing 20% FCS and, 20 h later, cells were washed and cultured for a further 24 h in serum-free medium until lysis.

2.4.2.3 RNA Interference

Transfection of 21-nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO, USA) for targeting endogenous genes was carried out using Oligofectamine (Invitrogen) and 4.2 µg siRNA duplex per 6-well plate as previously described (Elbashir et al., 2001).

24 h after transfection, cells were serum-starved and assayed 3 d after transfection. Highest efficiencies in silencing target genes were obtained by using mixtures of siRNA duplexes targeting different regions of the gene of interest.

2.5 Protein Analytical Methods

2.5.1 Lysis of Cells with Triton X-100

Prior to lysis, cells grown to 80% confluence were treated with inhibitors and agonists as indicated in the figure legends. Cells were washed with cold PBS and then lysed for 10 min on ice in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/mL aprotinin. Lysates were precleared by centrifugation at 13000 rpm for 10 min at 4°C.

2.5.2 Determination of Protein Concentration in Cell Lysates

The "Micro BCA Protein Assay Kit" (Pierce, Sankt Augustin) was used according to the manufacturer's recommendations.

2.5.3 Immunoprecipitation and in vitro Association with Fusion Proteins

An equal volume of HNTG buffer was added to the precleared cell lysates that had been adjusted for equal protein concentration. Proteins of interest were immunoprecipitated using the respective antibodies and 20 µL of protein A-Sepharose for 4 h at 4°C. Alternatively, lysates were subjected to in vitro associations with either 3 µg of GST-Grb2 (Daub et al., 1997) or 2 µg of GST as control pre-bound to 30 µL of gluthathione-agarose beads. Precipitates were washed three times with 0.5 mL of HNTG buffer, suspended in 2× SDS sample buffer, boiled for 3 min, and subjected to SDS-PAGE.

2.5.4 SDS-Polyacrylamide-Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook, 1990). The following proteins were used as molecular weight standards:

Protein	MW (kD)	Protein	MW (kD)
Myosin	205.0	Ovalbumin	42.7
ß-Galaktosidase	116.25	Carboanhydrase	29.0
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozyme	14.4

2.5.5 Transfer of Proteins on Nitrocellulose Membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade, 1982) for 2 h at 0.8 mA/cm2 using a "Semidry"-Blot device in the presence of Transblot-SD buffer. Following transfer proteins were stained with Ponceau S (2 g/l in 2% TCA) in order to visualize and mark standard protein bands. The membrane was destained in water.

2.5.6 Immunoblot Detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25% gelatin for at least 4 h. The membrane was then probed with primary antibody (typically overnight). Antibodies were diluted 1:500 to 1:2000 in NET, 0.25% gelatin. The membrane was washed 3x 20 min in 1x NET, 0.25% gelatin, incubated for 1 h with secondary antibody and washed again as before. Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films or a digital camera unit. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 h at 50°C. Stripped membranes were blocked and reprobed with different primary antibody to confirm equal protein loading.

2.5.7 In Gel Digestion of the Proteins and Mass Spectrometry

Protein samples were separated on a NuPAGE® 4%-12% Bis-Tris gel (Invitrogen), following the manufacturers instructions, with up to 200µg of protein loaded on one lane. The gel was stained with Colloidal Blue (Invitrogen) to visualize the protein content. Each lane was cut into several pieces, depending on the experiment 2-6 slices, for gel- enhanced liquid chromatography-mass spectrometry (GeLC-MS) and reduced, alkylated and trypsin digested (Shevchenko et al. 1996).

Shortly, thoroughly cut gel pieces (~1mm^2) were transferred to 50% ethanol/25mM Ammonium bi carbonate (ABC) buffer and incubated on a shaker at room temperature until gel pieces were achromatized. After, gel pieces were dehydrated in 100% ethanol and dried in speed-vacuum centrifuge (Speed-Vac, Eppendorf Inc.). The dried gel pieces were reduced with 10mM DTT in 50mM ABC buffer for 1h at 56°C. Liquid was aspirated and gel pieces were soaked with 55mM IAA in 50mM ABC buffer in the dark for 45min at room temperature. Following, gel pieces were washed in 50mM ABC buffer for 20min before dehydration in 100% ethanol. The dehydration/re-hydration procedure was repeated and gel pieces were dried in the speed-vac. Proteins were finally digested by over night incubation in trypsin 50mM ABC buffer. Amount of trypsin varied depending on the experiment from 1/50-1/100 (w/w) of the protein mass. The next day, trypsin was quenched by adding 100% TFA to acidify the sample and the supernatant was kept. Peptides were extracted by 2x 30% ACN/3% TFA and 2x 100% ACN, and ACN was removed by evaporation in the speed-vac.

2.6 Biochemical and Cell Biological Assays

2.6.1 Stimulation of Cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 48 h cells were treated with inhibitors and agonists as indicated in the figure legends, washed with cold PBS and then lysed for 10 min on ice. In some cases cells were transfected 24 h after seeding and serum-starved two days following transfection before being stimulated as indicated above.

2.6.2 ERK1/2 Phosphorylation

For determination of ERK1/2 and Akt phosphorylation, approximately 20 µg of whole cell lysate protein/lane was resolved by SDS-PAGE and immunoblotted using rabbit polyclonal phospho-specific ERK/MAPK antibody. Akt phosphorylation was detected by protein immunoblotting using rabbit polyclonal anti-phospho-Akt antibody. Quantification of ERK1/2 was performed using the Luminescent Image Analysis System (Fuji). After quantification of ERK1/2 phosphorylation, membranes were stripped of immunoglobulin and reprobed using rabbit polyclonal anti-ERK1/2 or rabbit polyclonal anti-Akt antibody to confirm equal protein loading.

2.6.3 ERK/MAPK Activity

HA-ERK2 or endogenous ERK2 were immunoprecipitated from lysates obtained from six well dishes using 0.5 µg of anti-HA antibody or 0.4 µg of anti-ERK2 antibody, respectively. Precipitates were washed three times with HNTG buffer, and washed once with kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 200 µM sodium orthovanadate). Kinase reactions were performed in 30 µL of kinase buffer supplemented with 0.5 mg/mL myelin basic protein, 50 µM ATP and 1 µCi of [γ-32P] ATP for 10 min at room temperature. Reactions were stopped by addition of 30 µL of Laemmli buffer and subjected to gel electrophoresis on 15% gels. Labeled MBP was quantitated using a Phosphoimager (Fuji).

2.6.4 Autokinase Assay of Syk Activity

Syk was Immunoprecipitated by using anti-Syk antibody (4D10, Santa Cruz) from the freshly prepared lysates and washed thoroughly with HNTG buffer (6X). Immunoprecipitated samples were equilibrated with the reaction buffer (50 mM HEPES (pH 7.6), 10 mM $MnCl_2$, 2 mM $MgCl_2$, 100 mM $NaVo_3$). Then 2 mM ATP (cold) and 5 µl [γ-^{32}P] dATP was added to the immunoprecipitation mix with 40 µl of the reaction buffer. The reaction was performed at 30°C in constantly shaking condition. The reaction was stopped after 10 minutes by adding the lammeli buffer to the reaction mix. The sample was then resolved on the SDS-PAGE gel and blotted on the nitrocellulose membrane. The radioactive signal was detected with phosphorimager.

2.6.5 Flow Cytometric Analysis of Cell adapted from Prenzel et al., 1999.

In brief, cells were seeded, grown for 20 h and in some cases retrovirus infected as indicated. Upon serum-starvation for 24 h cells were treated with inhibitors and growth factors as indicated. After collection, cells were stained with ectodomain-specific antibodies against HB-EGF, TGFα or AR for 45 min. After washing with PBS, cells were incubated with FITC-conjugated secondary antibodies for 15 min and washed again with PBS. Cells were analysed on a Becton Dickinson FACS calibur flow cytometer.

2.6.6 Incorporation of 3H-Thymidine into DNA

U373-MG cells were seeded into 12-well plates (1.5 x 10^4 cells per well). Upon serum deprivation for 48 h, cells were subjected to pre-incubation with inhibitors before ligand treatment. After 18 h incubation, cells were pulse-labelled with ^{3}H thymidine (1 µCi/mL) for 4 h, and thymidine incorporation was measured by trichloroacetic acid precipitation and subsequent liquid-scintillation counting.

2.6.7 Migration

MDA-MB-231 cells in exponential growth were harvested, washed and re-suspended in standard medium without FCS. Cells were pre-incubated with either DMSO (control) or the inhibitors for 20 min. 2x10^5 cells were seeded into polycarbonate membrane inserts (6.5 mm diameter and 8 µm pore size) in 24-transwell dishes. The lower chamber was filled with standard medium without FCS containing the chemoattractant. Cells were permitted to migrate for 6 h. Following incubation, non-migrated cells were removed from the upper surface of the membranes. The cells that had migrated to the lower surface were fixed and stained with crystal violet. The evaluation of migrated cells was performed by counting the cells using a microscope.

Analysis of cell motility of SCC9 cells was performed as described before (Gschwind et al., 2002) using a modified Boyden chamber. 24 h after transfection with siRNAs SCC9 cells were seeded into polycarbonate membrane inserts (6.5 mm diameter and 8 µm pore size) in 24-transwell dishes at 1 x 10^5 cells/well in the presence or absence of agonist. The lower chamber was filled with standard medium without FCS containing 10 µg/mL fibronectin as chemoattractant. Cells were permitted to migrate for 36 h. Following incubation, non-migrated cells were removed from the upper surface of the membranes. The cells that had migrated to the lower surface were fixed and stained with crystal violet. The stained cells were suspended in 10% acetic acid; absorbance at 570 nm was measured in a micro-plate reader.

2.6.8 MTT Assay

In a 96-well flat bottom plate (Nunc, Naperville, Ill.) approximately 2,000 cells/100 µl of cell suspension were seeded. Upon serum-starvation for 24 h cells were incubated with inhibitors and growth factors as indicated for another 24h. MTT, a tetrazolium dye (3-[4, 5-dimethylthiazol-2-

y1]-2, 5-diphenyltetrazolium bromide; thiazolyl blue, SIGMA, St. Louis, MO) was added to each well to a final concentration of 1 mg/mL MTT. Plates were incubated in the presence of MTT for 4 Hrs. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which is soluble (DMSO, acidic acid, SDS) and absorbance was read at 570 nm on an micro-plate reader.

2.6.9 Apoptosis Assay

NCI-H292 lung cancer cells were seeded and grown for 20 h. Upon serum-starvation for 24 h cells were treated with Δ^{9}-Tetrahydrocannabinol as indicated for 6h. Cells were collected in assay buffer (1% sodium citrate, 0.1% Triton X-100) containing propidium iodide (PI) and incubated at 4°C for 3 h. Nuclear DNA staining was analysed on a Becton Dickinson FACS calibur flow cytometer.

2.7 Statistical Analysis

Student's t-test was used to compare data between two groups. Values are expressed as mean ± standard deviation (s. d.) of at least triplicate samples. $P < 0.05$ was considered statistically significant

3 Results

Syk Tyrosine Kinase has gained importance in cancer as a suppressor of invasive and aggressive nature of tumour cells. In this study, the experiments are focused on the functional analysis of Syk in the progression of breast cancer and glioblastoma. The first part elucidates the expression of Syk and its cellular localisation in breast cancer and glioblastoma cell lines. The second part describes the molecular characterization of Syk signalling, mediated upon growth factor stimulation or treatment with DNA damaging agent. Furthermore, the effect of Syk on physiological responses driven by growth factors or DNA damaging agents is also described. In the last part, Syk dependent protein interactions are shown and analysed for their biological relevance.

3.1 Syk Expression, Localisation and Signalling Properties in Human Cancer Cell Lines

Syk expression is correlated with suppression of tumour growth and invasiveness in breast cancer (Coopman et al., 2000; Toyoma et al., 2000; Wang et al., 2004). To correlate the expression of Syk with invasiveness of breast cancer cell lines, the cell lines were cultured and lysed to extract the total protein. As shown in Fig. 9, the breast cancer cell lines are grouped based on their Syk expression. Syk expression is remarkably lost in many of the cell lines described metastatic by ATCC including classical metastasis model cell lines MDA-MB-231 and MDA-MB-435s.

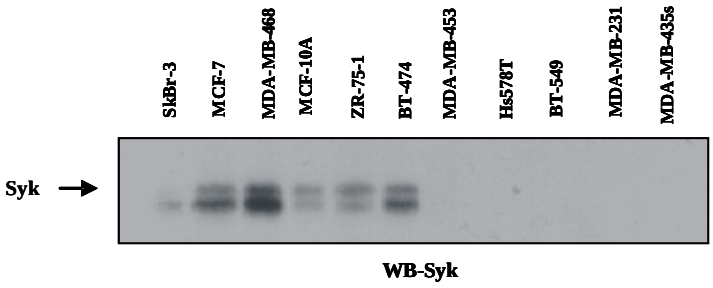


Figure 9 Syk expression in different breast cancer cell lines. Different breast cancer cell lines were utilised to analyse the Syk expression. Cells were lysed and total cell lysates were resolved on the SDS-PAGE and probed by anti-Syk antibodies.

Moreover, Syk expression is also reduced to an undetectable level in nine of the ten glioblastoma cell lines tested by western blotting (Fig.10). Amongst the glioblastoma cell lines analysed, it was observed that the only Syk expressing cell line is SF-767.

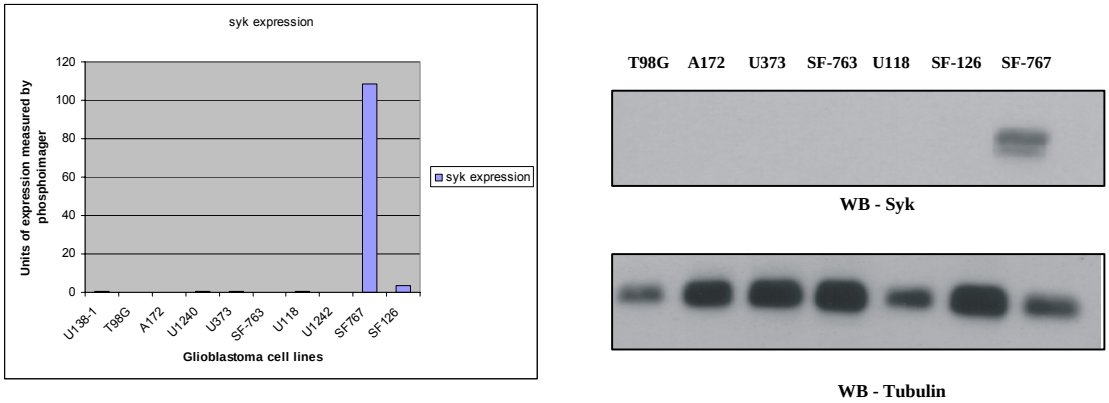


Figure 10 The expression analysis of Syk in Glioblastoma. Syk expression in ten cell lines of Glioblastoma was tested by microarray (fig. 7a). Seven cell lines were further tested for the protein expression by utilising cell lysates.

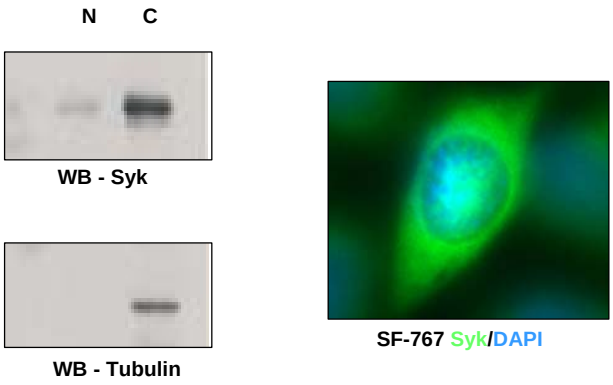


Figure 11. Syk is partially translocated to the nucleus. SF-767 cells were subjected to the cellular fractionation and immuno staining by using anti Syk antibody. The green fluorescence is reflected from Syk and the blue region is stained by DAPI and represents nucleus. N designates the nuclear fraction and C the cytoplasmic fraction.

Furthermore, Syk maintains high phosphorylation levels in SF-767 as it remains tyrosine phosphorylated to a great extent (Fig. 27). Syk phosphorylation in breast cancer was undetectable in phosphotyrosine blots and can only be detected in autophosphorylation assays (Fig.12). The localisation of Syk was analysed in the cancer cell line SF-767 by using immunofluorescence assay and western blotting of nuclear fractions. Syk was found to be partially localised in both the nucleus and the cytoplasm (Fig. 11). Syk was found to be translocated into the nucleus in the

breast cancer cell line MDA-MB-231 after ectopic expression of Syk which is confirmed by western blot analysis of nuclear fractions of MDA-MB-231 cells (Fig. 12). As Syk phosphorylation is not detectable by phosphotyrosine antibody in breast cancer cells, the cytoplasmic and nuclear fractions were subjected to autokinase assays using radioactive [γ ^{32}P] ATP in an *in vitro* kinase assay. The activity of Syk in nuclear and cytoplasmic extracts was found to be comparable. Therfore, it could be concluded from here that Syk is similarly active in the nucleus as it is in the cytoplasm.

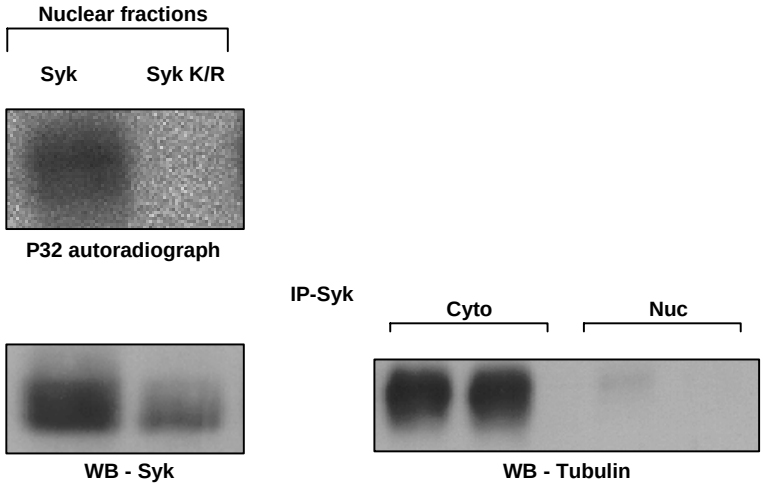


Figure 12 Syk kinase activity is not altered in the nucleus. MDA-MB-231 cells were transfected with pCDNA3-Syk and pCDNA3-Syk K/R (kinase inactive) constructs for their stable expression in these cells. Syk or Syk K/R expressing clones were further subjected to cellular fractions and assayed for kinase activity of Syk in an autokinase assay by utilising radioactive [γ ^{32}P] ATP. Nuc designates the nuclear fraction and Cyto the cytoplasmic fraction.

3.2 Syk Expression in MDA-MB-231 and MDA-MB-435s Leads to Reduced Invasivity

The breast cancer cell line MDA-MB-231 is highly invasive and Syk is abrogated in these cells due to the methylation of its promoter (Yuan et al., 2001; Goodman et al., 2003; Dhillon et al., 2004; Wang et al., 2004; Wang et al., 2005; Yuan et al., 2006 Muthuswamy et al., 2006). Therefore, ectopic expression of Syk and a kinase inactive mutant (Syk K/R) was achieved in this cell line to study its role in invasiveness. Syk expression and activity of Syk and Syk K/R expressing cells was tested by an *in vitro* autokinase assay and western blotting using anti-Syk

antibody (4D10, Santa cruz) (Fig. 12). Syk and Syk K/R expressing cells were seeded in matrigel, which consists of Extracellular Matrix (ECM) constituents, to study their invasive growth. MDA-MB-231 cells transfected with pcDNA3 vector alone (Mock) were grown in matrigel. Their growth in matrigel resembled branching which is marked with the movement of the cells out of the cellular cluster in a streamline manner. Syk K/R expressing cells also showed similar behaviour as of Mock expressing MDA-MB-231 cells but Syk expressing MDA-MB-231 cells were grown in matrigel in a strikingly different amoeboid like rounded morphology and showed no clustering (Fig. 13). The branching of the cells in the matrigel depends on the migration potential of cells and their ability to degrade matrigel constituents. Moreover, matrigel outgrowth of the cancer cells is a widely accepted model to study metastatic behaviour of cells. Therefore, lack of branching phenotype of Syk expressing MDA-MB-231 cells points to the lack of migratory abilities and their inability in degrading the ECM. These properties can be critical in the body during metastatic progression of cancer cells.

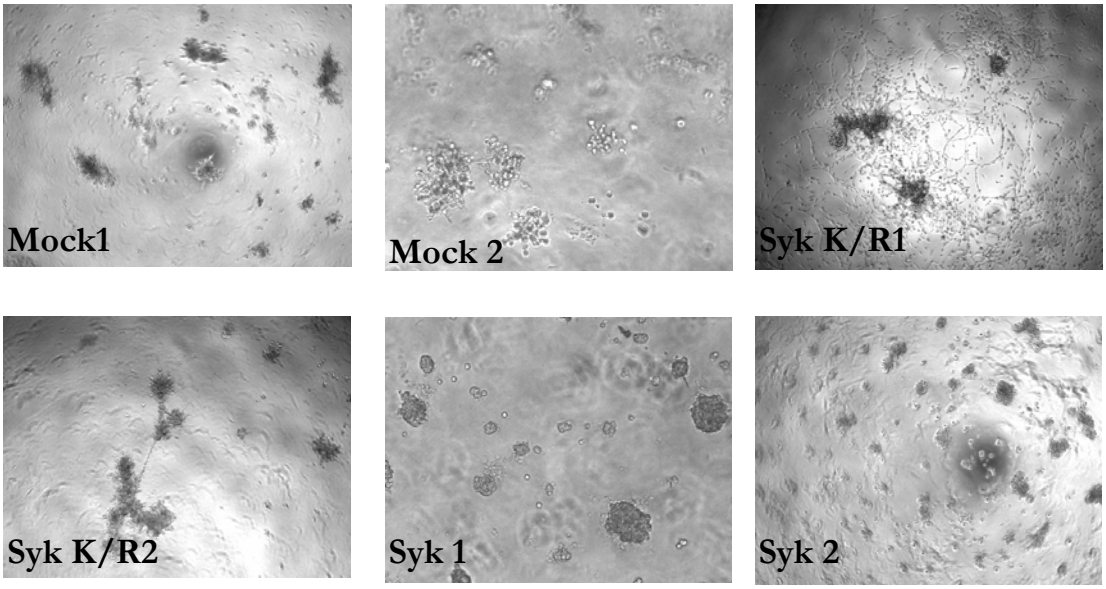


Figure 13 Matrigel outgrowth of MDA-MB-231 cells with stable expression of Syk. MDA-MB-231 cells were transfected with Syk, Syk k/R and vector alone and were seeded in equal number on matrigel.The pictures were taken after 24 Hrs of growth in normal culture conditions.

A Similar behaviour could also be observed with MDA-MB-435s breast cancer cells, strengthening the observations obtained from MDA-MB-231 (Fig. 14). As mentioned above, the behaviour of branching and streamline movement has been a qualitative sign of invasiveness and

therefore, it could be inferred from this experiment that ectopic Syk expression in the invasive breast cancer cells weakens their invasive potential when compared with Mock and K/R expressing cells.

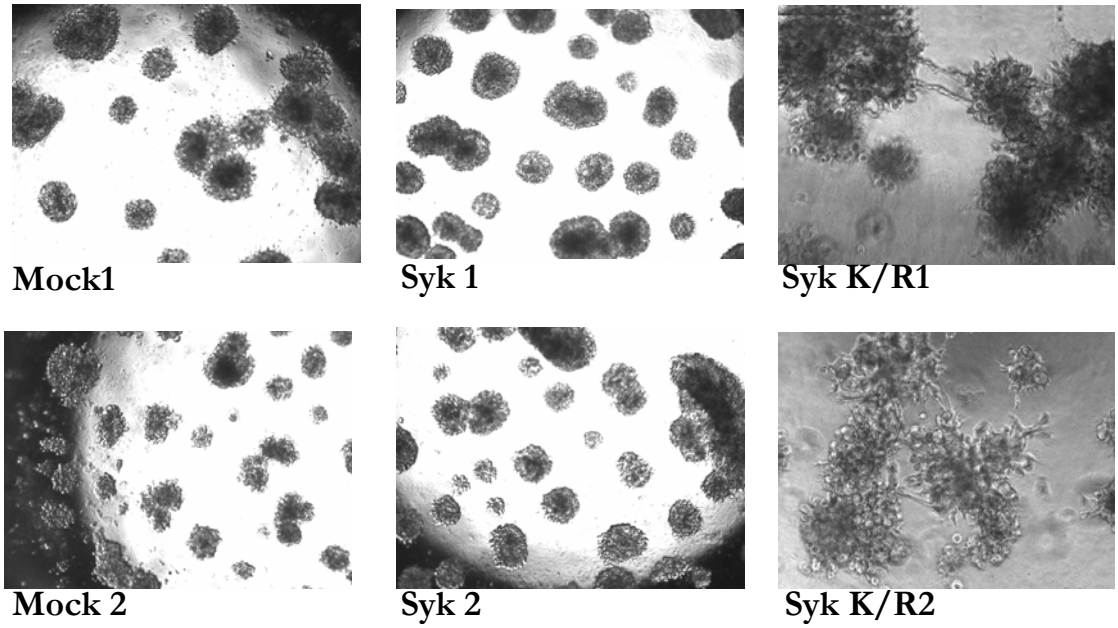


Figure 14 Matrigel outgrowth of MDA-MB-435s cells with stable expression of Syk. MDA-MB-435s cells were transfected with pcDNA3-Syk, pcDNA3-Syk K/R or vector alone and were seeded in equal number on matrigel.The pictures were taken after 24Hrs of growth in normal culture conditions.

3.3 Syk expression in MDA-MB-231 Counteract the Macrophage Stimulated Invasivity

Macrophages are cells of myeloid origin and infiltrate the growing tumour as an inflammatory response to it. Macrophages are associated with metastasis and have been shown to provide anti-apoptotic and angiogenic potential to the growing tumour (Lewis CE & Pollard J, 2006). A macrophage cell line (MAD-NT which stands for Macrophage line Adherent and Differentiated – Non Terminaly) was derived from HL-60 (an Acute Myeloid Leukemia cell line from ATCC) by subculturing methods. Briefly, the spontaneously differentiating adherent HL-60 cells were selected by continuous passaging (Pjotr Knyazev, unpublished). To test if MDA-MB-231 cells are responsive to macrophages, MAD-NT conditioned medium was applied to MDA-MB-231 cells growing in matrigel. Invasivity of MDA-MB-231 was highly enhanced when MAD-NT conditioned medium was applied to them and they showed increased branching as compared to

MDA-MB-231 cells growing in normal medium in matrigel. To study if Syk expression in MDA-MB-231 could also suppress the invasiveness observed in matrigel, Syk expressing MDA-MB-231 and Syk K/R expressing MDA-MB-231 cells were subjected to similar experimental conditions as mock transfected MDA-MB-231 cells. Syk K/R expressing cells showed similar behaviour as mock transfected cells but Syk expressing cells did not display any branching (Fig. 15).

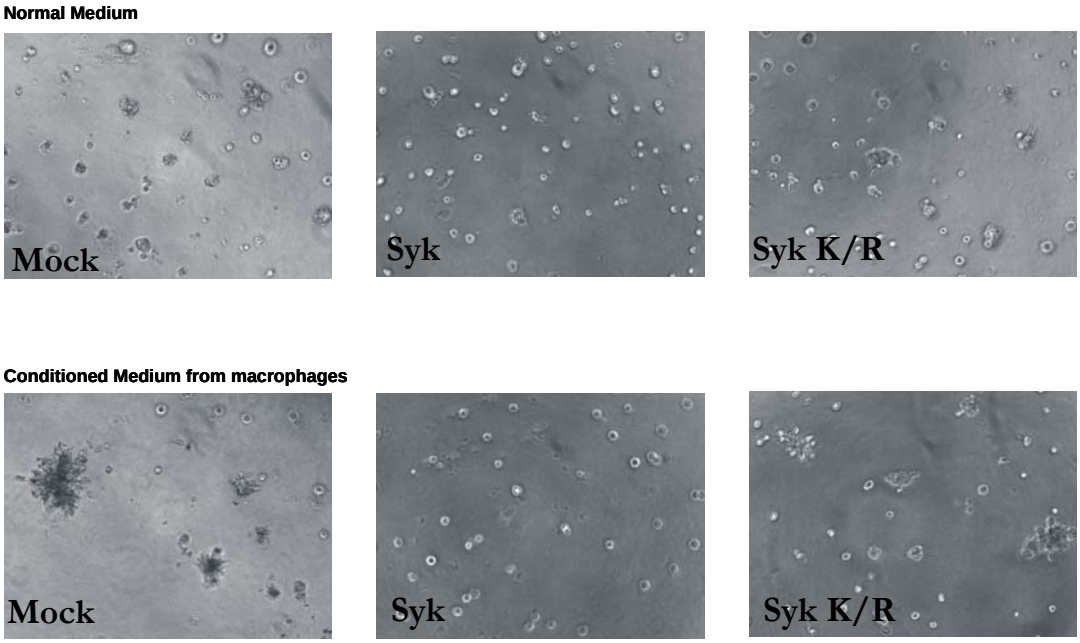


Figure 15 Macrophage dependant matrigel outgrowth of MDA-MB-231 cells with stable expression of Syk. MDA-MB-231 cells were transfected with pcDNA3-Syk, pcDNA3-Syk K/R or vector alone and were seeded in equal number on matrigel.The pictures were taken after 24 Hrs of growth in normal culture conditions. To test the effect of macrophages, conditioned medium derived from MAD-NT cells was applied on the MDA-MB-231 cells growing in matrigel. As it can be seen the Syk expressing cells shows clusterd cells in comparision to the spreaded and branching behaviour shown by Mock or K/R expressing cells. Moreover, the supplement of MAD-NT conditioned medium stimulates the invasiveness of the Mock or Syk K/R expressing cells

3.4 Syk Expression Leads to Modulation of Gene Expression

As it was observed in previous experiments, Syk was partially translocated to the nucleus. This prompted the question whether Syk acts as a transcription modulator. Interestingly, it has been mentioned in previous studies that Syk translocation to the nucleus could be an essential requirement for its role as a tumor suppressor (Wang et al., 2003; Wang et al., 2005). To test the

role of Syk in gene regulation Syk and Syk K/R expressing clones of MDA-MB-231 were utilised to extract total RNA and transcribe it into radioactive cDNA by using [α-^{33}P] dATP. The ^{33}P labeled cDNA samples from mock transfected or Syk and Syk K/R expressing MDA-MB-231 cells were subjected to macroarray analysis (see material and methods)

Down regulated Genes	Up regulated Genes
APRIL – A Proliferation Inducing Ligand	**MST – 1, Mammalian Sterile 20 Kinase-1**
MMP1 - Matrix Metalloprotease-1	**CK18 – Cytokeratine 18**
MMP14 – Matrix Metalloprotease 14	**PAI – Inhibitor of Plasminogen Activator**
MKP-3/Dusp-6 – MAP Kinase phosphatase-6	
PP1a and PP2a – Protein phosphatase-1a & 2a	

Table 2 The list of genes with modulated expression in the MDA-MB-231 cells with stable expression of Syk. The changes in gene expression were measured by using the AIDA vision programme from the phosphoimager plates. Genes differences in Syk expressing MDA-MB-231 as compared to Syk K/R or Mock expressing cells were selected as modulations occurred due to Syk expression.

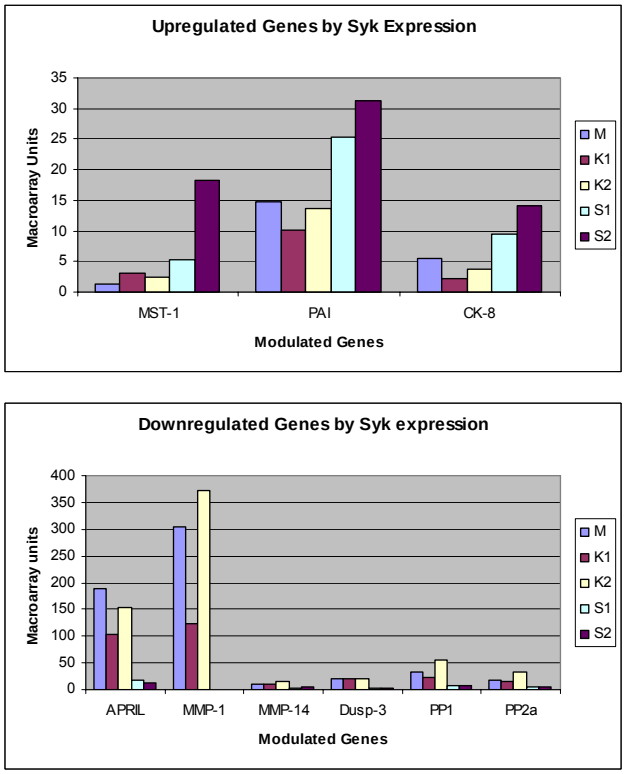


Figure 16 The genes modulated by the Syk expression in MDA-MB-231. The MDA-MB-231 stably expressing Syk and Syk K/R constructs were utilised for Macroarray analysis. The histogram shows the expression levels of genes which showed simmilar pattern in Mock and Syk K/R but differs in Syk expressing cells.

Syk expressing MDA-MB-231 showed modulation in gene expression when compared with the mock transfected or Syk K/R expressing MDA-MB-231 cells. The genes which were modulated by Syk expression are enlisted in table 2. Interestingly, the genes listed in table 2 are known to be involved in tumour progression and *in vitro* cell invasivity. Furthermore, Matrix Metalloprotease 1 (MMP1) was of interest in this analysis as it is a proven agonist of invasiveness and metastasis in cancer (Boire et al., 2005). Therefore, MMP1 expression in the cells expressing Syk and its kinase inactive mutant was further evaluated by RT-PCR (Fig. 17). It was observed that Mock transfected and Syk K/R expressing MDA-MB-231 or MDA-MB-435s cells express high levels of MMP1 whereas expression was significantly reduced in the Syk expressing MDA-MB-231 or MDA-MB-435s cells.

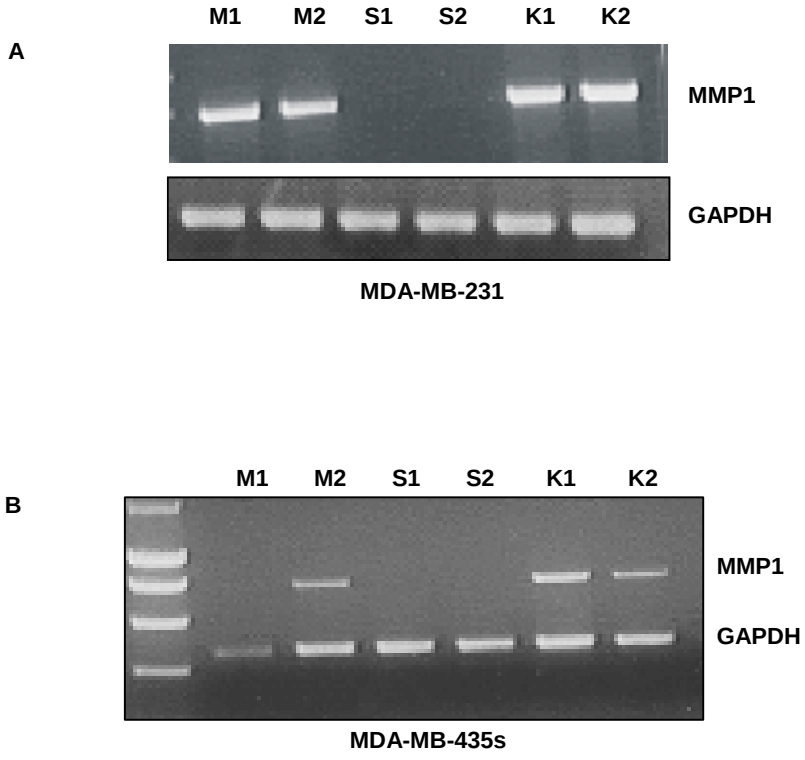


Figure 17 Modulations in MMP1 expression in MDA-MB-231 and MDA-MB-435s cells upon Syk overexpression: MDA-MB-435s (A) and MDA-MB-231 (B) cells overexpressing Syk (S), Syk k/R (K) and Mock (M) constructs were utilised for extraction of total RNA which was further subjected to semiquantitative PCR analysis of MMP1 transcripts.

3.5 MMP1 Inhibition in MDA-MB-231 is Independent of EGFR Signalling

Once we confirmed changes in gene expression, the next question to answer was the molecular mechanism for this phenomenon. It was shown previously that Syk can attenuate EGFR signalling in normal breast epithelium (Ruschel and Ullrich, 2004). Therefore, it was important to investigate if Syk mediated inhibition of MMP1 expression also coincides with EGFR signalling. EGFR signalling, as it has been described earlier, leads to MAPK activation which relays the growth factor signal to the nucleus to modulate gene expression changes and cell cycle progression (Citri and Yarden, 2006). Therefore, both EGFR and MAPK signalling were blocked using the tyrphostins AG1478 and PD98059 respectively to test their effect on MMP1 expression by RT-PCR in MDA-MB-231 cells. Piceatannol which is a relatively specific inhibitor of Syk was used to confirm the role of Syk in the MMP1 down regulation. Interestingly neither EGFR inhibition nor MEK1 inhibition could suppress MMP1 expression at various time points as shown in Fig. 18. Moreover, Syk inhibitor could not raise the MMP1 expression to the basal levels in Syk expressing MDA-MB-231 cells which might reflect either the non-specificity of the inhibitor or consequences of Syk overexpression.

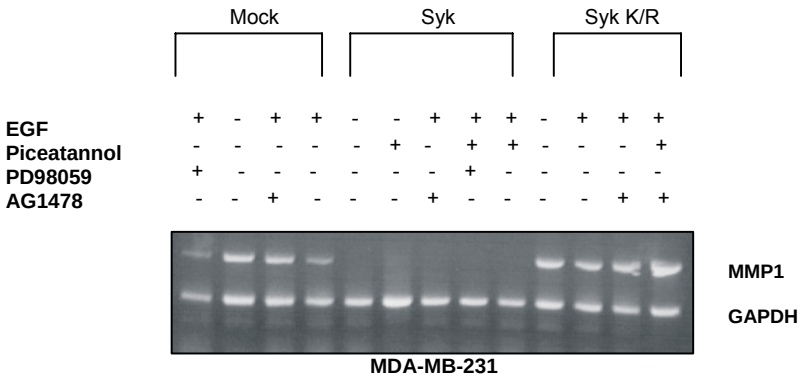


Figure 18 Pharmacological inhibition of the EGFR pathway does not affect MMP1 expression. The MDA-MB-231 cells overexpressing pcDNA3-Syk construct were treated with different inhibitors. EGFR pathway was blocked by inhibition of EGFR (AG1478, 250 nM) or by inhibition of MAPK pathway (PD98059, 100 nM). The Syk inhibitor (Piceatannol, 10 μM) was also utilised to assess the role of Syk activity in the regulation of MMP1. The cells were utilised to extract total RNA which was later utilised to test the expression of MMP1.

In fact, Syk expressing MDA-MB-231 cells do not show a reduction in EGFR activation or its signalling via the MAPK pathway although EGFR basal activity was reduced (Fig. 24).

3.6 Doxorubicin Induces Rapid Tyrosine Phosphorylation of Syk

Doxorubicin is one of the most common chemotherapeutics used in breast cancer treatment. This anthracycline is a DNA damaging agent and causes inhibition of Topoisomerase II apart from other effects including its intercalation in between DNA bases (Cutts et al., 2005). To test if Syk expression causes sensitivity of breast cancer to the Doxorubicin, Syk expressing MDA-MB-231 cells were treated with 1µg of Doxorubicin for 24 Hrs. Cells were then lysed and subjected to immunoprecipitation with anti-Syk antibodies and were later probed with phosphotyrosine antibodies by immunoblot analysis. As shown in Fig. 19, Syk samples show very high phosphorylation signals as compared to the kinase inactive Syk K/R mutant. Moreover, Syk and Syk K/R protein levels increase upon Doxorubicin treatment. To test if the Syk tyrosine phosphorylation is an early event, Doxorubicin was added to the culture for different periods of time. Syk was found to be activated within 4hrs of Doxorubicin treatment.

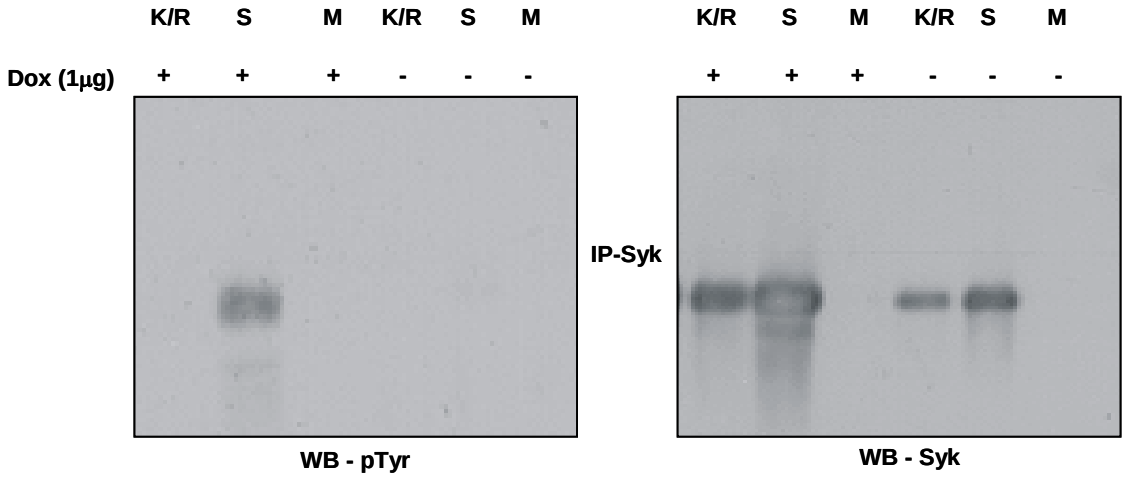


Figure 19 Doxorubicine induces Syk phosphorylation in MDA-MB-231. MDA-MB-231 cells expressing Syk (S), Syk K/R (K/R) or Mock (M) constructs were treated with Doxorubicin for 24 hours. The cell lysates were utilised for Syk immunoprecipitation and tested for the phosphotyrosine levels by using 4G10 antibody against pTyr residues. To check the amount of Syk, the blots were reprobed with anti-Syk antibodies (4D10).

3.7 Doxorubicine-Induced Tyrosine Phosphorylation of Syk is a Result of its Increased Kinase Activity

Syk is a cytoplasmic tyrosine kinase which is known to autophosphorylate on tyrosine 525 and tyrosine 526 (Couture et al., 1997; Furlong, 1997). As shown in Fig. 20, wild type Syk gets phosphorylated upon doxorubicin treatment in contrast to Syk K/R.

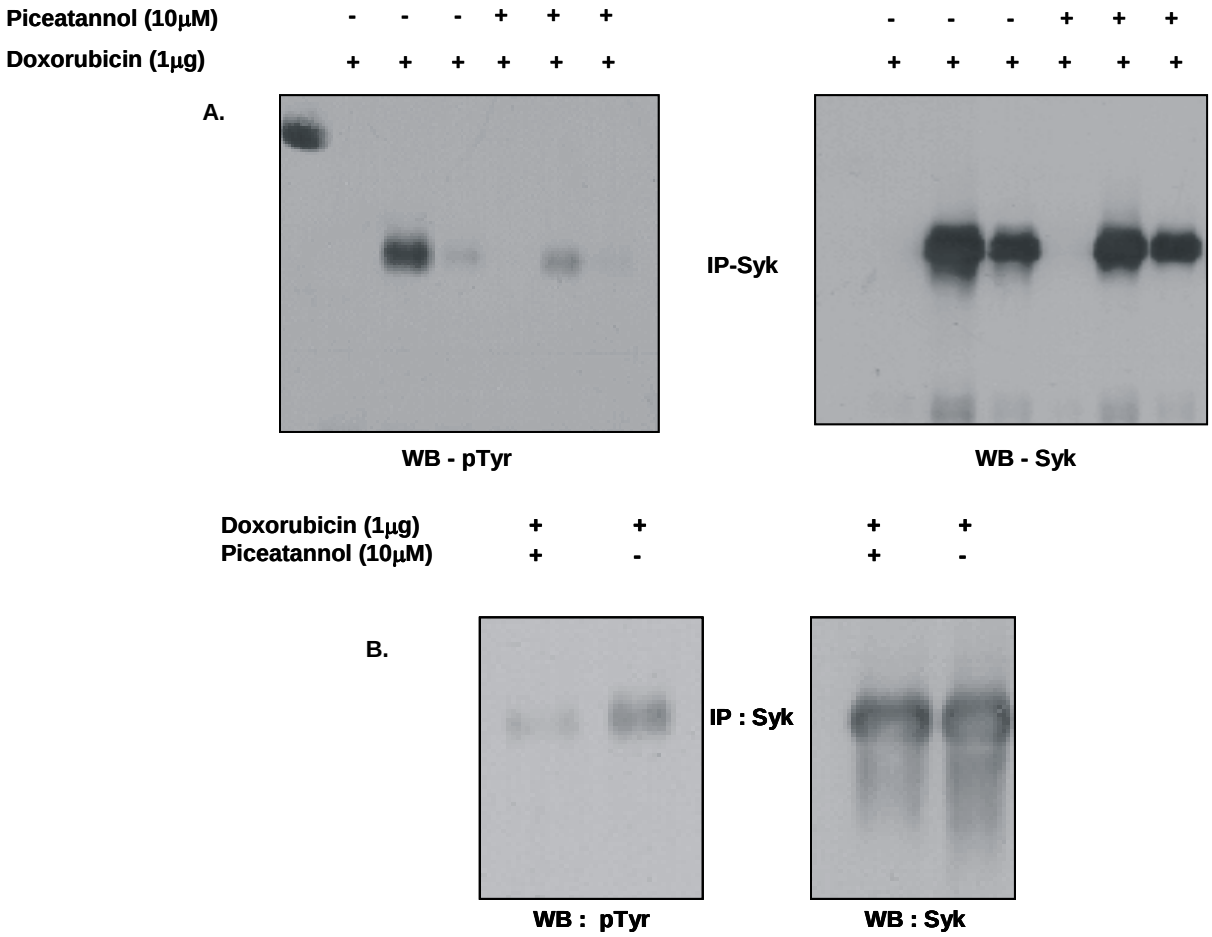


Figure 20 Doxorubicin induced tyrosine phosphorylation of Syk is due to autophosphorylation. Syk tyrosine phosphorylation upon Doxorubicin treatment was tested further by using piceatannol. The MDA-MB-231 cells overexpressing pcDNA3-Syk construct were pretreated with 10 μM of piceatannol for 30 minutes which was followed by Doxorubicin (1 μg) treatment for 24 Hrs (A) and 4 Hrs (B). The lysates were probed for phosphorylated Syk.

This suggests that Syk phosphorylation could be the result of autophosphorylation and thereby points at increased kinase activity upon Doxorubicin treatment. To further confirm this mechanistic point, the Syk inhibitor piceatannol was applied to the cells prior to the doxorubicin

treatment. This experimental treatment reduced the Syk phosphorylation signal significantly, strongly suggesting that Doxorubicin-induced phosphorylation is the result of autophosphorylation.

3.8 Doxorubicin Induces a Post Translational Modification of Syk Similar to Polyubiquitination

Doxrubicin treated Syk and Syk K/R lysates showed a higher molecular weight species when probed with anti-Syk antibodies, which was absent in mock MDA-MB-231 cells (Fig. 21). Syk has been shown to undergo polyubiquitination followed by degradation when activated in haematopoietic and myeloid cells (Sohn et al, 2003; Dangelmaier et al., 2005). Therefore, it was interesting to test in Syk expressing MDA-MB-231 cells, if Syk phosphorylation follows such a post-translational modification and degradation.

To test that, Doxorubicin treated Syk and Syk K/R expressing MDA-MB-231 cell lysates were subjected to immunoprecipitation with Syk antibodies. A high molecular weight protein was pulled down with anti-Syk antibody which was only present in Syk expressing MDA-MB-231 cells but not in Syk K/R or mock transfected MDA-MB-231 cells. It appeared therefore that the high molecular weight protein generated in response to Doxorubicin treatment is Syk as it was also detected in the control western blots probed by anti Syk antibody and did not express in mock transfected MDA-MB-231 cells (Fig. 21A).

Moreover, the putative high molecular weight Syk (≈ 116 kDa and ≈ 145 kDa) was also detected by pTyr antibody which suggests that the putative high molecular weight Syk is phosphorylated. Therefore, this result suggests a phosphorylation-dependent post-translational modification of Syk upon doxorubicin treatment. When the immunoprecipitates of Syk were probed with ubiquitin antibody, Syk was detected in the doxorubicin treated samples (Fig. 21B). This further suggests that Syk might undergo polyubiquitination upon Doxorubicin treatment.

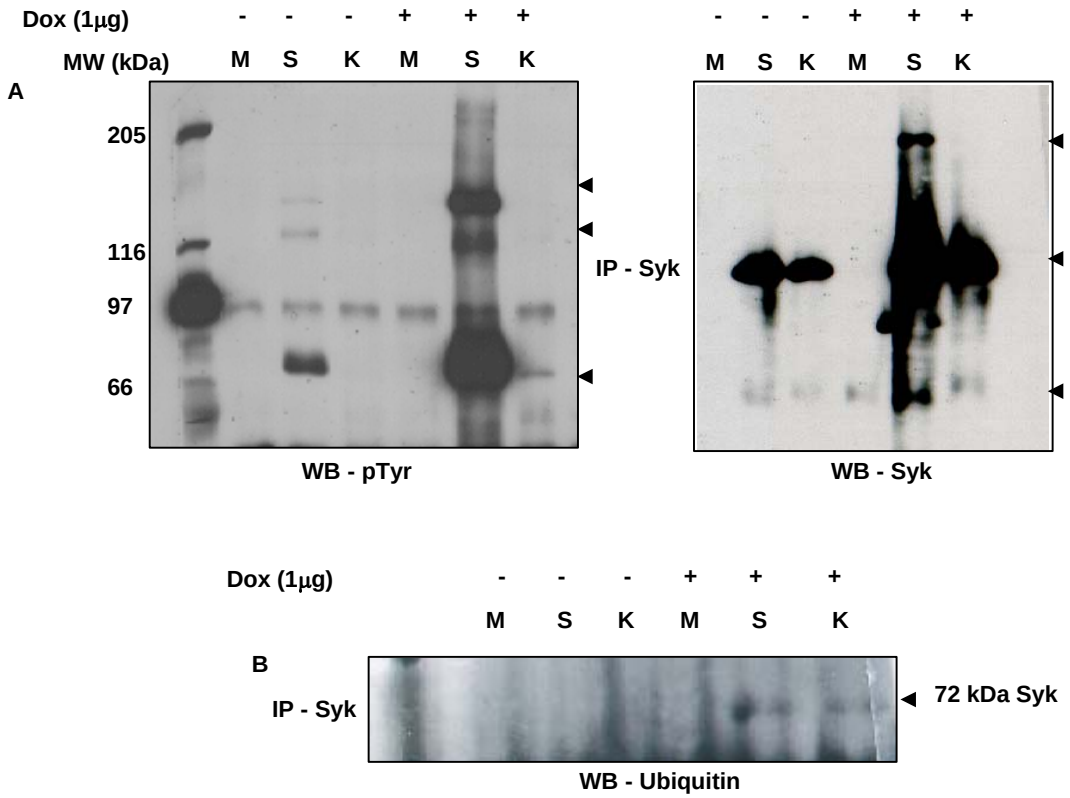


Figure 21 Syk undergoes high molecular weight modification upon Doxorubicin treatment: MDA-MB-231 cells overexpressing pcDNA3-Syk (S), pcDNA3-Syk K/R (K) and pcDNA3-Mock (M) constructs were treated with Doxorubicin for 24 hrs. The Syk immunoprecipitates were probed with anti pTyr, anti Syk (A) as well as anti Ubiquitin antibodies (B). The arrowheads denote Syk and its putative post-translationaly modified forms.

3.9 Doxorubicin Induced Syk Phosphorylation Potentiates Syk Interaction with EGFR

Doxorubicin treatment induces the phosphorylation of Syk which increases the possibility that Syk signalling and its interaction with its signalling partner could be induced upon Doxorubicin treatment. As it is shown in an earlier study (Ruschel and Ullrich, 2004), Syk interacts with EGFR and decreases its phophorylation. Therefore, it was important to ask whether Syk interacts with EGFR upon doxorubicin treatment in MDA-MB-231 cells. Immunoprecipitates obtained from Syk and Syk K/R expressing MDA-MB-231 cells, treated or untreated with doxorubicin, by using anti-EGFR antibodies were probed with anti Syk antibodies. As shown in figure 22, Syk coprecipitated with EGFR upon treatment of Syk expressing MDA-MB-231 cells with

Doxorubicin. Moreover, this interaction seemed to be dependent on Syk phosphorylation as Syk K/R was not coprecipitated by anti-EGFR antibodies.

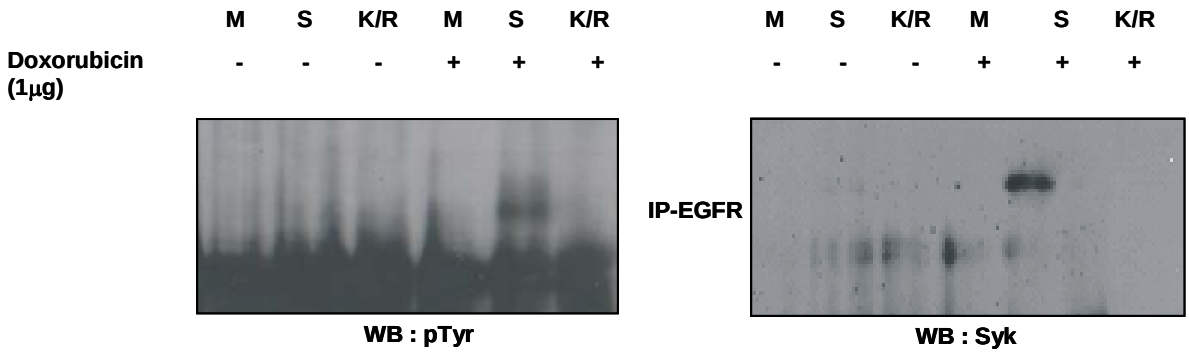


Figure 22 Doxorubicin induces Syk interaction with the EGFR. The MDA-MB-231 cells overexpressing pcDNA3-Syk (S), pcDNA3-Syk K/R (K/R), and pcDNA3-Mock (M) constructs were treated with Doxorubicin for 24 hours. The cellular lysates were utilised for the EGFR immunoprecipitations and probed with the phosphotyrosine and subseqquently with the Syk antibodies.

3.10 Syk Expression in MDA-MB-231 Results in Impeded Proliferation

Proliferation is an inherent property of tumour cells and causes the early growth of the tumour mass. Mitotic control abnormalities and loss of cell contact inhibition are critical for tumourogenesis. It has been shown in the primary tumour samples as well as in cell cultures that loss of Syk expression leads to a higher proliferation rate (Moroni et al., 2004). Therefore, Syk expressing MDA-MB-231 cells were tested for their proliferation potential in dependence of Syk expression. Syk expressing cells showed a reduction in their proliferation rate to almost 50% as compared to the mock transfected and Syk K/R expressing MDA-MB-231 cells. Additionally, Syk K/R did not show any significant increase in the proliferation rate than mock transfected MDA-MB-231 cells. This shows that Syk expression may indeed negatively influence the proliferation of breast cancer cells.

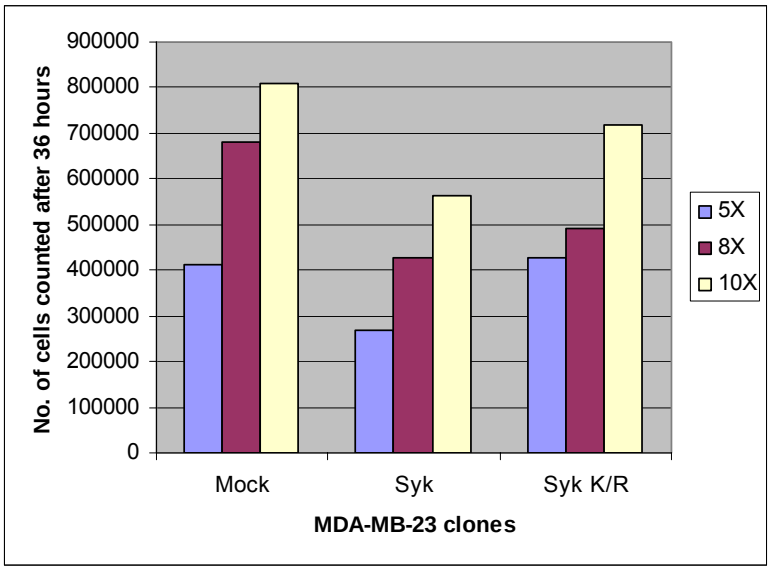


Figure 23 The proliferation potential of MDA-MB-231 is reduced upon ectopic Syk expression. The MDA-MB-231 cells expressing pcDNA3-Syk, pcDNA3-Syk K/R or pcDNA3-Mock constructs were seeded in three different densities i.e., 5X, 8X and 10X of 200,000 cells in 10 cm plates, and counted after 36 hours of culture in the Standard culture conditions (7% CO2, 37 °C).

3.11 Negative Regulation of EGFR by Syk is Enhanced by Doxorubicin-Induced Syk Activation

Syk expression in breast epithelium is shown to reduce EGFR activity and thereby reduces tumour cell proliferation as well as apoptotic potential (Ruschel and Ullrich, 2004) (Fig. 23). Therefore, once the gene expression changes and proliferation rate of Syk expressing cell lines was determined, the probable role of EGFR signalling in such changes was evaluated. Syk expressing MDA-MB-231 cells were subjected to treatment with EGF or Doxorubicin for different time intervals and tested for EGFR activation. As it can be seen in Fig. 24, the EGF treatment increased EGFR activation in mock transfected cells but to a significantly higher extent in Syk or Syk K/R expressing cells. Interestingly, Syk expression reduced only the basal levels of EGFR phosphorylation in MDA-MB-231 cells. As we showed earlier that Doxorubicin induced Syk activity in MDA-MB-231 cells, it was interesting to examine whether Syk activation is able to decrease EGFR activity further. Syk activation indeed was able to decrease the EGFR activity further in comparison to the mock transfected cells which surprisingly showed higher phosphorylation levels with Doxorubicin treatment.

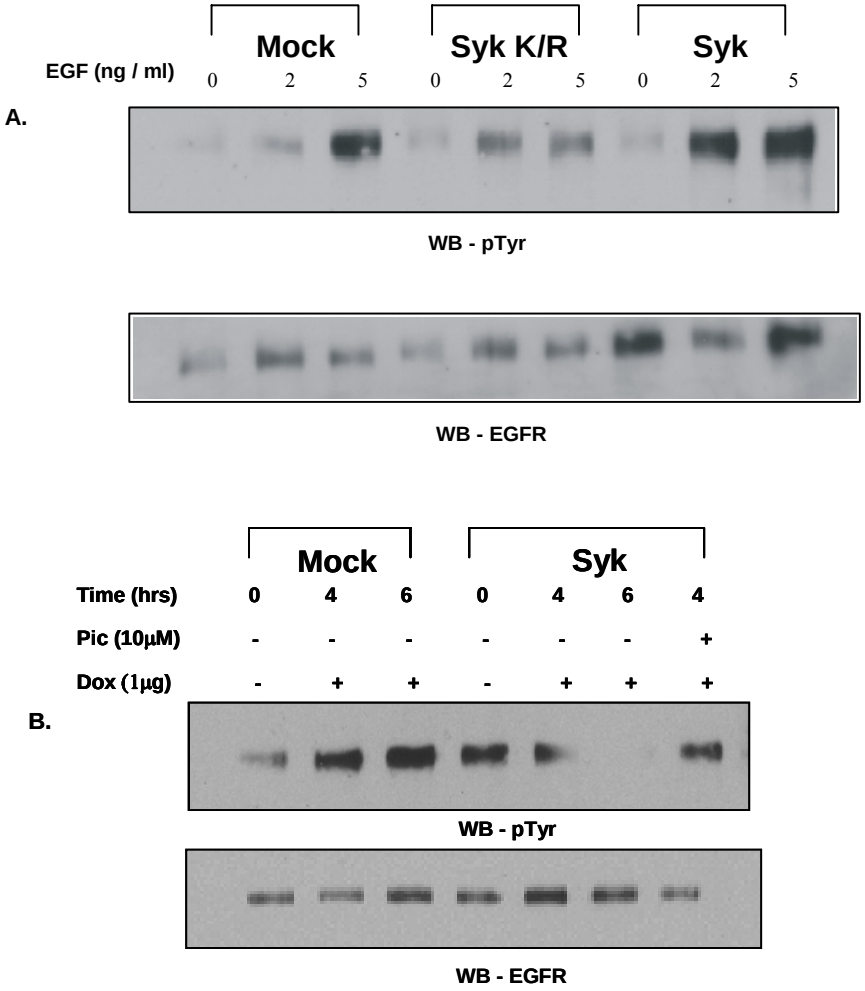


Figure 24 Effect of Syk expession on EGFR tyrosine phosphorylation with or without doxorubicin. MDA-MB-231 cells expressing Syk, Syk K/R and Mock constructs were treated with EGF (10 ng/ml, Fig. 24 A) and Doxorubicin (1 μg/ml Fig. 24 B) separately and tetsed for EGFR activation. The cell lysates were subjected to immunoprecipitation by anti EGFR antibody and probed with anti pTyr and anti EGFR antibodies.

To substantiate the role of Syk in EGFR dephosphorylation, MDA-MB-231 cells overexpressing Syk were pre-treated with piceatannol for 30 minutes. The doxorubicin treatment could not reduce the EGFR phosphorylation in piceatannol treated cells which underscores the role of Syk in EGFR dephosphorylation.

3.12 Syk is Activated in Glioblastoma by Various Growth Factors in dependence of High Cell Density

Cell density in tissue culture causes cell cycle arrest by various mechanisms including inactivation of RTKs e.g., EGFR (Perrais et al., 2007). Cell-cell contacts are formed by cadherins which are present on the cell membrane. These cadherins are also responsible for sensing cellular density and thereby mediating the signal to the cell in the form of activation of phosphatases or upregulation of cell cycle inhibitors e.g., p27 (Fagotto and Gumbiner, 1996; Abercrombie, 1979; Polyak et al., 1994). SF-767 is a fast growing glioma-derived cell line and gets rapidly confluent in culture. While studying the signalling aspects of Syk, it was observed that Syk is activated by various growth factors or lipid moieties when cells are sparsely grown (Fig. 25).

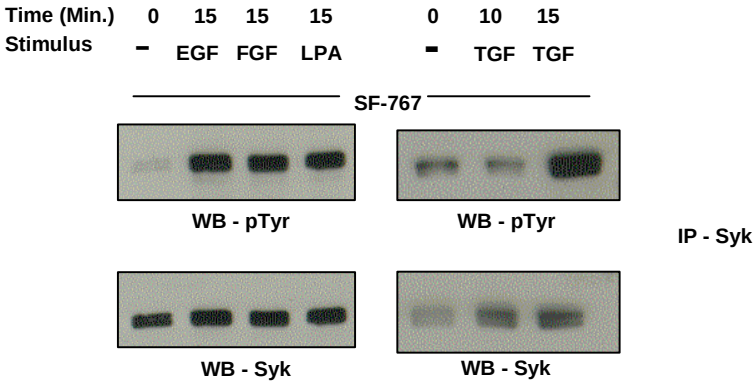


Figure 25 Syk is activated in glioblastoma by various growth factors. SF-767 cells were treated with EGF (20 ng/ml), FGF (100ng/ml), LPA (2.5 μm) and lysed for Syk immunoprecipitation. Immunoprecipitated samples were probed with anti-phosphotyrosine antibody.

Furthermore, high cell density cultures abrogate activation of Syk by any growth factors which suggest that cell-cell contact might play a role in signalling mediated by Syk (Fig. 26). TGF β is secreted by glioblastoma in an autocrine manner and suppresses tissue growth (Gold, 1999). Therefore, we investigated whether TGF β signalling affects Syk function. As shown in Fig. 25 Syk was activated by TGF β in SF-767 cells grown in serum deprived medium.

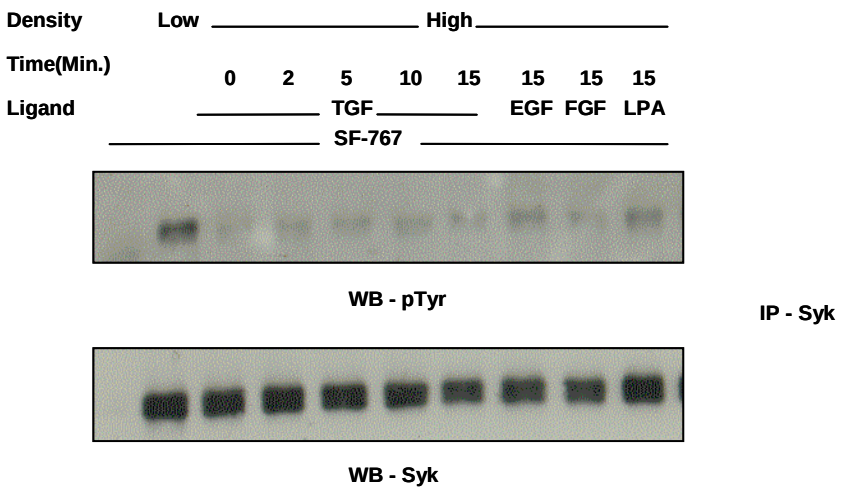


Figure 26 Syk activation is attenuated by high cell density. Glioblastoma cell line SF-767 was cultured at different cell density and treated with the various growth factors EGF (20 ng/ml), FGF (100ng/ml), LPA (2.5 μm) TGF-b (4nM). The cell lysates were immunoprecipitated by Syk antibody and probed with pTyr antibodies.

3.13 Syk Activation in Glioblastoma is Mediated by Src

Syk was found to be activated in the Glioblastoma cell line SF-767 in normal cell culture as well as in serum free medium (Fig. 25). Moreover, it is known that Syk may be activated by Src kinases in haematopoietic cells (Underhill and Goodbridge, 2007; Berton et al., 2005). To test if Syk activation is a result of Src activity, SF-767 cells were pre-treated with Src inhibitor (SKI-606 (Vichem, Budapest)) for 30 minutes followed by treatment of various growth factors and ligands (LPA (2 μM), EGF (50 ng/ml), FGF (50 ng/ml), TGF-b (4 nM). Cells were lysed and Syk phosphorylation was probed with phosphotyrosine antibody. Syk phosphorylation was found to be activated in a Src dependent manner as Src inhibition caused a significant decrease in Syk phosphorylation. Interestingly Syk phosphorylation was inhibited by FGF whereas TGF-b showed no significant increase in Syk phosphorylation. Therefore, it can be concluded from these results that various growth factors regulate Syk activation by Src family kinases (SFKs).

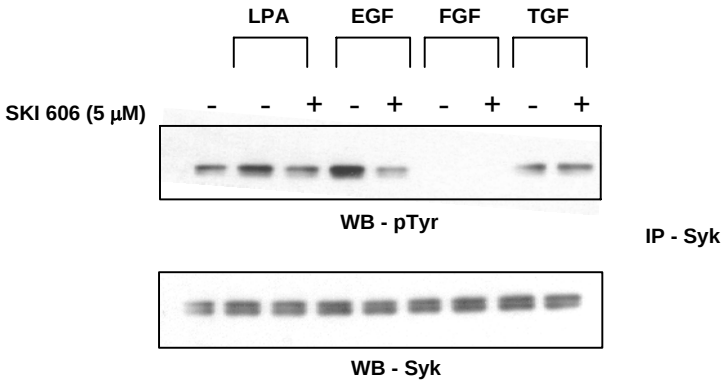


Figure 27 Syk activation is regulated by Src kinases. Syk activation in glioma cell line SF-767 was tested by probing the phosphorylated Syk in the LPA (2.5μM), EGF (50ng/ml), FGF (50ng/ml) or TGF β (4ng/ml) treated cells. To test if Src activity is essential in Syk activation, Src inhibitor SKI-606 (VICHEM) was applied to the cells at 5 μM prior to the stimulations.

3.14 Syk Abrogation by siRNA Increases EGFR Phosphorylation in Glioblastoma

Syk knockdown in breast epithelium led to an increase in the EGFR activity amd provided the anti apoptotic resistance against Reactive Oxygen Species (ROS) (Ruschel and Ullrich, 2004.). Therefore, it was important to examine if Syk abrogation in glioblastoma increases EGFR activity. To test whether Syk was knocked down by specifically designed siRNA (see material and methods) in the SF-767 glioblastoma cell line which express high levels of Syk and EGFR. Moreover, EGFR is highly active in this cell line which remains active even upon serum depletion for 24 hours. As it is shown in Fig. 28 Syk expression was significantly reduced by siRNA construct pRS-Syk2 directed against Syk. Once Syk knockdown was estabilished in SF-767 cells EGFR activity was tested. Reduced expression of Syk in SF-767 indeed raised the EGFR phosphotyrosine levels. Therefore, it could be concluded that Syk maintains the basal level of EGFR activity and its abrogation can lead to an increase in EGFR signalling (Fig. 28).

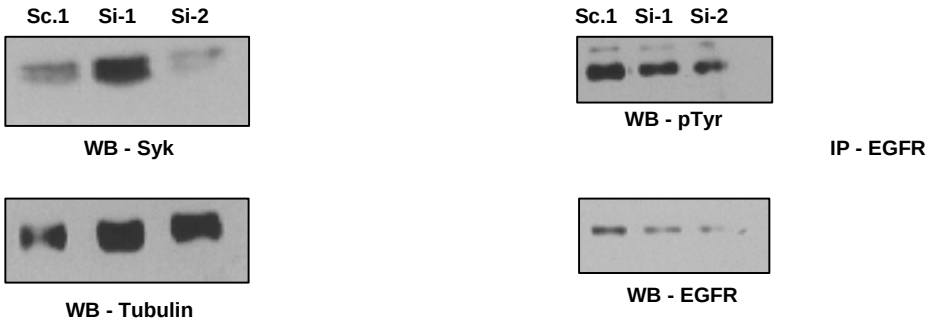


Figure 28 Syk knockdown increases EGFR tyrosine phosphorylation in SF-767 cells. Two different siRNA sequences (Si-1 and Si-2) were cloned in pRetro-Super vector (pRS) to stably knockdown the Syk expression in glioblastoma cell line SF-767. To test the EGFR phosphorylation upon knockdown of Syk expression, SF-767 cells stably expressing scramble siRNA (Sc.1), pRS-Syk1 (Si-1), or pRS-Syk2 (Si-2) were lysed and subjected to EGFR immunoprecipitation. The immunoprecipitated samples were later probed with pTyr antibodies.

3.15 LPA Induces Downregulation of EGFR Activity in Glioblastoma

Lysophosphatidic acid (LPA) is a lipid component in serum. It binds to the G-protein coupled receptors which are designated “Edg receptors”, and activates a variety of molecules such as PKC, PLC-γ or Src as well as transactivates the EGFR via triple membrane passing signal (TMPS) (Daub et al., 1996; Dorsam and Moolenaar, 2003). As LPA is abundant in serum, we asked if Syk activation is due to LPA signalling. To test this, SF-767 cells were starved by serum withdrawal and stimulated with LPA at different time points. Syk was activated by LPA treatment within 15 minutes (Fig. 25 and Fig. 26). Since Syk knockdown increases EGFR phosphorylation, we examined if LPA can reduce the EGFR phosphorylation state. Indeed EGFR was less phosphorylated when treated with LPA which reaches at its peak in 30 min and it reaches back to the basal level at 60 min of LPA treatment. Moreover, EGFR was decreased in its protein level in between 5 to 15 min of LPA treatment (Fig. 29).

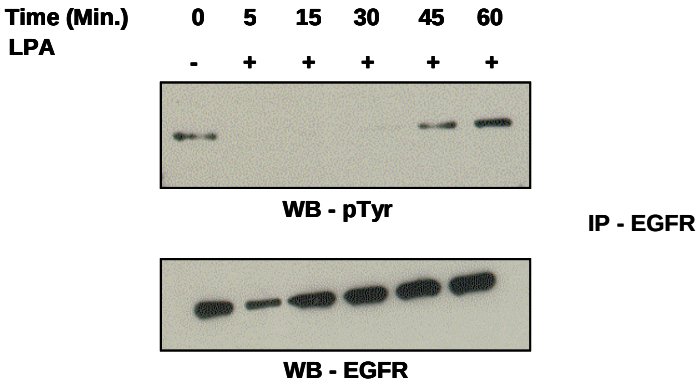


Figure 29 LPA attenuates the EGFR activity in Glioblastoma cells. Glioblastoma cell line SF-767 was treated with LPA (2.5μM) for different time intervals and cell lysates were utilised for EGFR immunnoprecipitation. The samples were probed with pTyr and EGFR antibodies.

3.16 LPA Mediated Attenuation of EGFR Activity is partially dependent on PKC and Syk

Hunter and colleagues (1984) showed that PKC mediates inhibition of EGFR by phosphorylating the Thr693 residue in the juxtamembrane domain (Hunter et al., 1984; Hubbard, 2004). The regulation of EGFR activity was explained by structural studies which showed that activation dependent phosphorylation of Tyr residues in the EGFR cytoplasmic tail can be altered by differing orientation of the activation loop in space (Hubbard, 2004). Moreover, downstream signalling of GPCRs is mainly mediated by Ca^{2+} release from the extracellular space or intracellular Ca^{2+} stores, Diacyl Glycerol (DAG) generation, PLC & PKC activation and activation of EGFR by transactivation pathway (Gschwind, 2002; Dorsam and Gutkind, 2007). In addition, GPCRs relay the signal to MAPKs majorly by PKC activation. As we observed EGFR attenuation by LPA treatment, it was conceivable that this could also be PKC driven.

To determine this model we utilised a Pan-PKC inhibitor Ro 31-8220 (Upstate Biotech., USA) at 100 nM to treat the cells prior to LPA treatment. Indeed, as shown in Fig. 30A PKC inhibition led to EGFR activation upon LPA treatment.

The downstream signalling pathway showed an even more interesting pattern. LPA treatment led to an increase in MAPK (Erk1/Erk2) activation, whereas Akt activation was not observed. Akt was activated upon PKC inhibition, whereas MAPK activation was unaffected. TGF-b signalling is not shown to have a cross-talk with EGFR signalling. To test the differences in the signalling

via EGFR dependent versus EGFR independent mechanism, TGF-b was applied to the cells. TGF-b led to Erk1/2 activation which was abolished upon PKC inhibition (Fig. 30 B). All together these results suggest that LPA regulates EGFR signalling by PKC.

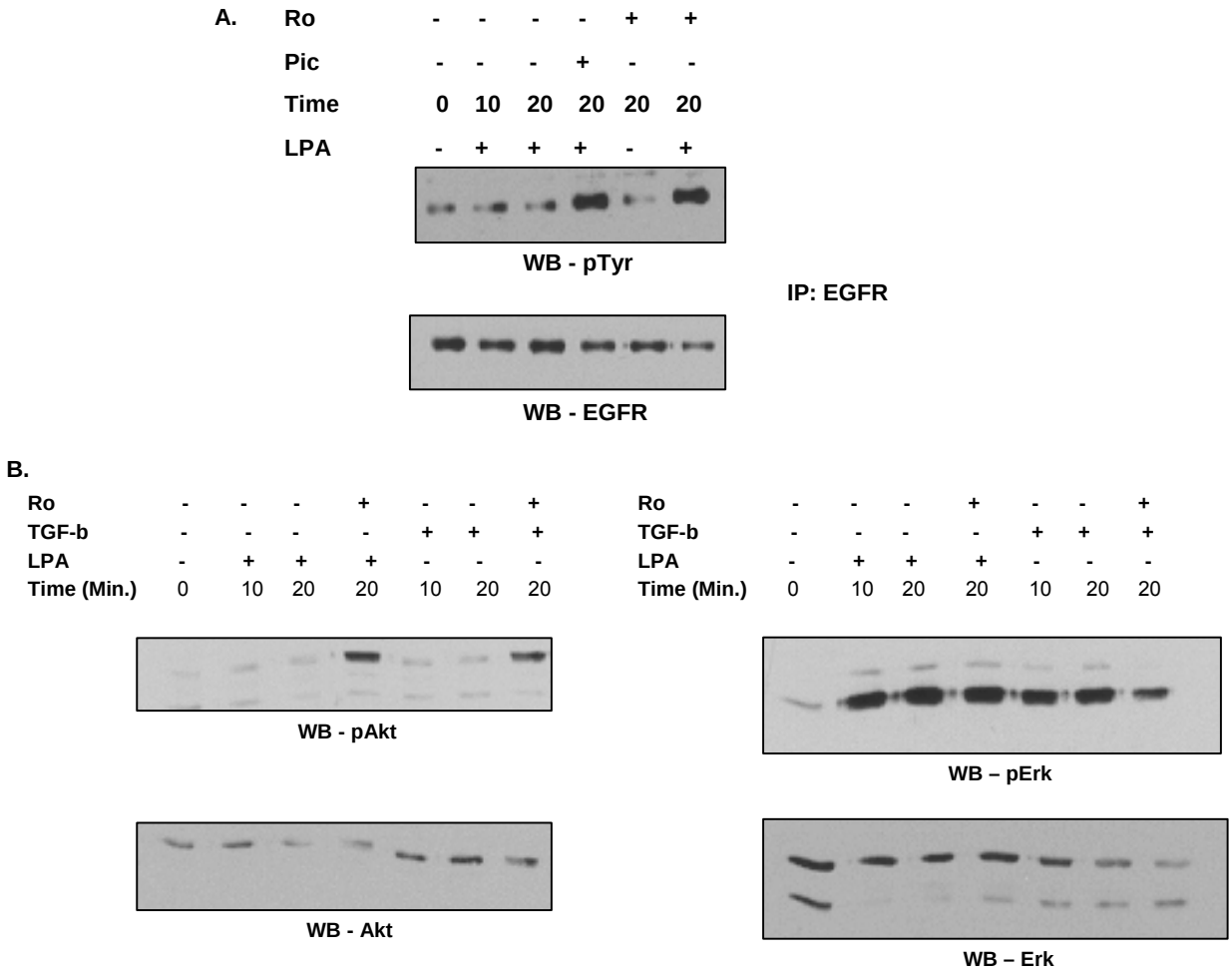


Figure 30 LPA mediated EGFR attenuation is via PKC. SF-767 cells were treated with LPA (2.5μM) at different time points. To observe the effect of PKC and Syk, their inhibitors (Piceatannol (Pic) at 10 μM for Syk & Ro 31-8220 (Ro) at 100nM for PKC) were applied to the cells 30 min. prior to the stimulation (A). To test the downstream signalling, TGF-b (4 nM) and LPA (2.5μM) was applied to the cells with or without pre-treatment of Ro 31-8220.

Since it was observed in previous experiments, LPA treatment induces Syk activation through Src, it was compelling to ask if Syk is involved in LPA mediated EGFR inhibition. Therefore the Syk inhibitor Piceatannol was applied on SF-767 cells prior to the LPA treatment and EGFR

activation was analysed. Syk inhibition was indeed causing a raise of EGFR phosphorylation upon LPA treatment (Fig. 30 A). Therefore, one may conclude that LPA induces the feedback inhibition of EGFR by PKC and Syk to maintain the EGFR activity in the cells.

3.17 Syk Interacts with Different Molecules Under Serum Depleted or -Supplemented Conditions

SF-767 cells express high amounts of tyrosine phosphorylated Syk. Moreover, Syk phosphorylation decreases upon serum withdrawl. While testing, Syk activation by several ligands in serum-supplemented or serum-depleted culture conditions, several molecules of different Molecular Weight (MW) could be seen, with the aid of Commassie Blue stain, in the Syk immunoprecipitated samples resolved on the SDS-PAGE (Fig.31). Moreover, these interactions seem to depend on the phosphorylation state of Syk as an increase in Syk phosphorylation tends to decrease some of the interactions whereas serum cultured conditions increases Syk affinity to other proteins.

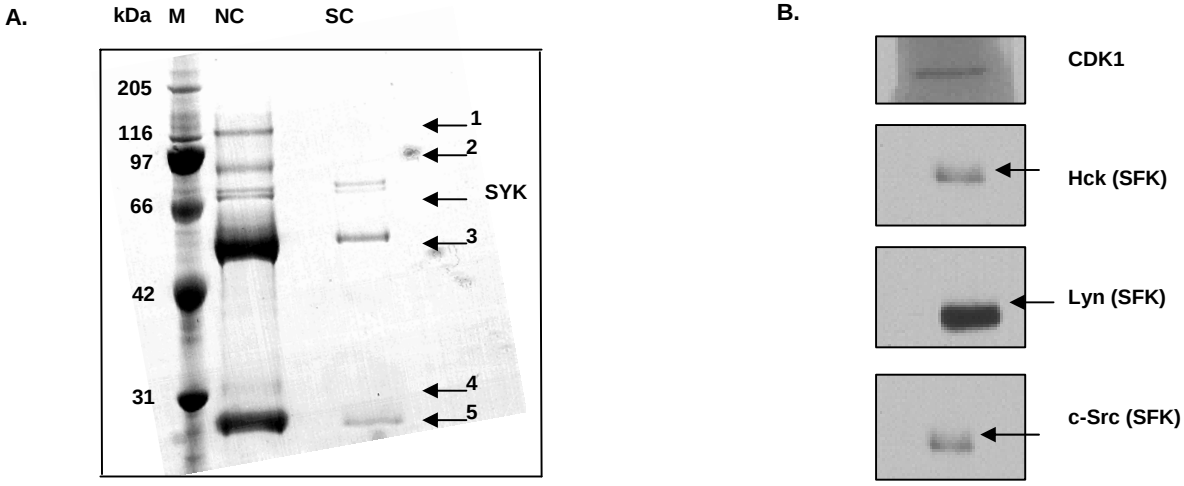


Figure 31 Syk interacts with different molecules in different culture conditions. Syk was immunoprecipitated from Serum supplemented (NC) or serum depleted (SC) SF-767 cells.The samples were resolved on SDS-PAGE gel which was stained with Commassie Blue stain (Fig 29 A). The different size molecules were sliced and pooled to identify by the mass spectrometery analysis. The identified molecules by mass spectrometry were further confirmed by western blotting using different antibodies (Hck (Santa Cruz), Lyn (Transduction Lab.), Src (Santa Cruz) and CDK1 (Santa Cruz) (Fig. 29 B). The arrows indicate the different size bands of the Commassie stained gel which were subjected to mass spectrometry.

To ascertain the identity of these proteins we utilised mass spectrometry. The Syk immunoprecipitations were resolved on the SDS-PAGE gel followed by staining with commasie blue. The protein bands which were selectively enriched in either starved or serum cultured conditions were excised from the gels and subjected to tryptic digestion. The digested peptides were then analysed by the mass spectrometer "ORBITRAP".

Some of the key Syk interacting molecules identified in the serum supplemented culture condition include the tyrosine kinases which are known to activate Syk e.g., Src family kinases. In fact Syk activation is compromised upon growth factor stimulation in the presence of Src inhibitor. The other molecules include the serine threonine kinases which could regulate cellular proliferation and genome maintenance, namely CDK-1 and CDK-2, CamKII, Casein Kinase, Cytidine Deaminase, and Nek-9.

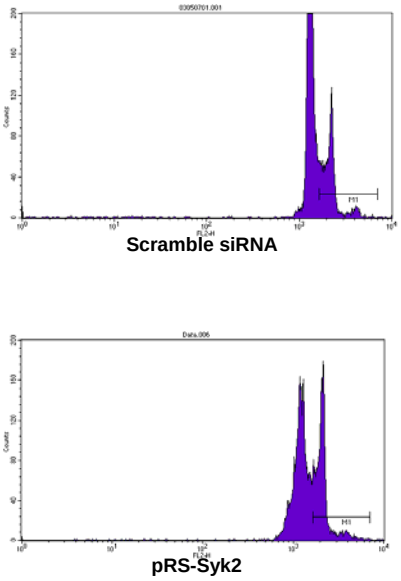
Position	Identified Protein	Mw
1.	**Nek-9 (Ser/Thr Kinase)**	**109 Kda**
	FAK-1 (Tyrosine Kinase)	**120 Kda**
	TAO-3 (Ser/ Thr Kinase)	**109 Kda**
2.	**AAK-1 (Ser/Thr Kinase)**	**90 Kda**
3.	**Hck (Src Family Kinase)**	**60 Kda**
	LYN (Src Family Kinase)	**60 Kda**
	YES (Src Family Kinase)	**60 Kda**
	Src (Src family Prototype)	**60 Kda**
	CamKII δ	**60 Kda**
	CamKII β	**60 Kda**
4.	**Annexin-1**	**39 Kda**
	CDK-1	**34 Kda**
	CDK-2	**34 Kda**
	Pyridoxal Kinase	**35 Kda**
5.	**Casein Kinase II**	**25 Kda**
	Cytidine Deaminase	**24 Kda**

Table 3 List of Syk interacting proteins identified by Mass spectrometric analysis: The proteins identified by mass spectrometry are enlisted on the basis of their respective position in the gel and molecular weight.

3.18 Syk Regulates Cell Cycle Progression

Syk expression in cells controls proliferation (Moroni M et al., 2004). Syk knockdown or ectopic expression in cells cause abnormal mitosis and mitotic catastrophe (Zyss, et al., 2005). Therefore, finding CDK1 in the analysis of Syk interaction partners by Mass-spectrometery is an important observation. CDK-1 is shown to be negatively regulated by threonine phosphorylation at Thr14 and tyrosine phosphorylation on Tyr15 residue which inhibits its activity.

A.



B.

Sample No.	Scramble	pRS-Syk2
1	28.36%	36.64%
2.	30.98%	38.86%

Figure 32 Syk knockdown in the SF-767 cells increases G2/M population. Syk expression was knockdown by stable expression of Syk siRNA construct pRS-Syk2 in SF-767 cells. SF-767 cells expressing pRS-Syk2 as well as pRS-Scramble were subjected to the FACS analysis to assay the cell cycle distribution among them. The Fig. 32 B shows the percentage of cells in M1 gate which represents the G2/M population of SF-767 cells.

To test the role of Syk in CDK-1 mediated cell cycle progression, SF-767 cells with stable knockdown of Syk expression were tested for the cell cycle distribution. SF-767 cells with stable knock down (SF-767-siSyk) were generated by using Syk specific siRNA construct pRS-Syk2 (see Fig. 28 and material and methods). SF-767-siSyk were cultured at a low density and starved for 16 hours prior to Propidium Iodide treatment for cell cycle analysis using fluorescence-activated cell sorter (FACS). The asynchronous cells grown in the normal cultured conditions showed a significant shift to the G2 phase of the cell cycle with Syk knockdown in comparison to the cells expressing scramble siRNA. Moroever, when we tested the cells grown in serum

depleted conditions, the cells expressing Syk siRNA showed no significant increase in G2 phase of cell cycle in comparison to the scrambled siRNA control.

4 Discussion

Carcinogenesis is a multistep process where accumulation of genetic aberrations contribute self sufficiency for growth factors, antiapoptotic potential, angiogenic potential, and unlimited proliferation potential to the cancer cells harbouring them (Hanahan and Weinberg, 2000). These changes are later translated into abnormal proliferation and colonising activity of cancer cells. Based on the abrogation of Syk in many tumours by hypermethylation of its promoter, it is implicated as a tumour suppressor (Yuan et al., 2001; Goodman et al., 2003; Dhillon et al., 2004; Wang et al., 2004; Wang et al., 2005; Yuan et al., 2006 Muthuswamy et al., 2006). Elusiveness of Syk function in tumours poses a common formidable question of correlations i.e., whether Syk abrogation is a cause or the result of tumour progression. In this study molecular biology approaches were employed to evaluate Syk as a possible causative agent in the tumour initiation and its progression. Moreover, this study is an attempt to identify the Syk interacting molecules to solve some specific problems such as finding the key molecules involved in EGFR regulation in the epithelial cancers.

4.1 Syk is Abrogated in Epithelial Cancers

It was first observed by Coopman and colleagues that Syk, a known haematopoietic kinase, is aberrantly expressed in breast cancers (Coopman et al, 2000). Moreover, they correlated the loss of Syk in breast cancer cells to their invasive phenotype, as many invasive cancer cell lines of breast cancer origin tend to loose Syk expression. They went further to claim that loss of Syk is one of the crucial events in making the cells competent for being invasive. In their study, they expressed Syk in an invasive breast cancer cell line MDA-MB-435s which originally lack Syk expression and injected these Syk expressing cells in nude mice to study their metastatic behaviour. They could show that Syk expressing MDA-MB-435s cells colonised lung with a lower efficiency than the cells that did not express Syk. They reasoned their observation by demonstrating Syk interactions with microtubules and hypothesised that Syk might be involved in regulation of mitosis. Their observations are further strengthened by another study conducted in a breast cancer cell line MCF-7 where it was shown that Syk co-localises with centrosomes and forced expression of Syk causes abnormal cell division (Zyss, et al., 2005).

In the present study, we have identified glioblastoma as another cancer type where Syk expression is either reduced or abrogated (Fig. 10). On the other hand it has been reported that glioblastoma shows either overexpression or deletion of EGFR gene as one of the major genetic abnormality (Zawrocki and Biernat, 2005). Therefore, Glioblastoma may serve as an interesting model to understand the role of Syk in the regulation of EGFR. The methylation of Syk promoter may potentially abrogate the expression of Syk in glioblastoma as frequently observed in other human cancers as well (Yuan et al., 2001; Goodman et al., 2003; Dhillon et al., 2004; Wang et al., 2004; Wang et al., 2005; Yuan et al., 2006 Muthuswamy et al., 2006).

4.2 Syk in the Nucleus: With a Purpose?

The present work focused on the localisation of Syk in the nucleus with a purpose to study if Syk could relay signals to the nucleus to regulate gene expression. Therefore, we first investigated whether Syk is active in the nucleus by performing *in vitro* kinase assays of Syk immunoprecipitated from nuclear fractions of a breast cancer cell line MDA-MB-231. Indeed Syk was found to be active in the nucleus with autophosphorylation activity comparable to its cytoplasmic counterpart (Fig. 12). The finding of active Syk in the nucleus leaves many questions open. First and most important of all is whether entry of Syk into the nucleus is regulated by different signals such as growth factors, stress etc. or is it a spontaneous phenomenon. There are controversial reports published recently regarding the biochemical nature of Syk translocation to the nucleus. An interesting observation by Dai JL and colleagues (Wang et al., 2003.) implicates alternative splicing as the major mode of regulation on Syk nuclear localisation and claims that the unspliced form of Syk, which enters into the nucleus, is responsible for the tumour suppression. In fact, they showed the presence of a NLS in the sequence that undergoes splicing and therefore, renders short form excluded in the cytoplasm. Additionally they showed the correlation of short form of Syk with aggressive tumours. In contrast to their observation, there is a recent study that shows that nuclear localisation of Syk is dependant on the initial amino acid sequence of the tyrosine kinase domain of Syk instead of the above described NLS (Zhou et al., 2006).

In this study we tried to investigate, leaving aside the regulation involved in the nuclear transport of Syk, the functional significance of Syk in the nucleus. Our results revealed (Fig. 13, Fig. 14 and Fig. 15) that Syk mediated attenuation of invasivity in MDA-MB-231 cells could be related

to the gene expression changes due to the ectopic Syk expression. Moreover, these results were further tested to understand the nature of these modulations in gene expression by using pharmacological inhibition of many of the key signalling pathways involved in those gene regulations (Fig. 18). It could be interpreted from the obtained results that Syk modulated gene expressions are priamarily due to the nuclear localisation of Syk as many of the cytoplasmic signalling pathways were ineffective on the Syk dependent gene expression changes. Furthermore, Syk kinase activity was also found to be important in the suppression of invasiveness and the gene expression changes (Fig 13 & Fig. 14). This observation in particular is worth investigating further as the tyrosine phophorylation could act as a regulation of the activity of transcription factors as well as opening up of the chromatin. In fact, a study from Dai JL and co-workers showed that Syk interacts with the HDAC and acts as a transcriptional suppressor (Wang et al., 2006).

4.3 Regulation of Syk Activity by DNA Damaging Agents: A Possible Role in the Maintenance of Genetic Integrity.

A hallmark of invasive cell is their intrinsic property of resistance to apoptotic stress generated from the environment (Hanahan and Weinberg, 2000; Stupack et al., 2006). Therefore, it was a matter of interest whether Syk expression increases the sensitivity of the cancer cells to undergo apoptosis under stress imposed due to the abnormal conditions (e.g., evading out from the tissue or DNA damage). It is shown that Syk over expression in MCF10A, a breast epithelial cell line increases the apoptotic rate when cells are subjected to an oxidative stress (Ruschel and Ullrich, 2003). Moreover, Syk knockdown in similar conditions decreases the rate of apoptosis which is confirmatory of the role of Syk in inducing apoptotic signalling pathways under stress. Current study adds to these observations by showing that Syk is involved in stress signalling as the DNA damaging drug Doxorubicin increases Syk phosphorylation as well as expression levels (Fig. 19). It is worth noting that Syk localises to the centrosomes which is the site of many cell cycle checkpoint kinases (Zyss et al., 2005). It is also shown that Syk ectopic expression causes the abnormal mitosis and an increase in aneuploidy. In that context, observation of Syk activation upon treatment of DNA damaging agents becomes interesting. Moreover, DNA damaging agent, Doxorubicin used in this study is a commonly used chemotherapeutic in cancer treatment. However, resistance towards Doxorubicin has been frequently observed which is partially due to

the selection of the doxorubicin resistant cancer cells. Recent data from high throughput gene expression analysis of tumour samples from cancer patients suggest a shift in gene expression pattern in resistant cancer cells which might be beneficial for the cancer cells in different therapeutic treatments (Potti A et al., 2006). The gene expression changes observed in the resistant tumours involves up regulation, down regulation or complete abrogation of gene expression. As many developed cancers showed Syk abrogation or decreased Syk expression, it is interesting to hypothesise that one of the genetic abnormalities in chemotherapeutic resistant tumours could be the loss of Syk expression. Therefore, it warrants further studies to investigate the response of Syk positive as well as Syk negative tumours towards commonly used chemotherapeutics as well as other developing drugs. Such studies will be beneficial in segregating the groups which may respond to a given chemotherapeutics. In other words, Syk expression in tumours could have correlation with their responsiveness to the drugs. In fact, Syk expression in tumour is correlated with a better prognosis and survival (Dejmek et al., 2004). Once such results are obtained, they can be further utilised for genetic diagnosis of cancer patients before undertaking a particular chemotherapeutic treatment.

4.4 Regulation of Syk in Glioma by Growth Factors and Other Ligands

Syk is an enigmatic signalling molecule in epithelial cells where its upstream regulators are largely unknown. More importantly no growth factor or secreted ligand is shown to stimulate or suppress the Syk activity in epithelial cancers. Integrins are shown to activate Syk in a breast cancer cell line MCF-7 and blocking of integrin interaction with collagen is shown to reduce Syk activity (Dejmek et al., 2004). Therefore, it leaves a major caveat in understanding the role of Syk in cancer progression. Moreover, the physiological functions of Syk are limited to be observed only by Syk knockdown or its ectopic expression in the cell lines, which leaves the regulation of the process poorly studied. In this study we could identify some of the very important ligands of epithelial cells as agonist of Syk in glioma. These ligands regulate proliferation (EGF, FGF), migration (EGF, LPA) and differentiation (TGF β). The finding of new agonists in one cell type (e.g., glioblastoma (Fig. 26 and Fig. 27) and their inefficiency to carry the same function in another (e.g., breast cancer) suggests that cellular context is important in Syk signalling. Moreover, SFKs apparently activate Syk upon growth factor stimulation because

Syk activation could be suppressed by Src inhibitor treatment (Fig. 27). Various members of SFKs are also detected by mass spectrometry screen which suggest that Syk interacts with SFKs in order to receive the activating signal (fig. 31 and Table 3). It is also worth noting here that Syk activation in epithelial cells could be based on different theme than haematopoietic cells, where ITAM phosphorylation on the activating receptors (e.g., BCR, TCR, FcR etc.) is a prerequisite. Syk recognise and interacts with the phosphorylated ITAM motifs followed by its activation via SFKs (Yaghini et al., 2007; Underhill and Goodbridge, 2007). As ITAMs are not a general feature of epithelial cells but are carried to epithelial cells by viral agents, it is attractive to hypothesise that Syk interacts with SFKs in epithelial cells without the aid of ITAM (Lanier, 2006; Grande and Ross et al., 2006; Grande et al., 2006). Moreover, it is noteworthy here that ITAM bearing molecules are oncogenic in epithelial cells therefore it is important to consider them before analysing the role of Syk in tumour suppression (Lanier, 2006; Grande and Ross et al., 2006; Grande et al., 2006).

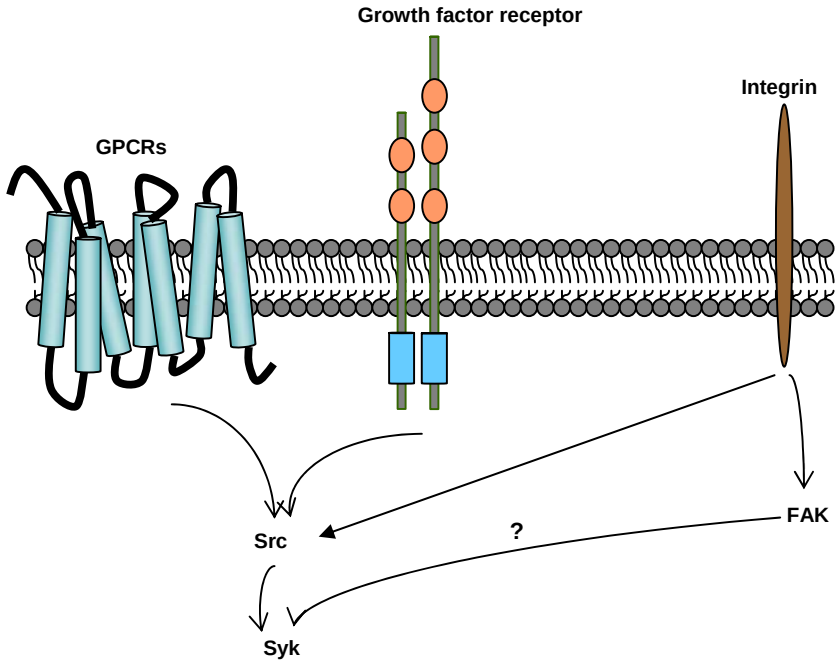


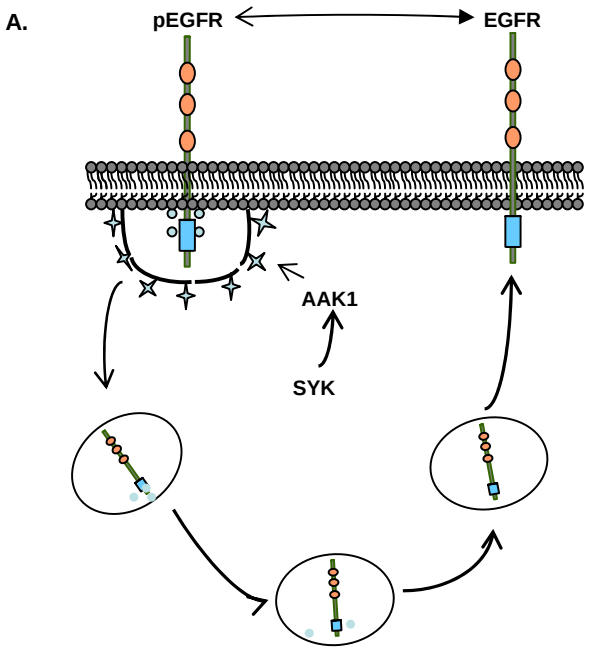
Figure 33 Model of Syk activation by various agonists. GPCRs, Growth factor receptors, and adhesion molecules activate SFKs. The src associates with Syk in the cell and activate it by phosphorylating it. Focal adhesion kinase (FAK) is shown to be downstream activator of signalling and therefore, serves as prospective kinase to activate Syk by integrins or other adhesion molecules.

Furthermore, this study points towards the role of receptor protein tyrosine phosphatases (RPTPs) in regulation of Syk, as higher confluency in cell culture leads to decrease in Syk activity. It has been shown in various studies that RPTPs are involved in sensing the cell-cell contact and cell contact dependent signalling (Peles et al, 1998). Therefore, it is interesting to study the regulation of Syk activation or inhibition by the signals generated due to cell-cell contact and to investigate its role in contact inhibition or metastasis in the normal epithelium or cancer cells respectively.

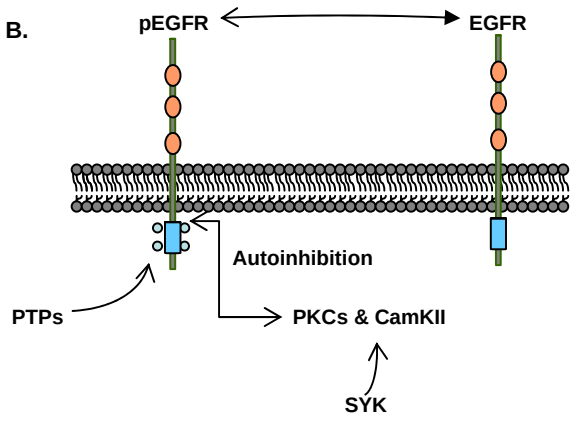
4.5 Syk as a Negative Regulator of EGFR

In previous studies Syk has appeared as a regulator of proliferative signal (Moroni et al., 2004; Ruschel and Ullrich, 2004). It was shown earlier that Syk negatively regulates EGFR in breast epithelium and therefore, regulates apoptosis (Ruschel and Ullrich, 2004). Another clue that Syk could be regulating proliferation in breast cancer comes from some recent studies conducted on breast cancer tumour samples using immunohistochemistry (Moroni et al., 2004). They could show that Syk was lost in the tumour samples of high proliferative index (increased Ki67 staining, a proliferation marker). Similar results were obtained in our study, as knockdown of Syk increases the phosphorylation of EGFR in glioblastoma whereas a decrease in EGFR phosphorylation was observed upon ectopic expression of Syk in a breast cancer cell line MDA-MB-231. Moreover, ectopic expression of Syk in MDA-MB-231 cells reduced their proliferation potential. These results suggest that there might be an important regulatory mechanism for maintaining EGFR activity by Syk tyrosine kinase prevalent in several cell types. As it can be observed in the results that EGFR phosphorylation is weakly inducible, it is more likely that Syk maintains the basal activity of EGFR instead of inducing its dephosphorylation. Two models of EGFR regulation are described below which could serve as probable mechanistic model (Fig. 32) based on the internalisation or autoinhibitory phosphorylation of EGFR. AAK1, a serine threonine kinase involved in internalisation of membrane receptors, was found as an interaction partner of Syk in the mass spectrometry results in normal culture conditions. Therefore, it is plausible that Syk regulates the internalisation of receptors and other membrane molecules. AAK1 is a Ser/Thr Kinase which is shown to be responsible for the internalisation of Transferrin receptor (Conner and Schmid, 2002; Conner et al., 2003). Furthermore, it is shown that AAK1 phosphorylates the AP-2 subunit of clathrins which is supposed to be regulatory in clathrin coating of the internalisation vesicles. Therefore, Syk interaction with AAK1 could be highly

interesting with respect to EGFR phosphorylation and regulation. Second model described below is based on autoinhibition of phosphorylation of EGFR at juxtamembrane threonine residue of EGFR (Hunter et al., 1984). It is well known that EGFR is autoinhibited by PKC mediated theronine phosphorylation at Thr693 residue. Moreover, Syk is shown to be interacting with PKC in haematopoietic and endothelial cells (Pula et al., 2005; Kawakami et al., 2003). Therefore, it is of great interest to test if Syk increases or maintains the feedback regulation of EGFR activity via PKC. Moreover, it is interesting to study if the signals which induce differentiation are also mediated by Syk. In other words it would be important to understand the physiological significance of Syk in modulating these signals. Therefore, it is interesting to ask if Syk potentiate migratory or differentiating signals by reducing proliferation.



Internalisation and recycling model



Autoinhibition Model

Figure 34 Different mechanisms which regulate EGFR activity in the cell. Activated EGFR is swiftly internalised by endocytosis and impaired endocytotic mechanism can increase the pEGFR on cell surface. AAK1 is a serine/Threonine Kinase involved in the regulation of endocytosis of membrane molecules by Clathrin coated pits. Internalised receptors are either targeted to proteosomal degradation or recycled back to the membrane after desensitisation (stripping of the ligands and phosphoryl residues) (A). Receptor activity is also regulated by autoinhibition. PKC is shown to phosphorylate at juxtamembrane residue of EGFR and causes its autoinhibition (B). As these molecules also interact with Syk; it is plausible that Syk inhibit EGFR phosphorylation by one of the above mechanisms.

It is noteworthy here that Syk is induced by migratory (LPA) as well as differentiating signals (TGF β) (Fig. 25). Moreover, these signals also reduce EGFR phosphorylation in Syk dependent or independent manner. Furthermore, ectopic expression of Syk in breast cancer as well as glioma reduces the proliferation rate. Reduction in proliferation is commonly observed during and after differentiation (Massague, 2000). Therefore, it is interesting to hypothesise that Syk mediated reduction in growth factor signalling promotes differentiation signals further.

4.6 Signalling Network with Syk Taking the Centre Stage; a Proteomic Based Approach

Syk has been elusive in epithelial cells due to the lack of mechanistic models which corresponds to the phenotypic observations obtained by genetic manipulations of Syk gene in cells or animals. Proteomic approaches could play crucial role in understanding the signalling regulated by a given molecule. It has been shown in the past that interactions between different molecules could determine the fate of a signalling pathway. Therefore, identification of Syk interaction partners by unbiased proteomic based approach was a key interest of this study. Selection of cell line was critical in this approach as molecular interactions are easier to detect depending upon protein

expression levels and inducible post-translational modification. Moreover, the inducible nature of post-translational modification can be further exploited by scrutinising the interacting partners based on certain cellular state upbringing a modified protein or increasing the nascent protein. Therefore, we chose SF-767 cell line of glioblastoma origin as this cell line expressed high amount of total protein including high levels of Syk expression. Moreover, Syk phosphorylation could be induced by various signals e.g., TGF, FGF, LPA etc. The immunoprecipitation of Syk protein was carried in two different cellular states i.e., normal cell culture conditions with FCS containing medium and starved cellular state with FCS deprived medium. Interestingly, Syk interacted with different molecules at varying affinity in these two culture conditions. For example Syk interacts with SFKs, FAK, CamKII, and CDKs in the normal culture conditions whereas associates with actomyosin complex in the starvation or LPA stimulated conditions. Moreover, these interactions were reversible and could be induced or suppressed by changing the cellular state by removing or adding FCS. Understanding the functional relevance of these interactions with Syk could be helpful in deciphering the signalling pathways which include Syk involvement. For instance, this study showed that Syk interaction with SFKs is important for basal activity of Syk, as well as its activation by growth factors or other signals.

4.7 Differential LPA Signalling is Due to PKC Action in Glioblastoma

The LPA signalling has been reported to regulate various physiological fates that can be either proliferative or migratory depending on the cellular context (Mills snd Moolenaar, 2003). Moreover, it has been shown that LPA stimulation could lead to apoptotic induction in the neuronal cells (Ye et al., 2002) Therefore, LPA signalling regulates various cellular functions and gets even more complicated due to its role in EGFR transactivation. Herein we showed that LPA could induce rapid attenuation of EGFR activity which depends on PKC. LPA, normally a transactivating signal for EGFR, induced the EGFR autoinhibition by PKC in glioblastoma. PKCs are activated by various growth factors and GPCR ligands and phosphorylate EGFR at Thr693 residue at juxtamembrane position (Hunter et al., 1986) apart from activating MAPKs. Threonine phosphorylation of EGFR by PKC is shown to be autoinhibitory. Therefore, by utilising PKC inhibitor, the role of PKC in maintenance of EGFR basal activation could be analysed. It is attractive to hypothesise that GPCR signals regulate the signal generation based on

PKC occupancy of EGFR which could differ in different cellular context. Moreover, the role of PKC in generating complexity in the GPCR and growth factor signalling become more interesting by analysing the downstream signals. It could be observed in Fig. 30 that LPA induces Erk1/Erk2 activation which is presumably pro-proliferative signal. But in the presence of PKC inhibitor the activation of Erk was unchanged but instead an additional Akt signal was generated. Akt is activated by PI3K in cells in response to various growth factors. Moreover, PI3K signalling is multidimensional and causes several physiological changes such as migration, apoptosis, proliferation, growth etc. Furthermore, PI3K signalling is highly potent in the cells expressing ErbB-3/HER-3 which represents multiple PI3K binding sites (Schulze et al., 2005). Therefore, it is tangible to hypothesise that the additional Akt signal generated by PKC inactivation stems from HER-3/ErbB-3 heterodimerization with EGFR.

4.8 Regulation of Cell Cycle Progression by Syk

Considering the role of Syk in proliferation in cancer cells, it is important to understand the role of Syk in the regulation of cell cycle. In fact it was observed that Syk knockdown caused increase in G2/M population in glioblastoma cells. This change in cell cycle could be either due to the cell cycle arrest in G2/M phase or by early exit of the cells from the G1/S phase. Both causes can be detrimental to the genome and can cause increase in proliferation. Moreover, the finding of CDK1 interaction with Syk increases the possibility that Syk regulates the cell cycle progression during mitosis. CDK1 is known as the master switch for mitosis phase of cell cycle and its timely activation and inhibition is important for the cell cycle progression (Nigg, 2001). Tyrosine phosphorylation of CDK1 is inhibitory and crucial for mitotic entry as well as exit. Therefore, the delayed mitotic phase by Syk knockdown could be the event occurred as a result of Syk-CDK1 interaction.

4.9 Regulation of Syk Activity by Growth Factors

Syk is reflected as a mitotic regulator from the experiments conducted in this study. Moreover, the major growth factor signalling is destined to bypass the negative regulation imposed on the cell cycle to accelerate the proliferation. Therefore, the regulation of Syk by growth factors is of extreme interest. It can be observed that growth factors positively regulate Syk activity, when applied at a very high concentration which is mediated by Src. However, Syk activity is

negatively regulated by the growth factors, when applied at lower concentration. Therefore, there seems to be a bimodal regulation of Syk which might depend on the intensity of the extracellular signal as well as its duration. The suppression of Syk activity by growth factors might be responsible for the release of negative regulation of proliferation imposed by Syk.

5 Summary

The main findings of this study are:

1. Syk regulates expression of genes which are crucial for invasivity of breast cancer cells. Syk activity was found to be induced by DNA damaging agent, Doxorubicin.

2. Ectopic expression or knock-down studies show Syk as a crucial regulator of physiological processes like proliferation in breast cancer cells and cell cycle progression in glioblastoma.

3. Syk expression is abrogated in aggressive glioblastoma cell lines.

4. Syk activity is regulated by different growth factors and ligands in glioblastoma cell lines which is dependent on Src family kinases (SFKs).

5. Mass spectrometric analysis identified various interacting partners of Syk, which belonged mainly to kinase family of proteins.

Combined together these findings reinforce the role of Syk as an important tumor suppressor in breast and glial cells. Therefore, further investingations along these lines are of special clinical relevance.

Zusamenfassung

Die wichtigsten Ergebnisse dieser Arbeit:

1. Syk reguliert die Expression von Genen, die von entscheidender Bedeutung für die Invasivität von Brustkrebs-Zellen sind. Zudem konnte gezeigt werden, dass Syk durch das DNA interkalierende Agens Doxorubicin aktiviert wird.

2. Mittels ektopischer Expression sowie durch RNA-Interferenz Analysen wurde die regulative Rolle von Syk in wichtigen physiologischen Prozessen wie Proliferation (Brustkrebs) und Zellzyklus (Glioblastom) gezeigt.

3. In agressiven Glioblastomen wird Syk vermindert exprimiert.

4. Die Aktivität von Syk wird in Glioblastom Zellen in Abhängigkeit von Src Kinasen durch verschiedene Wachstumsfaktoren sowie Liganden reguliert.

5. Mit Hilfe massenspektrometrischer Untersuchungen konnten verschiedene Interaktionspartner von Syk, die großteils zur Familie der Kinasen gehören, identifiziert werden.

In ihrer Gesamtheit verstärken diese Ergebnisse die Funktion von Syk als wichtigen Tumosuppressor in Brustkrebs- und Glioblastom-Zellen. Es ist daher von besonderer klinischer Bedeutung, die hier aufgezeigten Funktionen und Regulationsmechanismen weiter zu untersuchen.

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Publications

- **Sushil Prasad Sati,** Saurabh Kumar Singh, Nirbhay Kumar, and Amit Sharma. (2002) Extra terminal residues have a profound effect on the folding and solubility of a *Plasmodium falciparum* sexual stage-specific protein over-expressed in *Escherichia coli*. *Eur J Biochem*. 2002 Nov;269(21):5259-5263.

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