STAT3 IN EGF RECEPTOR-MEDIATED FIBROBLAST AND HUMAN PROSTATE CANCER CELL MIGRATION，INVASION AND APOPTOSIS

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STAT3 IN EGF RECEPTOR MEDIATED FIBROBLASTS AND HUMAN PROSTATE CANCER CELLS MIGRATION AND INVASION AND APOPTOSIS

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Growth factor-induced migration is a rate-limiting step in tumor invasiveness. The molecules that regulate this cellular behavior would represent novel targets for limiting tumor cell progression. Epidermal growth factor (EGF) receptor (EGFR)-mediated motility, present in both autocrine and paracrine modes in prostate carcinomas, requires de novo transcription to persist over times greater than a few hours. Therefore, we sought the specific signaling pathways that directly alter cellular transcription. We confirmed that STAT3 directly associates with, and is activated by EGFR in DU-145 and PC3 human prostate carcinoma cells in addition to the model NR6 fibroblast cell line. This correlated with electrophoretic motility shift of STAT3-selective oligonucleotides. Inhibition of STAT3 activity by antisense or siRNA down-regulation or expression of a dominant-negative construct limited cell motility as determined by an in vitro wound healing assay and invasiveness through a matrix barrier. The expression of constitutively activated STAT3 in the absence of EGF did not increase the migration. Together these data indicate that STAT3 is necessary but not sufficient for EGFR-mediated migration. An initial gene array detected a number of candidate operative molecules; the protein levels of both ENA/VASP, a repressor of cell motility, and caspase 3, a nexus of apoptotic signaling, were down regulated by EGF in a STAT3-dependent manner. Preliminary data show that EGF requires STAT3 functioning to inhibit the induction of apoptosis

in the two human prostate cancer cell lines. This suggests that STAT3 signaling may be contributing to tumor progression in a second manner by rendering the cells resistant to death. Together, the sum of these findings suggest that STAT3 signaling may be a new target for both limiting prostate tumor cell invasion and enabling the tumor cells to be killed.

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PREFACE

I dedicate this to my loving parents, Xiao-An Wu and Yi Zhou, for the encouragement and support they have given me throughout my life.

I am greatly appreciative of my mentor Alan Wells, who has patiently taught and steered me during my scientific training. For their insight and guidance, I would also like to thank the members of my thesis committee at the University of Pittsburgh: Jennifer R Grandis, Jian Hua Luo, Thomas Smithgall and Razor Zarnegar. I would especially like to thank past and present members of the Wells Lab for their helpful advice and support over the past 5 years: Philip Chang, Jeffrey Chou, Angela Glading, Akihiro Iwabu, Jareer Kassis, Priya Kulasekaran, Asmaa Mamoune, Latha Satish, Hidenori Shiraha, Kate Sullivan, Kien Tran, Diana Whaley, Clayton Yates, and Cecelia Yates.

1.0 INTRODUCTION

Cancer progression results from complex and still riddling myriad of physiological and pathological events. Among them, the ability to break tissue and matrix barriers and establish growing masses within normal tissue represents the most fundamental characteristics of dissemination. Such spread causes the vast majority of morbidity and mortality from cancer.

Histological analyses in *de novo* human tumor specimens and animal tumor models shows cancer cells invading into adjacent healthy tissues or breaching a basement membrane to access a vessel for dissemination; this local movement is termed tumor invasion [1]. The processes of invasion includes de-adhesion and penetration of surrounding matrix. Metastasis, which often has invasion as the first step, is the ability of the tumor cell to then move to, and grow in a new site. The difference between normal invasive growth and invasion is that normal cells end up with polarized structures while tumor cells infiltrate into surrounding tissue [2].

Central to invasion is the ability of the tumor cell to actively move into the surrounding matrix and tissue. Cell motility is tightly controlled by growth factors and cytokines during organogenesis, inflammation and wound healing, while it appears to become dysregulated during tumorgenesis. This lack of control and direction results in invasion. The initiation and maintenance of this aberrant motility is important to understanding the transition to tumor invasion [1].

Many growth factors have had their increased levels in tumors correlated with tumor invasiveness [3]. As an example, EGFR signaling is upregulated in over half of the invasive gliomas compared to almost none of the non-invasive gliomas [4] [5]. EGFR is highly expressed in a variety of tumors including bladder, breast, colorectal, esophageal, gastric, head and neck, non-small cell lung cancer, ovarian, pancreatic and prostate cancers, and in most cases this

correlates with tumor invasion or metastasis [6]. How the EGFR signaling cascade works in those cells is the key to understanding metastasis and invasiveness of tumor cells.

1.1 PROSTATE CANCER

Adenocarcinoma of the prostate is the most common malignancy of the male genitourinary tract and is a significant health problem. In Europe the incidence of prostate cancer is 30 per 100, 000 males, whilst in the USA rates of 178 per 100 000 have been reported [7]. This makes it the most common malignancy in American men. Localized prostate carcinomas exist in most of elderly males, but the most of those carcinomas are asymptomatic and medically important [8]. For this reason, the research of tumor proliferation and dissemination are even more important of this disease than of other cancers.

1.1.1 Background of Prostate Cancer

The major risk factors of prostate cancer including age, race, family history and maybe diet. The chance of getting prostate cancer is highly correlated with a man's age. Most patients get prostate cancer when older than 55 [9] (Fig 1-1). Rates of prostate cancer vary widely across the world. It is least common in South and East Asia, more common in Europe and most common in the United States. In the United States, prostate cancer is more common in African American men than in white men. The chance of getting prostate cancer is also found related to family history [10]. Some studies suggest that dietary amounts of certain foods, vitamins, and minerals can also contribute to prostate cancer risk.

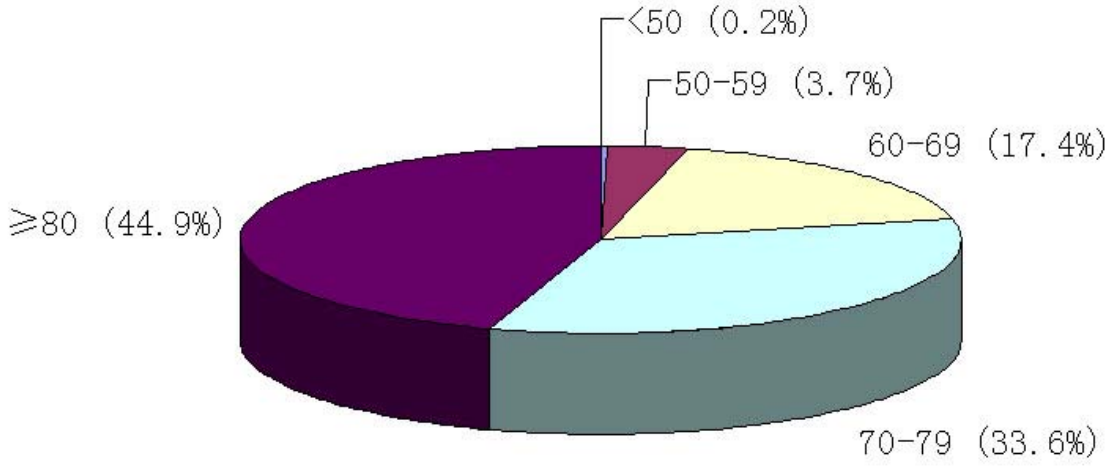


Figure 1-1. Age distribution of deaths from prostate cancer in the United States. (From American Family Physician, Lefevre, 1998, modified)

1.1.2 Diagnosis of Prostate Cancer

The most commonly used method to diagnose and evaluate prostate cancer is the PSA (Prostate Specific Antigen) test though it is far from perfect. PSA is a serine protease produced by prostate, its normal function is to liquify gelatinous semen after ejaculation. The only test which can fully confirm the diagnosis of prostate cancer is a biopsy.

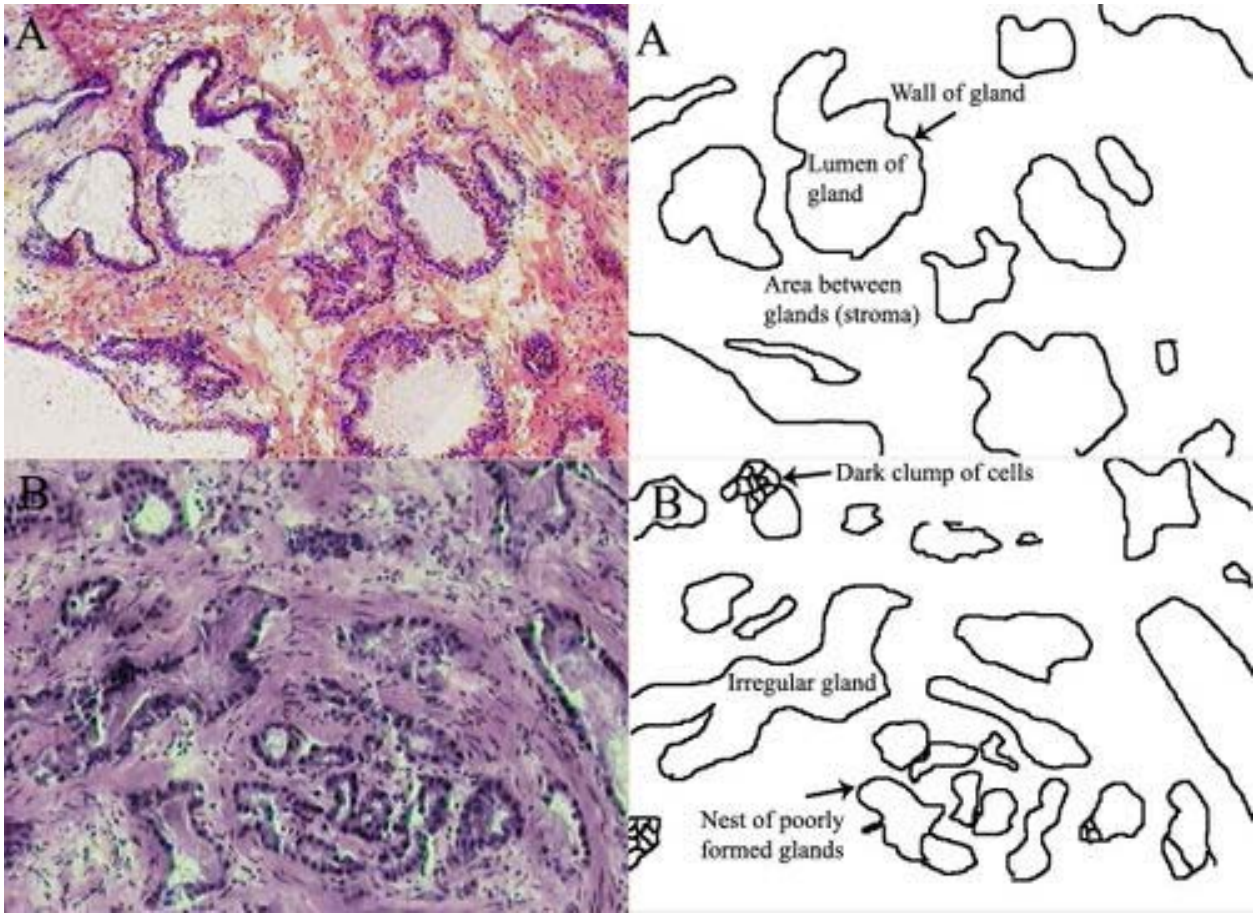


Figure 1-2. Biopsy pictures of normal and prostate cancer tissue.

Normal prostate (A) and prostate cancer (B). In prostate cancer, the regular glands of the normal prostate are replaced by irregular glands and clumps of cells. (From NIH website)

1.1.3 Therapy of Prostate Cancer

Treatment for prostate cancer includes but not limited to surgery, radiation therapy, cryosurgery and hormonal therapy. Surgical removal of the prostate (also called prostatectomy) is a common treatment mainly for early stage prostate cancer. Radiotherapy is also widely used in prostate cancer treatment. However, once the tumor has spread, such local tumor removal has little impact on the overall outcome or course of disease. Thus, the disseminated tumors need innovative and systemic approaches.

Hormonal therapy is to block prostate cancer cells from getting dihydrotestosterone (DHT) by medicine or surgery. DHT is a prostate produced hormone and is required for the growth and spread of most prostate cancer cells. Blocking DHT can inhibit prostate tumor growth. Early in the prostate carcinoma, such physical or chemical castration leads to regression of the tumor masses. However, this rarely cures prostate cancer as androgen independence develops within a year or two. Thus, we have focused on the tumor-intrinsic events, in androgen-independent prostate carcinomas, as novel approaches to halting the progression of this disease.

1.1.4 EGFR mediated signalling pathway and Prostate Cancer

EGFR plays a pivotal role in the metastasis and proliferation of prostate cancer cells. Local invasion leads to much morbidity and metastasis contributes most of the mortality in patients. It has been long known that tumors originating in different sites metastasize to different locations. Prostate cancer is usually clinically silent until metastatic disease produces symptoms. Prostate tumor cells metastasize preferentially to bone marrow, especially to bone in the central spine [11], and to the liver, though these are usually clinically silent [12] [13] [14]. The bone metastases, being osteogenic cause pain, and are often the earliest clinical signs of tumor spread [15] [16]. Prostate cancer is thought to spread by lymphatic and hematogenous vessels. The larger and less differentiated the primary tumor, the higher the incidence of metastases. The prostate cancer cells can spread to bone, lung liver, and kidney via blood vessels during the late stage of the disease.

It is well known that growth factors stimulate the growth of tumors. In the case of prostate cancer, TGF-α was found to stimulate the cancer cell growth via the cell surface EGFR

[17] [18]. In the normal prostate stromal-derived TGF-α supports the prostate epithelium in a paracrine fashion. Both TGF-α and EGF are positive regulators for prostate cancer [19] [20], promoting growth and invasiveness. Several human prostate cancer cell lines, including PC3, DU145 and LNCaP cells, have autocrine loops consisting of EGFR and one or more of its ligands [21] [22]. The autocrine activation of EGFR by TGF-α and/or EGF promotes prostate tumor cell growth [23] [24] [25] and invasion and metastasis [26] [27].

1.2 EGF RECEPTOR AS A MOLECULAR IN SIGNALLING

Epidermal growth factor or EGF is a growth factor that plays an important role in a lot of cell processes, which inclding growth, proliferation, migration, adhesion, apoptosis, angiogenesis and differentiation. Human EGF is a 6045 Daltons single chain protein with 53 amino acid residues derived from a large (1207 amino acids) integral membrane protein precursor [28]. EGF is the first described member of a family of related but distinct ligands that bind to the same receptor. Other members of this growth factor family which binds to EGFR include TGF-α (transformation growth factor α), HB-EGF, vaccinia growth factor, amphiregulin and neuregulin [29] [30] [31] [32] [33]. All of these ligands contain a conserved EGF-like domain and synthesized as transmembrane precursor proteins [34]. Those ligands, especially TGF-α, were found up regulated in many human cancers [35] [36] [37].

Epidermal growth factor receptor or EGFR is a member of ErbB receptor family, a subfamily of four closely related receptor tyrosine kinases: EGFR (HER1 or ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4) [38]. EGFR is a 170 KDa transmembrane

glycoprotein with 1186 amino acids [39]. It has an extracellular domain with ligand binding sites and an intracellular domain with tyrosine kinase activity [40]; the other members of this family are similarly constructed. These receptors can homo- and hetero-dimerize to transmit signals (Figure 1.1). For instance HER2 has no known ligand and gets activated upon dimerizing with other members. HER3 does not have an active kinase domain, but rather serves to be phosphorylated by the other members of the EGFR family [41].

When the ligand binds to the EGFR, it induces a conformational change in the extracellular domain of EGFR and the receptor assembles into dimers. This greatly increases the EGF receptors' intracellular tyrosine kinase activity. The activated EGFR kinase catalyses the transfer of the γ-phosphate of bound ATP to its own or the other's C-terminal domain on specific tyrosine residues and also other substrates [42]. On activation, EGFR can pair with another EGFR to create a homodimer or pair with another member of the ErbB receptor family to form a heterodimer. Differences in the C-terminal domains of the ErbB receptors and the heterodimers they make results in different repertoires of signaling molecules that activated (Fig 1-1). There are also some compensating differences between the bindings of different ligands to EGFR [43]. The EGFR signaling affects apoptosis, differentiation, adhesion and, most evidently, cellular migration and proliferation [44].

Recent structural work shows that the activated EGFR is an asymmetrical dimer, with one kinase domain inducing allosteric activation of the other [45].

Various proteins can be complex with or phosphorylated by EGFR, which implies that EGFR activation can results in simultaneous activation of multiple pathways. The most well studied EGFR induced pathways include Ras/MAPK, STAT, and PLC-γ. Activated EGFR can bind to Grb2/Sos complex, which constitutively binds to Ras and resulting in the exchange of

Ras-bound GDP for GTP and hence Ras activation [46]. Activated Ras in turn activates Raf-1 [47] and then Erk-1 and Erk-2. PLC-γ binds directly to the phosphorylated EGFR and is activated by it [48]. Activated PLC-γ then moves to the membrane and cleaves PIP_2 (phosphatidylinositol bisphosphate) into IP_3 (inositol 1,3,5-trisphosphate) and DAG (1,2-diacylglycerol). This hydrolysis of PIP_2 releases actin binding proteins that alter the cytoskeleton [49]. Further more, EGFR can also cross-talk with heterologous receptors activated by other inducers [50].

After performing its function, the EGFR signal is inactivated two ways. If the ligand comes off the receptor, phosphatases rapidly remove the phosphotyrosines which shuts off the kinase activity. The greater part of inactivation likely occurs through endocytosis of the receptor-ligand complex. The ligand binding receptor are then either degraded or recycled to the cell membrane again [38]. Among the other ErbB family receptors, HER2 attracts most of the attention from researchers. It is a major partner of EGFR in forming heterodimers [51]. Although HER2 is not a receptor for EGF, it decreases the rate of ligand dissociation from EGFR, [52] and activated heterodimers are more stable with HER2 in the complex [53]. This leads to prolonged and heightened signaling [54].

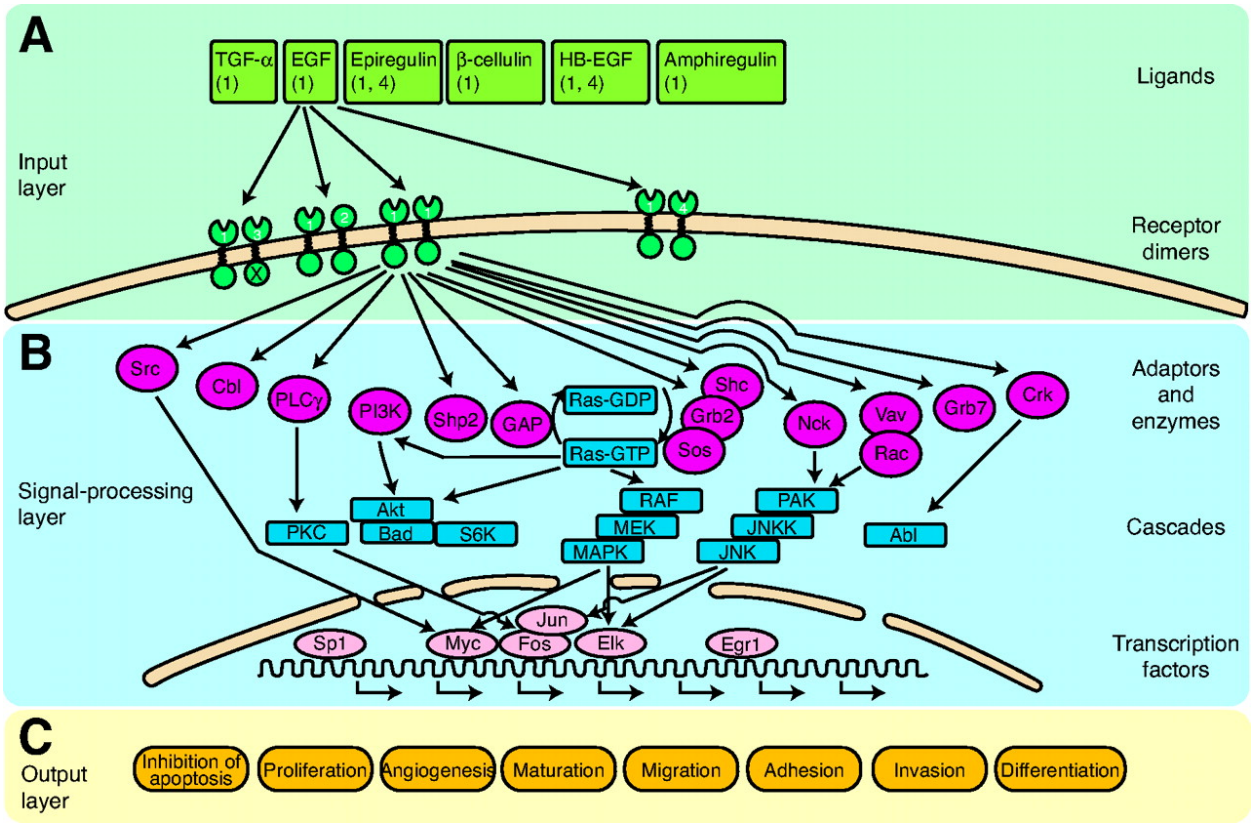


Figure 1-3. Schematics of EGFR signalling pathway.

A) Input level. The ligands and the dimerized receptors. Numbers indicate the respective high affinity HER receptor. HER1 (1), HER2 (2), HER3 (3) and HER4 (4). B) Signal transduction level. Not all the signalling pathways are shown here. C) Output level. Some target genes for EGFR signalling. (From The Oncologist, Arterga 2002)

1.3 EGF RECEPTOR AND CANCER

Aberrations in growth factor signaling pathways are strongly connected with cancer. Extracellular growth factors/growth factor receptors are essential for tumor invasiveness and metastasis as well as proliferation. The growth factor receptor most often found up-regulated in human tumors that have progressed to the invasive and metastatic state is the EGF receptor [55]. It is already demonstrated that the EGFR family of receptor tyrosine kinases lies at the beginning of many signal transduction pathways that regulate cell adhesion, migration, proliferation and differentiation [44]. Previous studies demonstrated that EGFR signaling in tumor cells causes enhanced motility/invasion, increased proliferation and decreased apoptosis, which are all critical to carcinogenesis.

EGFR is found over expressed in many different solid human tumors, including non-small cell lung, breast, gastric, head and neck, bladder, ovarian, esophageal, glioblastomas, colorectal, pancreatic and prostate [56] [57] [58] .High EGFR expression correlates with severe tumor stage, higher invasiveness of the tumor cells [59], resistance to normal therapies [60] [61], and poor prognosis [62] [63] [64].

The dysregulation of EGFR expression may come from mutations. A number of EGFR mutants have been found in tumors (Table 1) [65]. The most thoroughly studied EGFR mutant is EGFR vIII, in which exon 2-7 are missing. This truncated receptor has constitutive if low level activity and has defective downregulation [66] [67]. It has been detected in breast, medulloblastomas, and ovarian and non-small cell lung cancer [68].

In addition to mutations, other mechanisms also cause aberrant EGFR regulation, such as ligand autocrine/over expression, hetero-dimerization with other ErbB receptors, especially HER2, and transactivation by heterologous signaling pathways. EGFR signals may be enhanced by high levels of ligands. Co-expression of EGFR and its ligands can result in activation of an autocrine loop which leading to dysregulation of EGFR [36] [69] [70].

During signal termination, activated EGFR is endocytosed. A ubiquitin ligase called Cbl is required in this process. EGFR homodimers are more stable in the mildly acidic endosomal environment and remain bound to Cbl, while EGFR-HER2 heterodimers are less stable and cause Cbl to dissociate from the receptor complex, and the receptor is not degraded [71] [72]. Many data show that cancers with high expression of both EGFR and HER2 have a worse prognosis than cancers that only have high expression of one of the receptors [73] [74] [75] [76].

Various methods have been applied to evaluate EGFR expression in human tumor and normal tissues. None of them is consistently employed in all the research groups and this making the comparison of results from different labs difficult. Some commonly used techniques including immunohistochemistry (IHC), Western, Northern, RT-PCR, fluorescence *in situ* hybridization and quantitative PCR.

From decades of work, a variety of data supports the view that the EGFR is a relevant target for cancer therapy (Figure 1-2). So far, two main therapeutic approaches have been exploited to inhibit the EGFR. The first is the monoclonal antibodies that against EGFR [77] [78]. Those antibodies bind to the EGFR with affinity similar to normal EGFR ligands like EGF and TGF-α, compete with those ligands for receptor binding, and block EGF or TGF-α induced activation of EGFR tyrosine kinase. A second method targeting EGFR is the small molecular inhibitors of the EGFR tyrosine kinase enzymatic activity (TKIs) [79] [80]. Most of these

molecules are reversible competitors with ATP for binding to the intracellular catalytic domain of the tyrosine kinase.

Type	Alteration in sequence	Reference
EGFR vI	Translation starts at aa 543	[81]
EGFR vII	Deletion of aa 521–603	[82]
EGFR vIII	Deletion of aa 6–273	[83]
EGFR vIII/_12–13	Deletions of aa 6–273 and 409–520	[84]
EGFR vIV	Deletion of aa 959–1030	[85]
EGFR vV	Truncation at residue 958	[85]
EGFR.TDM/2–7	Tandem duplication of 6–273	[86]
EGFR.TDM/18–25	Tandem duplication of 664–1030	[86]
EGFR.TDM/18–26	Tandem duplication of 664–1014	[87]

Table 1. Mutations of the EGFR detected in tumor cells.

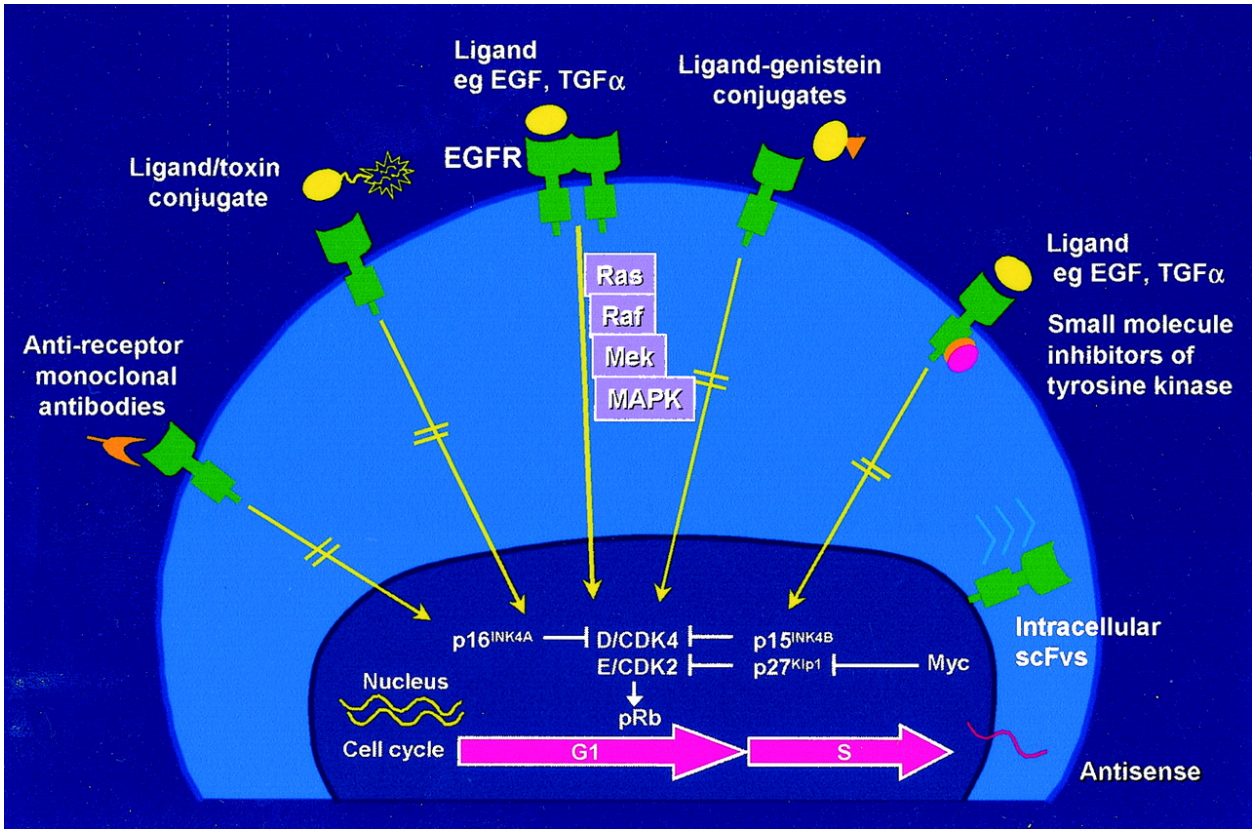


Figure 1-4. EGFR signalling pathway and approaches to inhibiting the EGFR.

Monoclonal antibodies against EGF receptor and small molecule inhibitors of tyrosine kinase are the two main groups that targeting the blockade of EGFR signalling pathway. (From Clinical Cancer Research, Ciardiello, 2001)

1.3.1 EGFR and Tumor Cell Invasion

Tumor invasion into surrounding tissues is the main reason for severe morbidity and mortality in many cancers, especially prostate, bladder, head and neck and esophagus [88]. Metastases cause 90% of human cancer deaths [89]. The removal of the primary tumors is accessible by surgery and radiological therapy, but local extension beyond the physiological borders can make patients impotent or engender adverse effects. The major events of metastasis include the invasion of adjacent tissues, intravasation, transport through the lymphatic and hematic system, arrest at a remote site and growth in a secondary organ (Figure 1-3) [90].

Early studies found that a number of retroviral oncogenes are derived from peptide growth factors and their receptors [88]. EGFR is the most frequently identified among those signaling molecules that over expressed in a wide variety of human tumor cells. Most epithelial cells express EGFR as well as its ligands like EGF and TGF-α. To prevent forming autocrine loop, the cells segregate the growth factors and their receptors by releasing them from different polarities [91] [92]. When the epithelium transformed with broken cell-cell junctions, the segregation disappears and results in autocrine stimulation [93]. Studies on different human tumor cells showed upregulated EGFR expression in invasive tumors compared to their non-invasive counterparts, which including glioblastomas, bladder and gastric carcinomas [4] [5] [94] [95]. For instance, ErbB2 is an important biomarker that is over expressed in 15-30% of human breast cancer and associated with poor prognosis [89]. More studies indicated that invasiveness of many tumor cells is modulated by EGFR mediated signals at least in vitro and in animal models [96] [26] [97] [98] [99].

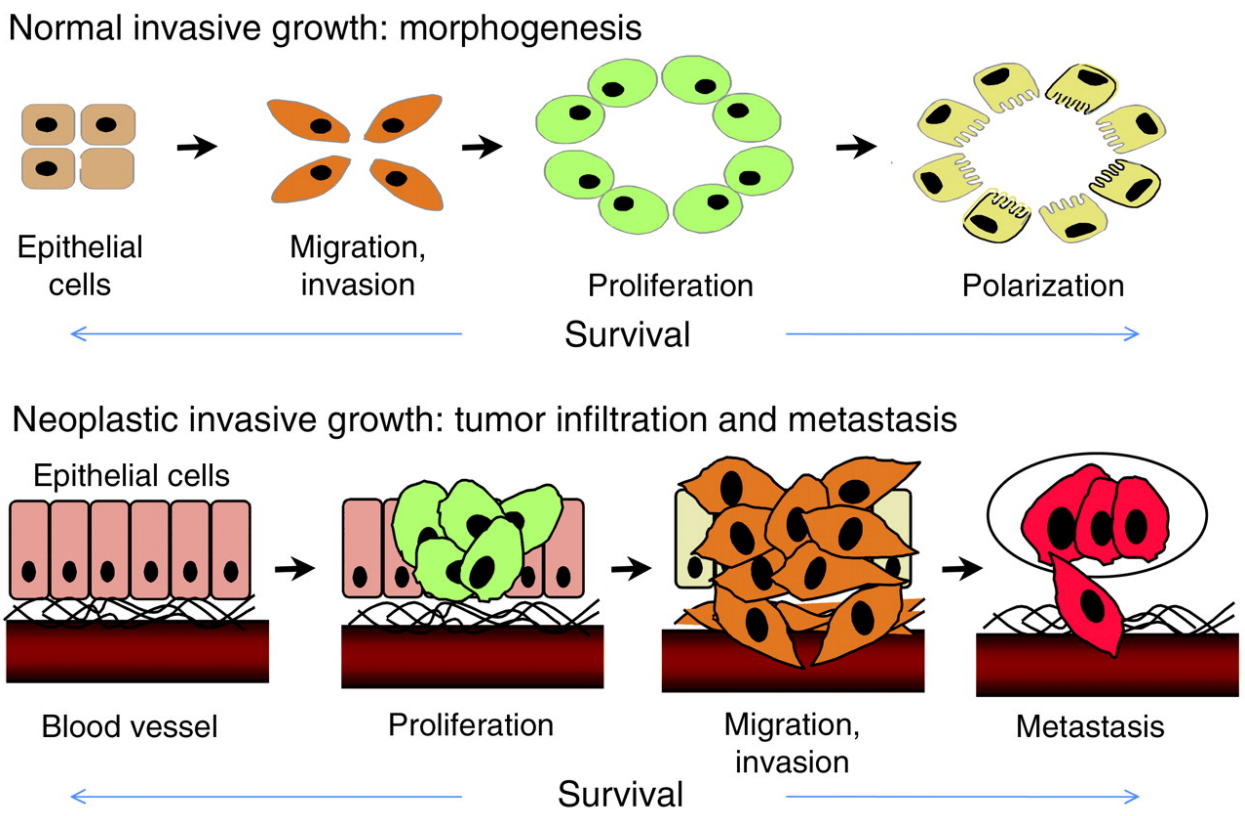


Figure 1-5. The invasive growth program under physiological and pathological conditions.

Invasive growth results from dissociation and migration, cell multiplication and survival. Normal cells use invasive growth to colonize new territories and forming new organs in development while tumor cells forming metastasis. (From JCI, Comoglio, 2002)

A critical protein needed in the EGFR mediated migration is PLC-γ. Wells' group demonstrated that overexpressing EGFR in DU145 human prostate carcinoma cells promoted EGF receptor dependent invasiveness both *in vitro* and *in vivo* [26] [27]. In addition, PLC-γ is necessary for the EGFR mediated motility [100] in human prostate tumor cells that over express EGF receptors. PLC-γ actuates motility by hydrolyzing PIP_2 into IP_3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol) and releasing actin binding proteins such as gelsolin, cofilin, and profilin [49] [101]. A second key switch for EGFR mediated motility is m-calpain. Activation of m-calpain is required for the deadhesion of tail retraction [102] [103]. How these molecules function in cell motility will be discussed below.

1.3.2 EGFR and Tumor Cell Proliferation

It is well known that EGFR signaling is highly correlated with cell proliferation. The EGFR autocrine pathway contributes not only to cancer cell migration/metastasis, but also to proliferation, and decreased apoptosis [104]. In normal physiology, paracrine signaling of TGFα from fibroblasts to the endothelial cells promotes angiogenesis and to soft organ epithelial cells maintains the epithelial layer and heals any breaks. The aberrant activity of members of EGFR family has been shown to play a critical role in the cancer development and progression. EGFR combined with constitutive, elevated expression of *c-myc* leads to abrogated cell cycle regulation in an in vitro mammary epithelial cell model system [105]. The involvement of EGFR was recognized in breast cancer [106] [107], head and neck squamous cell carcinoma [108] [109], and prostate cancer [110].

Normal cells require mitogenic growth signals for entering an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind to distinctive

classes of signalling ligands. Dependence on growth signalling offers a strict control method to make cells only proliferate when in an appropriate environment. On the contrast, tumor cells generate many of their own growth signals, which greatly reduce the dependence on a normal tissue micro-environment and contribute to the unlimited growth of cancer cells. For instance, in human prostate tumor cells, a TGF-α/EGFR autocrine loop is built and thus liberating the tumor cells from normal growth regulation.

1.4 SIGNALLING CELL MIGRATION INCLUDES ENA/VASP

Directed cell migration is one of the most critical aspects of a functional living cell. It choreographs the morphogenesis of the embryo during development, and in adult cell migration is central to homeostatic processes such as immune response and the repair of injured tissues. The dysregulation of cell motility can cause tumor invasion, chronic inflammatory diseases, failure of wound healing and many other diseases [111] [112]. Cell migration is a dynamic, cyclical process (Figure 1-6). A cell first extends a protrusion at its front which attaches to the surface the cell is migrating, then moves the cell body forward toward the protrusion, and finally it releases the attachments at the cell rear as the cell continues to move forward [112].

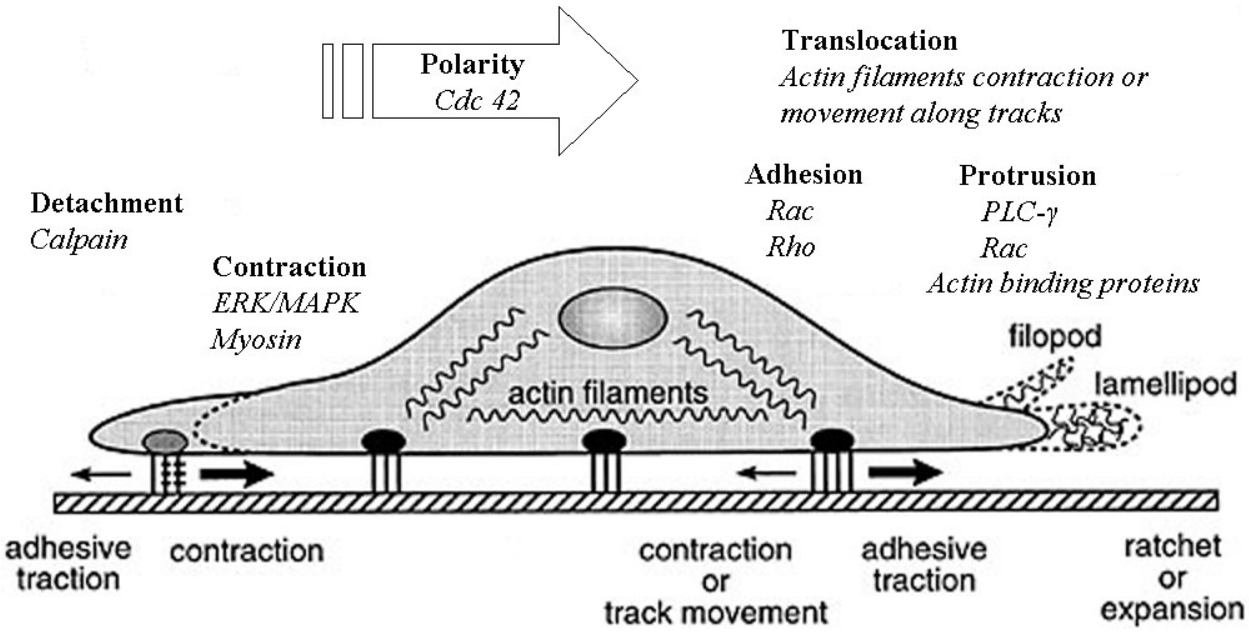


Figure 1-6. Cell motility events.

A schematic of the major steps involved in migration. (From Lauffenburge and Horowitz, 1996 and modified). The various aspects of cell migration are listed and given with their mainly associated signaling molecules. First, a cell extends its lamellipods, and one of them becomes the main extension, which adheres to an adjacent surface. Secondly, a rearrangement of cytoskeleton happens, simultaneously with cellular morphological disruption and the flow of activated kinases towards the extension. Thirdly, the rear side of the cell contracts to the extension and is released from the surface.

1.4.1 Signalling Cell Migration

Cell migration is initiated by external signals, which are recognized by specialized receptive proteins in the cell membrane. There are various signalling pathways and proteins involved in the process of cell migration from polarization to extension to rear release.

EGFR signaling initiates migration in many cells. EGFR effects cell migration through numerous downstream signaling pathways. When EGFR get activated, it dimerizes and auto-phosphorylates the specific tyrosine residues that induce these pathways by binding via SH2 domains [113]. The phosphorylated tyrosines on EGFR are recognized by the SH2 domains of PLC-γ and activate PLC-γ by phosphorylating it [114]. Activated PLC-γ then moves to the membrane [115] and cleaves PIP_2 (phosphatidyl inositol 3,4-bisphosphate) into IP_3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol). When PIP_2 is hydrolyzed, it releases many actin-binding proteins like gelsolin, cofilin, destrin, profiling and capping proteins from inhibition [116] [117] [118] [119] and thus making them available for the cytoskeletal reorganization and protrusion formation [49]. On the other hand, DAG can signal for PKC activation, which downregulates EGFR activity while IP_3 signaling releases Ca^{2+} from endoplasmic reticulum leading to the activation of Ca^{2+}- dependent enzymes like PKC and μ-calpain.

Another pathway from EGFR for cell migration is through ERK/MARK [120]. EGF signaling goes through Ras-Raf-MEK-MAPK/ERK and the last activates m-calpain by phosphorylating its Ser50 [121] [122]. Calpains are a family of calcium-dependent intracellular cysteine proseases. They cleave several substrate proteins that connect the cytoskeleton to the substratum [123] [124] [125] [126] [127] [128] [129], which demonstrates this pathway is required for focal adhesion disassembly and decrease in adhesiveness to the substratum. [102]

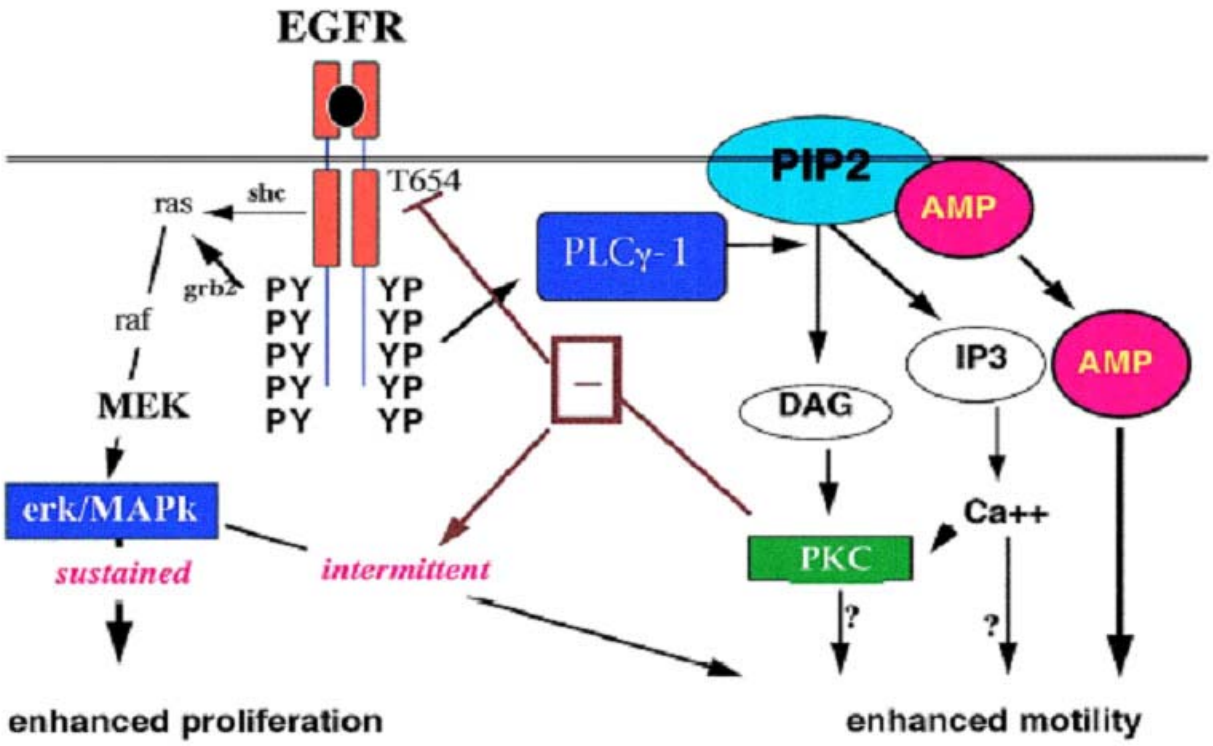


Figure 1-7. EGFR mediated cell motility pathways.

A schematics of EGFR mediated pathways regulating cell migration. EGF receptors phosphorylate PLC-γ and PI3K. Only surface EGF receptors mediate PIP_2 hydrolysis by PLC because PIP_2 is not accessible to internalized receptors.

As we just mentioned, PLC-γ activation by EGFR produces DAG, which activates the Ser/Thr kinase protein kinase C (PKC). PKC is a large family of at least 12 members [130]. PKC proteins phosphorylate a variety of substrates including signal transductional proteins [131] and motility-associated proteins [132] [133], making PKC an important player in migration signaling pathways. Increased levels of PKC have been found associated with malignant transformation in a number of cancers including lung, breast and gastric carcinomas. Previous work in our lab clarified the EGF induced signaling pathway of migration in fibroblast cells. We demonstrated that EGF stimulation increases myosin light chain (MLC) phosphorylation, which is a marker for contractile force, concomitant with protein kinase C, particularly PKCδ [134]. Many recent data suggest a crucial role that PKCδ may plays in breast cancer [135] [136], both for its invasion and proliferation [137]. One of the interesting characteristics of PKC is that PKC attenuates signaling from the EGF receptor [138]. It decreases the binding affinity of the EGFR and its ligands [139] and diminishes tyrosine kinase activity of the receptor [140]. These data imply a feedback attenuation mechanism in the EGFR induced cell migration regulation [141].

Lamellipod extension is the most obvious process in cell motility. This protrusion extends from the cell body to create new, distal adhesion sites. The leading edge of a lamillipod contains highly branched actin filaments [142]. At the leading edge, G-actin monomers are added to the barbed ends of actin filaments and removed from the pointed end [143], which pushes the membrane forward and leads to protursion at the leading edge. This cytoskeletal growth is made possible by actin binding proteins released by PIP_2 hydrolysis by PLC-γ. How these are presented at the front of the cell, even in the absence of a gradient, involves Cdc42 binding to PLC-γ and directing towards the leading edge [144]. This then denudes the front of

PIP_2. As m-calpain needs PIP_2 to be fully activated by EGFR, this asymmetry of PLC-γ results in de-adhesion occurring only at the cell's rear [145].

1.4.2 Arp2/3 Complex

The polarizing of actin in the lamellipod is regulated by proteins that bind to and modify actin. Arp2/3 (Actin-related protein 2/3) is a stable protein complex that responsible for branching [146] and nucleation [147] of actin filaments. It is localized to the leading protrusions of migrating cells [148] [149]. After being activated by WASPs (Wiskott-Aldrich Syndrome proteins), Arp2/3 nucleates actin by binding profilin, a protein bound to monomeric actin-ATP [150]. The actin-ATP is transported to the nucleated actin on Arp2/3, then loses its γ-phosphate group and becomes actin-ADP. The Arp2/3 complex caps the pointed ends of actin filaments, this is the molecular basis for the formation of highly branched actin filament network at the leading edge [151]. Most recent data demonstrated that mRNAs for the seven subunits of Arp2/3 complex are localized to the protrusions in fibroblasts, which supporting the hypothesis that Arp2/3 is targeted to its function site by mRNA localization [152].

1.4.3 Ena/VASP

Ena/VASP Proteins are actin-binding proteins that localize to actin stress fibres, filopodial tips and to the lamellipodial leading edge [153]. Enabled (Ena) was first found in 1990 as a genetic suppressor of mutations in *Drosophila* Ableson tyrosine kinase [154]. Ena was also found to function in several signaling pathways essential for axon guidance in the developing of

nervous system [155] [156]. Vertebrates have the other three members in this family, which are VASP (Vasodilator-stimulated phosphoprotein), Mena (Mammalian Enabled) and EVL (Ena-VASP like). They localize to focal adhesions, actin stress fibers, filopodial tips and lamellipodial leading edge [157] [158] [159]. VASP was also identified as a major protein kinase A substrate in platelets [160]. Ena/VASP family members share a conserved domain structure consisting of an N-terminal Ena-VASP-homology-1 (EVH1) domain, a central polyproline-rich core and a C-terminal EVH2 domain (Fig 1-9).

The studies on Ena/VASP these years got a number of seemingly conflict results. Deletion of the Ena/VASP binding sites within the bacterial protein ActA decreased actin dependent intracellular motility [161] [162], delocalization of all Ena/VASP proteins abolished reorganization of the actin cytoskeleton and impaired actin dependent phagocytosis [163] [164], and purified Ena/VASP proteins can stimulate the nucleation of actin filaments *in vitro* [165] [166] [167]. This was all interpreted as indications of a positive role for Ena/VASP in actin dependent process. On the other side, a dose-dependent decrease in movement is observed when Ena/VASP proteins are over expressed in fibroblasts and deletion of them results in increased cell movement [168]. Platelets without VASP expression shows increased collagen induced platelet aggregation [169]. Neutralization of Ena/VASP functions in neurons in the developing neocortex leads them migrate much farther than normal neurons [170]. All these data suggest a negative role for Ena/VASP proteins in actin dependent processes.

Although all those processes mentioned are actin dependent, they are not directly comparable. There is no persuasive evidence supporting Ena/VASP as nucleator of actin in living cells [171]. Recent study shows Ena/VASP proteins antagonize capping protein to inhibit actin polymerization at barbed ends *in vitro* [172], which suggested that Ena/VASP proteins

associate with actin filaments at or near the barbed end and protect them from being capped by capping protein. The depletion of Ena/VASP making the lamellipodial protrudes slower but persists longer, which actually makes cells move faster (Fig 1-9).

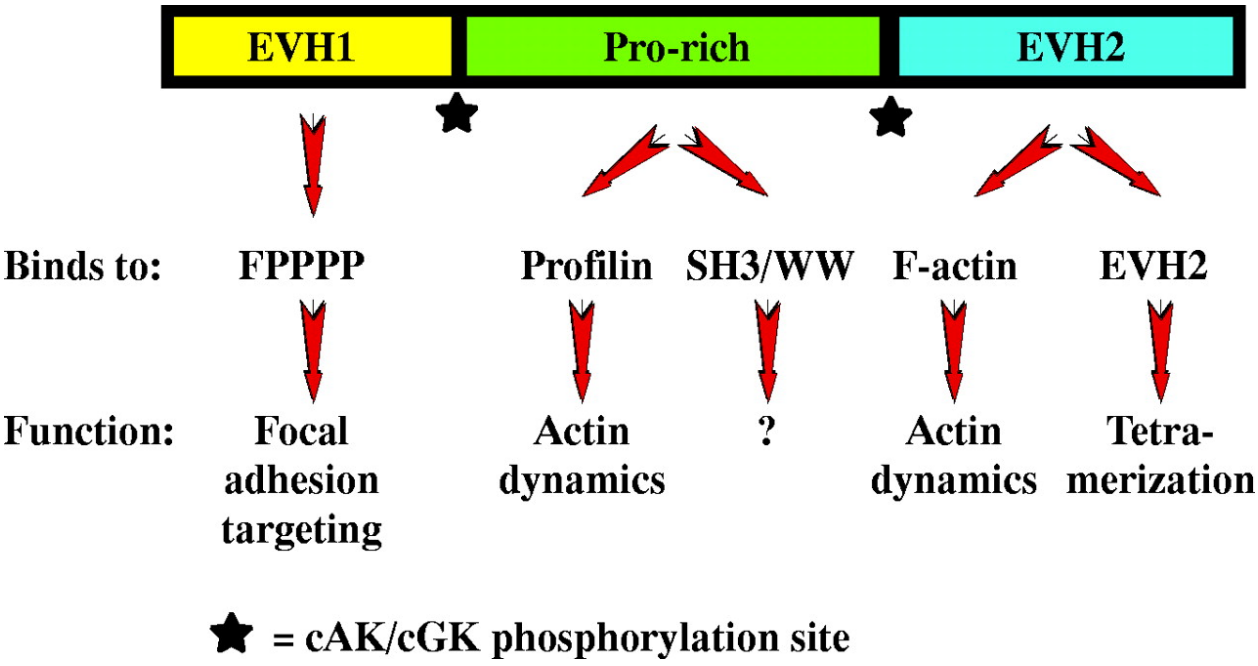


Figure 1-8. Domain structure of Ena/VASP family proteins.

Primary structure of Ena/VASP family proteins with their binding partners and functions. (From: Krause M. et al. 2002 J Cell Sci)

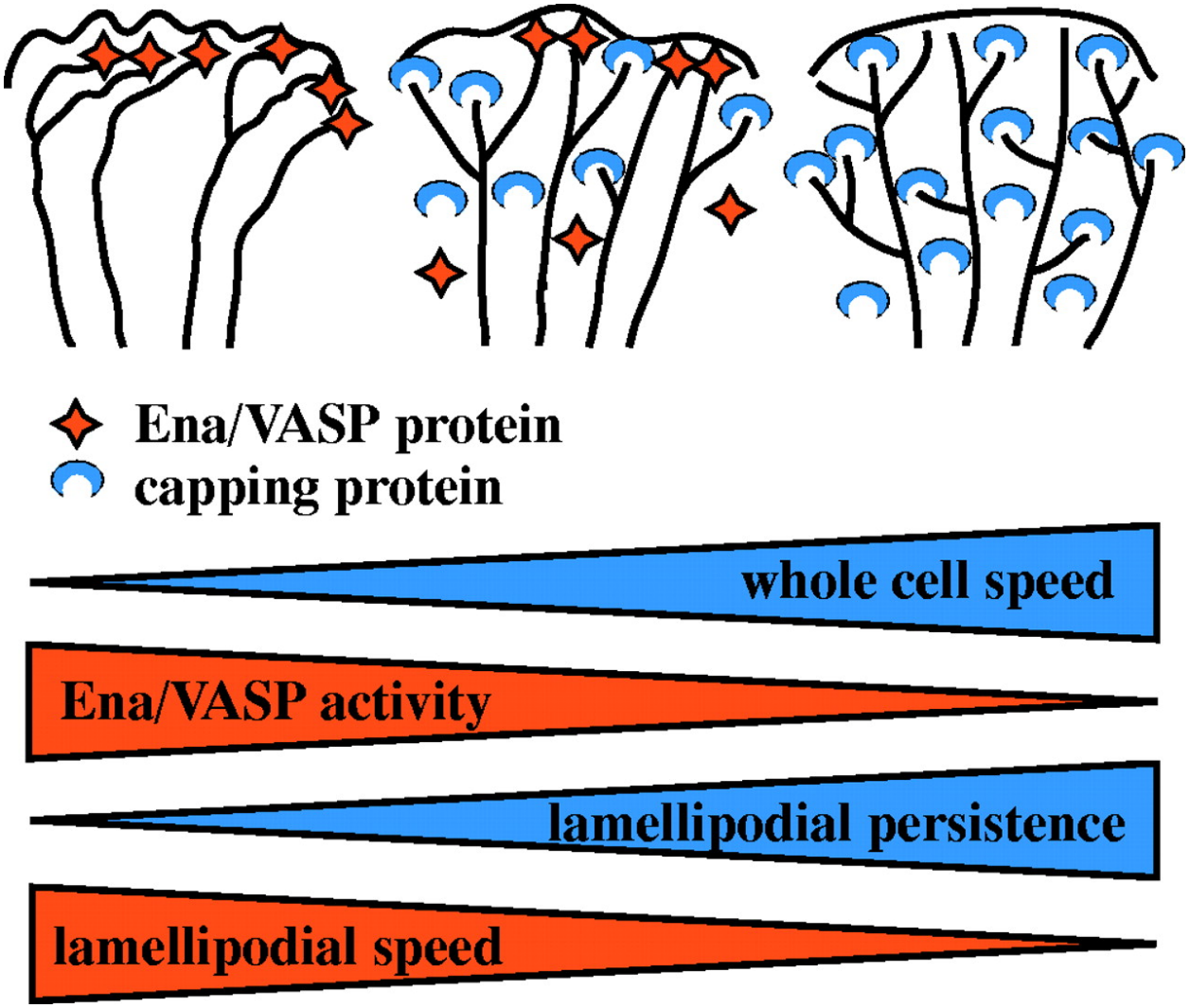


Figure 1-9. Antagonism between Ena/VASP and capping protein.

The antagonism between Ena/VASP and capping protein regulates lamellipodial protrusion and whole cell motility. Elevated Ena/VASP activity inhibits capping protein in the leading edge, resulting in longer and less branched actin filaments. This leads to higher lamellipodial protrusion velocity but shortened persistence. (From JCS, Krause, 2002)

1.4.4 Regulation of β-actin

Cells may also modulate migration by regulating localization of β-actin messenger RNA to sites of active actin polymerization [173] [174] [175]. This monomer is the building block of the actin cytoskeleton, and supply of the monomer regulates actin filament length and extension. Asymmetric localization of specific mRNAs generates cell polarity by controlling sites of translation and restricting the synthesis of its protein product to specific compartments of the cell [176] [177]. β-actin mRNA is localized near the leading edge where actin polymerization is actively promoting forward protrusion. A protein called ZBP1 (Zipcode binding protein 1) is necessary for the localization. This protein binds to a conserved 54-nucleotide element in the 3'-untranslated region of β-actin mRNA known as "zipcode". [178] [179] The β-actin zipcode sequence is essential for correct targeting of β-actin mRNA [180]. The mRNAs are transported on microtubules and actin filaments and anchored on actin filaments [181] [182] [183]. Most recent research found ZBP1 associates with the β-actin transcript in the nucleus and blocks initiation of translation in cytoplasm until the ZBP1-mRNA complex reaches its destination, where the protein kinase Src phosphorylates a tyrosine residue in ZBP1 that is required for binding to RNA and promotes translation [184] (Fig 1-11).

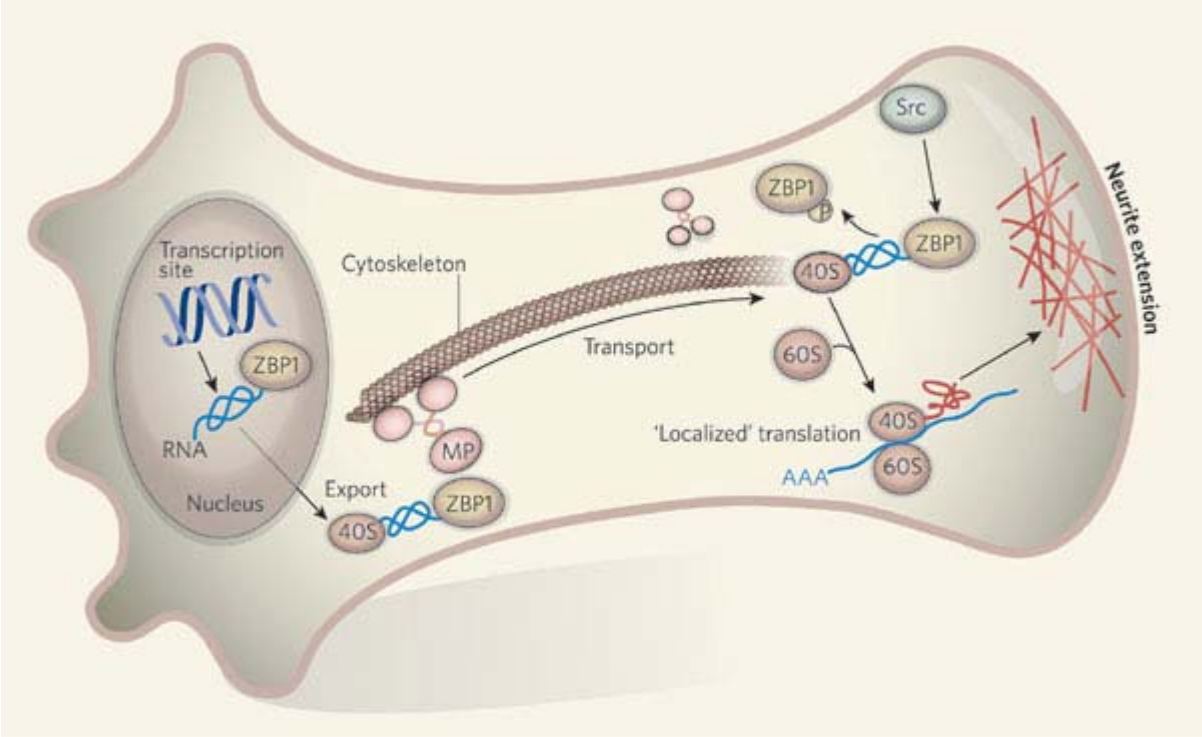


Figure 1-10. Regulation of localized β-actin mRNA translation in a polarized neural cell.

ZBP1 protein is regarded to control the transport of β-actin mRNA and its translation. ZBP1 escorts β-actin mRNA from nucleus to the cytoplasm, at where the ZBP1-β-actin mRNA complex binds to a motor protein and is transported along the cytoskeleton to the periphery. During transport, ZBP1 prevents the mRNA from being translated. (From Nature, Dahm, 2005)

1.5 SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION

One of the major signal transduction pathways required for proliferation and migration mediated by EGFR is the activation and translocation of STATs [185] [186]. De novo transcription is required for proliferation, and also for migration [100]. It is still unknown whether the transcriptional need in migration is for replacement of degraded proteins, increased levels of the motility machinery components, or specific regulatory factors. Therefore, I decided to ask whether this key transcriptional pathway was required in prostate cancer motility.

EGFR was found to directly activate STAT 1, STAT 3 and STAT 5. Our lab was among the first to demonstrate that EGFR activates members of the STAT family [187]. Activation of ERK MAP kinases and PI3-kinase are not sufficient for mitogenesis by growth factors, suggesting that an additional pathway such as STAT is needed [188].

1.5.1 Background of STATs

STAT (Signal Transducer and Activator of Transcription) is a family of transcription factors that are implicated in programming gene expression in biological events, including embryonic development, programmed cell death, organogenesis, innate immunity, adaptive immunity and cell growth regulation [189]. Seven mammalian STAT genes have been found now; they are STAT1, 2, 3, 4, 5a, 5b and 6, which are structurally conserved (Fig 1-12). All the seven members of the STAT family share several conserved domains. Those domains including an amino-terminal domain (interacts with the transcriptional co-activator and regulates nuclear translocation); a coiled-coil domain (forming predominantly hydrophilic surface, receptor binding, tyrosine phosphorylation and nuclear export); the DNA binding domain; a linker

domain; an SH2 domain (binds to specific phospho-tyrosine motifs); a tyrosine activation domain (circled P) and the transcriptional activation domain. The transcriptional activation domain (TAD) is conserved in function but not in sequence [190].

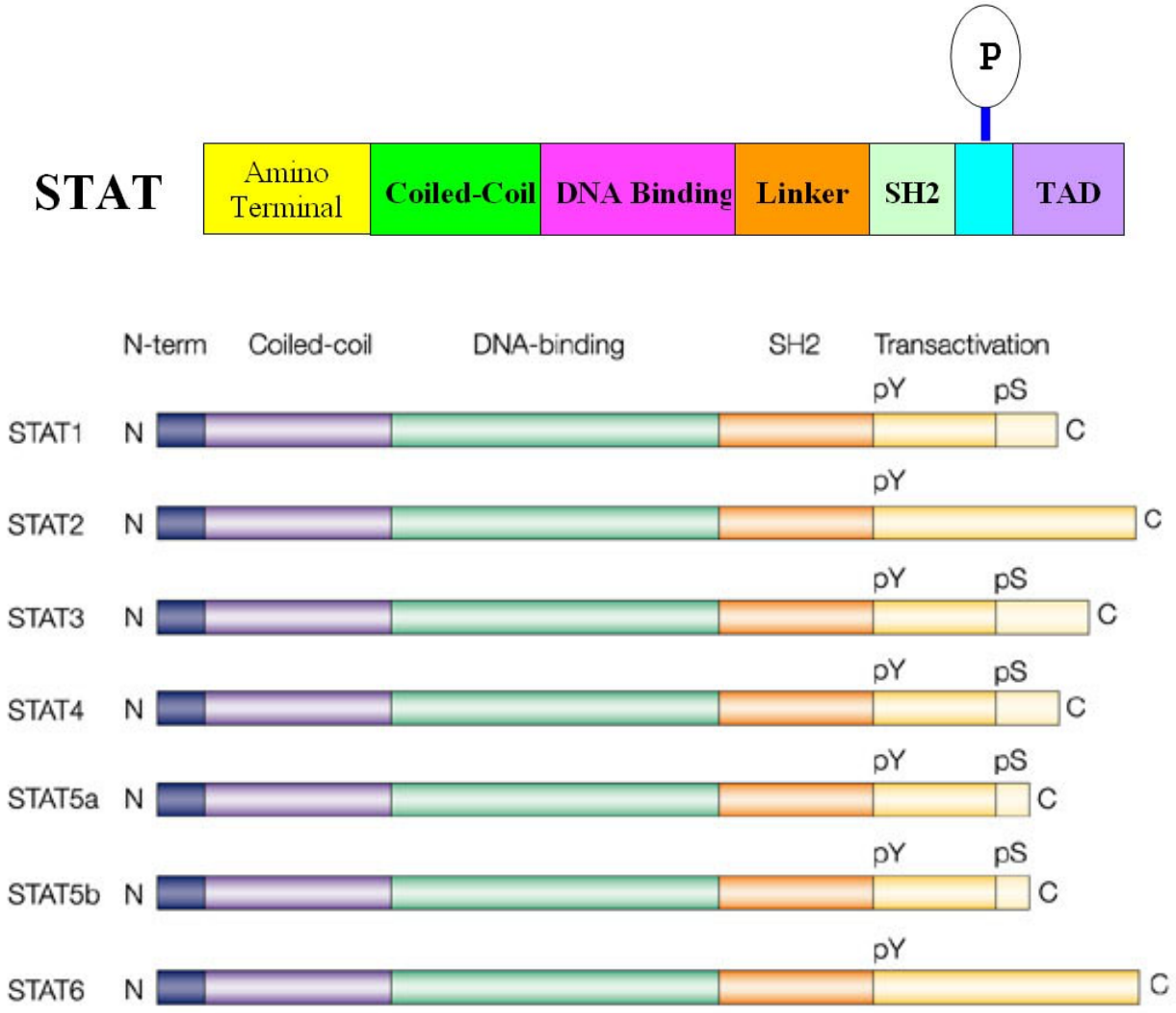


Figure 1-11. STAT structure and family of proteins.

The domain structure of the seven STAT family members is shown. The N-terminal domain mediates the interaction between two STAT dimers to form a tetramer. The coiled-coil domain is involved in interactions with regulatory proteins and other transcription factors. The DNA binding domain makes direct contact with STAT-binding sites in gene promoters with consensus core sequence. Reciprocal interaction of SH2 domain of one STAT monomer and the phosphotyrosine of another mediate dimer formation. (Nature Reviews of Cancer, Jove 2004)

1.5.2 Signalling Pathways that activate STATs

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway transmits information received from extracellular polypeptide growth factors, through transmembrane receptors, directly to target gene promoters in the nucleus, providing a mechanism for transcriptional regulation without second messengers [191]. The STAT signaling pathways were originally found in the cascade of normal cytokine receptors like interferon (IFN) and interleukin-6 (IL-6). Unlike growth factor receptors, most of the cytokine receptors are devoid of intrinsic tyrosine kinase activity. Instead, ligand engagement leads to activation of Janus kinase (JAK) family members, a group of receptor associated tyrosine kinases [192]. In some cases, SRC family kinases also involved [193]. Those kinases phosphorylate specific tyrosine residues on the cytoplasmic tails of cytokine receptors, thus providing docking sites for STAT monomer's SH2 (SRC Homology 2) domain. The recruited STAT monomers are phosphorylated on their specific tyrosine residues by the JAK or SRC kinases and form activated dimers through reciprocal phosphotyrosine-SH2 interactions between two monomers [192].

STATs can be activated by growth factor receptors with intrinsic tyrosine kinase activity. Those receptors include EGFR and PDGFR (platelet derived growth factor receptor) [194] [195]. These activate STAT directly without the intervention of JAK kinases [196]. However, the intrinsic tyrosine kinases also may cooperate with JAK kinases in the phosphorylation of STATs [197]. There is also non-receptor, cytoplasmic tyrosine kinases that signal through STATs, which include ABL (Abelson Leukaemia Protein) and SRC related kinases (Fig 1-13) [198].

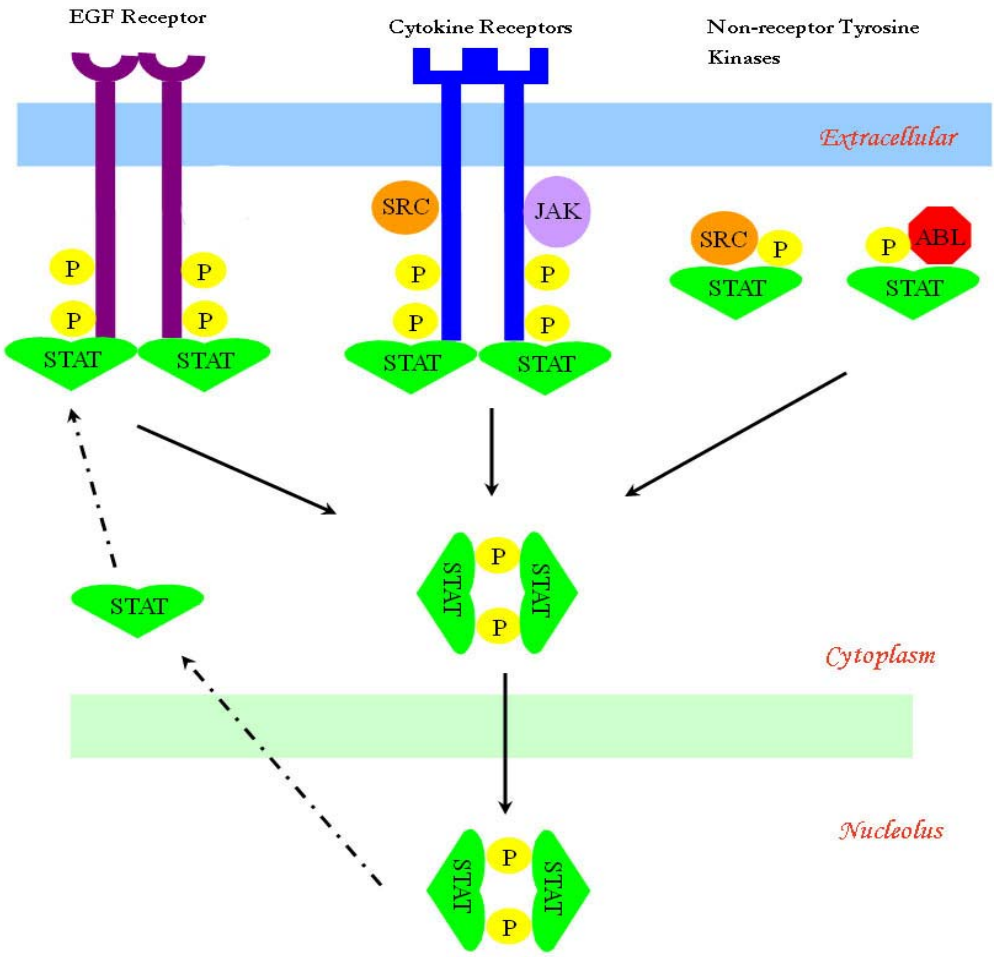


Figure 1-12. Signalling pathways of STATs.

Binding of growth factors or cytokines to their receptors results in the activation of intracellular receptor tyrosine kinase activity or receptor associated kinases such as JAK or SRC. These activated tyrosine kinases phosphorylate the receptor tails that in the cytoplasm, which provide a docking sites for the mono-STAT protein. Non-receptor tyrosine kinases, such as ABL, can phosphorylate STATs withorht receptor engagement. After been recruited, STAT monomers phosphorylate each other on special tyrosine residues and form activated dimers. The dimers translocate to the nucleus and directly regulate gene expression. (From Nature Reviews of Cancer, Jove, 2004 and modified)

1.5.3 STAT3 and Cancer

In many cancer cells, both cytokine and growth factor receptors become constitutively active due to autocrine or paracrine expression of their own ligands, mutations, failure of degradation or simply over expression of themselves. These aberrant changes can all cause the constitutive activation of STATs.

STAT3 activation is implicated in tumor invasion in head and neck squamous cell and other carcinomas [185] [186]. A significant correlation has been reported between the expression of nuclear STAT3 and breast cancer as compared to normal mammary tissues [199]. Interestingly, prostate tumor cells have been found to contain constitutively activated STAT3, and blockade of this activated STAT3 significantly suppressing the tumor cell growth [200] [201] [202]. A first clue implicating STATs in oncogenesis was the finding that STAT3 is constitutively activated in SRC transformed cell lines and interrupting STAT3 signaling blocks the transformation [203] [204] [205]. This is of key interest as a later study demonstrated that constitutively activated STAT3 mediate cellular transformation [206]. A survey of organ-confined prostate biopsies demonstrated a correlation between local aggressiveness and phospho-STAT3 staining [207]. These reports support hypothesizing that STAT3 signaling contributes to carcinogenic progression, in addition to increasing the cell number.

Constitutively activated STAT3 has been detected at high frequency in many human cancers (Table 2). Various signaling molecules and their receptors like IL-6, TGF-α, EGF and HGF (Hepatocyte Growth Factor) are involved in this process. Besides being a crucial part for a variety of oncogenic signaling pathway, STAT3 also participates in tumor cell growth, survival, angiogenesis and immune evasion. The first direct evidence that inhibition of STAT3 signaling in human cancer cells apoptosis came from the finding that increased expression of BCL-XL, an

anti-apoptotic BCL-2 family gene, is dependent on constitutively activated STAT3 [208]. More research data demonstrated that activated STAT3 increases the expression of c-Myc and cyclin D1, which are both critical to cell proliferation and suppress the expression of TP53 gene, the inducer of apoptosis [206] [209]. The same group also found STAT3 is a direct transcriptional activator of the VEGF (Vascular Endothelial Growth Factor) gene. Blocking STAT3 signaling inhibits the SRC and IL-6 induced VEGF up regulation [210]. Furthermore, inhibition of STAT3 in tumor cells was found to lead the production of inflammatory cytokines and chemokines, which in turn activate immune cells [211].

TUMOR TYPE SOLID TUMORS	ACTIVATED STAT PROTEIN	REFERENCE
Breast cancer	STAT1, 3, 5	[212]
Head and neck cancer	STAT1, 3, 5	[186]
Melanoma	STAT3	[213]
Lung cancer	STAT3, 5	[214]
Ovarian cancer	STAT3	[215]
Pancreatic cancer	STAT3	[216]
Prostate cancer	STAT3, 5	[217]
BLOOD TUMORS		
Multiple myeloma	STAT1, 3	[218]
HTLV-1-dependent leukemia	STAT3, 5	[219]
Acute myelogenous leukemia	STAT1, 3, 5	[220]
Chronic myelogenous leukemia	STAT5	[221]
Large-granular-lymphocyte leukemia	STAT3	[222]
Acute lymphoblastic leukemia	STAT5	[223]
LYMPHOMA		
EBV-related and Burkitt's lymphoma	STAT3	[224]
Cutaneous T-cell lymphoma	STAT3	[225]
B-cell non-Hodgkin's lymphoma	STAT6	[226]
Anaplastic large-cell lymphoma	STAT3	[227]

Table 2. Activation of STATs in human cancers.

1.6 CASPASES AND APOPTOSIS

Apoptosis is a programmed form of cell death, which is regulated in an orderly way by signal pathways under certain situations. Apoptosis is critical to cell growth regulation, development,

immune response as well as keeping a constant amount of cells for organs. A hallmark of cancers is the imbalance between cell proliferation and death, mainly by apoptosis, resulting in excessive cell number. For the slow growing prostate cancer, there is often more a deficit of apoptosis rather than rapid proliferation.

1.6.1 Apoptosis

So far, there are two main apoptosis pathways have been described: the death receptor induced pathway and the mitochondrial mediated pathway, or called extrinsic and intrinsic pathway according to the location of initial signaling [90]. Both of these pathways activate the executioner intracellular proteases, caspases.

Generally, there are two pathways for caspase proteins to be activated: one is the death signal induced and the other is mitochondrion mediated. In the first pathway, death signals as TNF (tumor necrosis factor) or FasL can be specifically recognized by their receptors and activate those receptors. Activated death receptors will interact with pro-caspases and induce their activation through oligomerization and auto-cleavage, leading to apoptosis [228]. On the other hand, procaspase can also be activated through a cytochrome C dependent pathway. After cytochrome C is released from mitochondria under cellular stress like DNA damage, it can directly activate some type of procaspase or form a complex called the apoptosome by oligomerizing Apaf-1 (apoptotic protease activation factor-1), then activate caspases [229] [230]. There are many regulators of mitochondria mediated apoptosis pathway, including BCL-2 and BCL-XL, which negative to apoptosis; and BAX and BAD, which is pro-apoptosis (Fig 1-14).

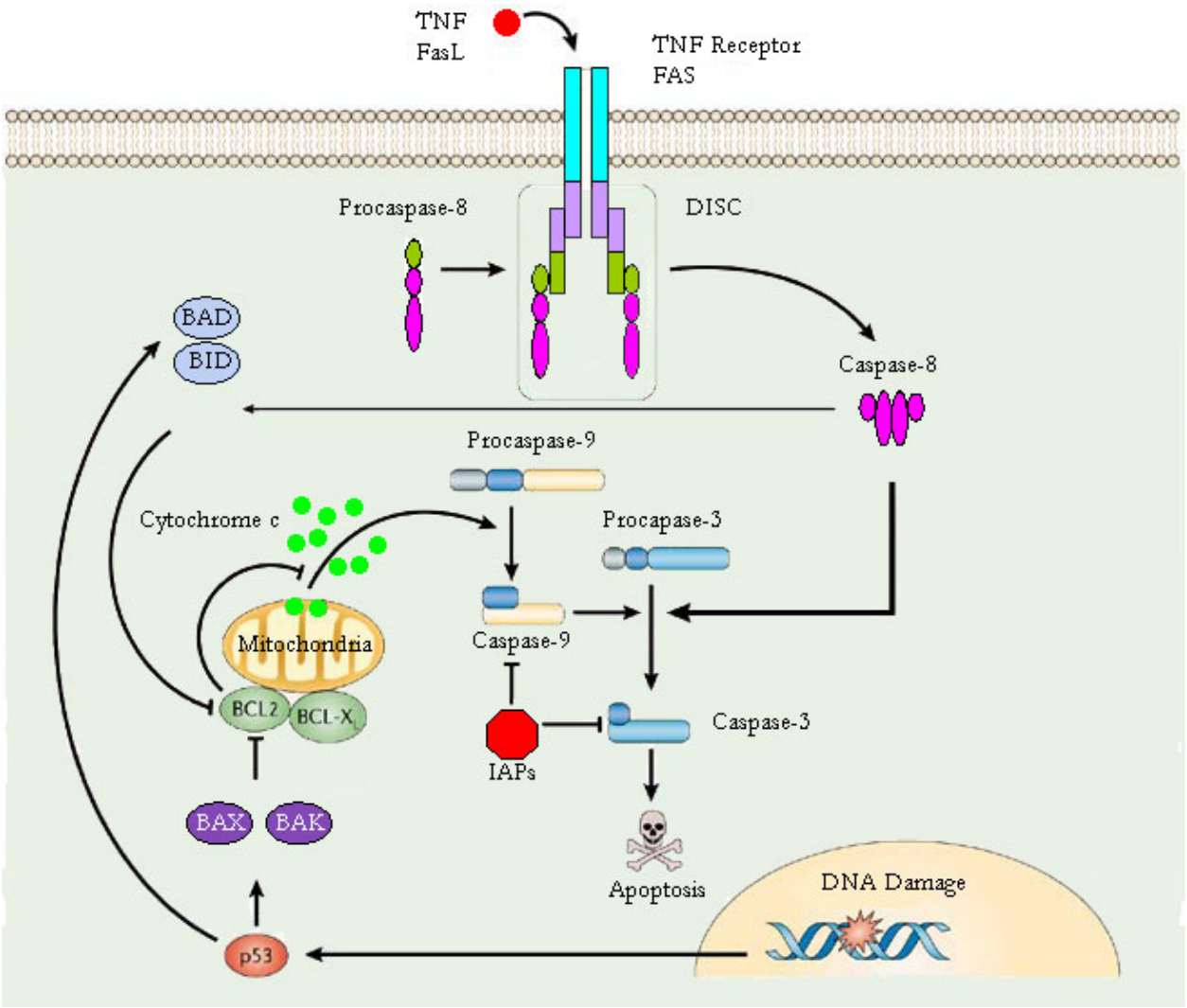


Figure 1-13. Schematic representing the core components of apoptosis pathways.

In the extrinsic pathway, TNF super family members including Fas Ligands binding to a death receptor and forming a death inducing signalling complex (DISC), which activate caspase-8. In the intrinsic pathway, cytochrome c released from mitochondria causes apoptosome formation and caspase-9 activation. Both caspase-8 and caspase-9 activate down stream caspases like caspase-3 and leading to apoptosis. (From Patric, 2006 and modified)

1.6.2 Caspase proteins

The initiation and execution of apoptosis requires a group of proteins that include signal transducers, receptors, gene regulators and enzymes. Among them, the caspase proteins are vital to the process of apoptosis [231].

Fourteen members of caspase family have been identified up to now, and all share some common properties. Caspases are aspartate-specific cysteine proteases and they all come from a zymogen precursor. Based on their function and homology in amino acid sequences, caspases are divided into three groups: apoptosis activators like caspase 2, 8, 9, 10 that contain a long prodomain at the N-terminus; apoptosis executioners such as caspase 3, 6, 7 that have a short prodomain and inflammatory mediators with caspase 1, 4, 5, 11, 12, 13 and 14 (Table 3).

Among all the apoptosis-related caspase proteins, Caspase 3 acts at the effector's stage of the whole signal cascade. It cleaves various of targets like PARP (poly ADP-ribose polymerase) and DFF (DNA fragmentation factor) that lead to cell death as well as cleaving Caspase 6, Caspase 7 and caspase 9 [232]. Also known as CPP32, Yama or apopain, Caspase 3 is a key factor in apoptosis execution. It is activated by cleavage from procaspase 3. It was found that a small molecule antagonists of XIAP (X-chromosome-linked inhibitor of apoptosis protein) that overcome inhibition of caspase-3 directly induced cell death in tumor cells while having little toxicity on normal cells, which indicates a critical role of caspase-3 in cancer cell apoptosis [233].

Subfamily	Role	Members
I	Apoptosis activator	Caspase-2
		Caspase-8
		Caspase-9
		Caspase-10
II	Apoptosis executioner	Caspase-3
		Caspase-6
		Caspase-7
III	Inflammatory mediator	Caspase-1
		Caspase-4
		Caspase-5
		Caspase-11
		Caspase-12
		Caspase-13
		Caspase-14

Table 3. Subfamily of caspase proteins.

2.0 MATERIAL AND METHODS

2.1 CELL LINES & MEDIUM

A murine fibroblast line was selected to define the transcriptional changes concomitant with EGFR-mediated cell motility. This was chosen as such fibroblast lines avoid the autocrine signaling through EGFR that is prevalent in carcinoma cells including those of the prostate [234]. The line chosen is a well characterized subline of NR6 cells that now expresses physiological levels of the EGFR [235]. These are derived from murine Balb/c 3T3 cells that were selected to not respond to EGF; these cells had silenced their EGFR transcription [236]. EGFR was expressed upon MLV-based retroviral transduction of a single gene copy with the selectable marker, neomycin phosphotransferase, driven from the env position using a separate promoter. Use of these cells allowed for isolation of EGFR signaling by comparison to the parental NR6 cells.

I chose to use two human prostate carcinoma cell lines to explore the role of the STAT3-driven transcription changes in tumor progression and survival. Androgen-independent cell lines were used as these represent the state at which prostate carcinoma is refractory to standard hormonal therapy [237] [238] and the situation that engenders the major part of prostate cancer mortality [239]. DU145 cells were selected as their in vitro and in vivo invasive behavior is well characterized [26] [27]. These were derived from a prostate adenocarcinoma metastasis to the brain [238]. These cells are only moderately invasive and have limited dissemination from orthotropic (intra-prostate) inoculation in mice. These cells were made more invasive and metastatic by over expression of full length human EGFR [26] [27]. DU145 present a mutant androgen receptor that is independent of exogenous testosterone [240]. The second cell line,

PC-3, was isolated from a prostate adenocarcinoma metastasis to the bone [237]. This line is considered highly aggressive and metastatic when inoculated in mice.

Human DU145 prostate carcinoma cells [238] over-expressing EGFR, DU145WT [26], were maintained in Dulbecco's minimal essential media (DMEM) (Mediatech) supplemented with 10% fetal bovine sera (FBS), L-glutamine (2mM), non-essential amino acids (0.1mM), sodium pyruvate (1mM) and 100U/ml of penicillin. To maintain expression from the transduced EGFR in the DU145WT cells, 350 mg/ml of G418 was added to the media. Human PC3 prostate cancer cells [237] were maintained in F-12 medium (GIBCO) supplemented with 10% FBS, L-glutamine (2mM), non-essential amino acids (0.1mM), and sodium pyruvate (1mM). NR6WT mouse fibroblasts expressing human EGFR [235] were maintained in Eagle's minimal essential medium alpha modification (MEMα) (Mediatech) supplemented with 10% FBS, L-glutamine (2mM), non-essential amino acids (0.1mM), sodium pyruvate (1mM), 100 U/ml of penicillin and 350 mg/ml of G418. All the cells were grown in 5% CO_2 at 37˚C.

2.2 RNA PURIFICATION

To purify the RNA for microarray analyses, the treated cells were washed by ice cold 1 X PBS twice. 1 ml of Trizol was added into each 10 cm plate and incubated at room temperature for 5 minutes. After incubation, the cells were scraped and transferred into a 1.5 ml centrifugal tube. Chloroform equal to 20% of the original volume of cells was added into the tube and shake vigorously by hand for 15 seconds. The mixture was incubated at room temperature for 2 minutes before going centrifuge at 12,000 rpm for 15 minutes. The upper aqueous after centrifuge was transferred into a fresh tube and 75% of the volume of isopropyl alcohol was added. The mixture was incubated at room temperature for at least 10 minutes before going

centrifuge again at 12,000 rpm for 15 minutes. The pellets were washed with 70% and then 100% ethanol, ended with air dry for 10 minutes [241].

2.3 MICROARRAY ANALYSES

To determine the genes transcribed downstream from EGFR/STAT3 signaling pathway, we performed transcription microarray analyses to monitor the gene expression changes in RNA levels. The experimental design was to determine which transcripts were both up after EGF exposure and then reverted to untreated levels when the cells were treated with STAT3 antisense oligonucleotides prior to EGF treatment. I chose the NR6WT fibroblast cell, which expresses physiological levels of the EGFR [235]. Use of these cells allowed for isolation of EGFR signaling from the confounding influence of growth factors and their receptors' autocrine loops in human prostate tumor cell lines.

The NR6WT cells were starved in MEMα media containing 0.5% FBS for 24 hours to quiescent the cells before treatment with 1nM of EGF and/or 10μM of mouse STAT3 antisense nucleotide for another 24 hours. The sequence of it is 5'-GTT CCA CTG AGC CAT CCT GC-3'. After the treatment, the RNA from those cells was purified with the protocol described in above. 5μg RNA from each sample was prepared for microarray analysis using the Affymetrix U74AV2 murine genome array chip that contains probe sets interrogating approximately 36,000 full-length mouse genes and EST clusters from the UniGene database of transcripts. 5 μg of purified total RNA were added into a 20 μl first strand reaction with 200 U of SuperScript II (Invitrogen) and 1 μg T7 primer [5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAG GCGG(T)24] in 1X first strand buffer (Invitrogen) followed by a 42°C incubation for 1 hour. Second strand synthesis was performed by adding 40 U of *E. coli* DNA polymerase, 2 U of *E.*

coli RNase H, and 10 U of *E. coli* DNA Ligase in 1 X second strand buffer followed by incubation at 16°C for 2 hrs. The second strand synthesis reaction was purified according to the manufacturer's protocol (Affymetrix). The purified cDNA was amplified according to manufacturer's protocol to produce 70-120 µg of biotin labeled cRNA [242].

Murine U74Av2 GeneChip probe arrays were pre-hybridized in a GeneChip Hybridization Oven 640 (Affymetrix) according to the manufacturer's protocol. 15 µg of labeled cRNA were fragmented in 30 µl 1X fragmentation buffer with 100 mM KOAc and 30 mM MgOAc at 95°C for 35 minutes. The fragmented labeled cRNA was resuspended in 300 µl 1X hybridization buffer containing 100 mM MES, 1 M [Na^+], 20 mM EDTA, 0.01% Tween 20, 0.5 mg/mL Aceylated BSA, 0.1 mg/ml herring sperm DNA, control oligonucleotide B2, and control transcripts bioB 1.5 pM, bioC 5 pM, bioD 25 pM, and cre 100 pM. 200 µl of the hybridization cocktail (containing 10 µg of labeled cRNA) were hybridized to GeneChip probe arrays according to manufacturer's protocol (Affymetrix).

The raw data from microarray were normalized with the Affymetrix Microarray suite 5.0, based on the housekeeping gene expression profile. Expression values were adjusted to the intensity of the expression value of the 100 housekeeping genes. This allowed us to normalize between runs.

2.4 MOLECULAR CLONING

To check if STAT3 effects alone are sufficient for EGFR mediated cell migration, I used a constitutively activated STAT3 mutant to transfect the NR6WT cells. A dominant negative mutant of STAT3 and empty vector were used as controls. In Stat3D, E434 and E435 of Stat3,

which is in the DNA binding domain, were replaced with alanines [243]. This construct competes for upstream activation signals but cannot promote transcription [244] [245]. The constitutively active mutant [206] of STAT3 was generated by a mutation of substituting cysteine residues for A661 and N663 of the Stat3 molecule, which allowed for sulfhydryl bonds to form between STAT3 monomers and render a STAT3 that dimerizes independent of upstream activation [206]. Both constructs were kindly provided to me by Dr. Jennifer Grandis. The mutant fragments were cut by Not I and Hind III endonucleases and inserted into pCEP4-MMTV expression plasmid that induced by dexamethasone and has hygromycin resistance [246]. The cloning was checked by DNA sequencing and immunoblot for the expression marker of HA-tag in dominant negative STAT3 and Flag in constitutively active STAT3 mutants.

2.5 ELECTROPORATION AND SELECTION OF THE CLONS

The constructed plasmid with STAT3 mutants were transfected into NR6WT cells by electroporation. Cells grown in a 10cm plate at around 50% ~ 60% confluence were trypsinized and the pellet was collected in a 15 ml conical tube by centrifugation at 1,000 rpm for 5 minutes at 4˚C. One milliliter of OptiMEM was added into the tube to resuspend the pellet. The resuspended cells were mixed with 40 µg of plasmid DNA and transferred into a 0.4 cm cuvette. (Bio-Rad, Hercules, CA) The electroporation was performed with a voltage 220 V and 950 µF of capacitance.

After electroporation, cells were transferred to a new cell culture plate with 11 ml fresh medium. Medium was changed after 24 hours to get rid of the dead cells and debris. Both the STAT3 constitutively active mutant with Flag-tag and the STAT3 dominant negative mutant

with HA-tag were selected by hygromycin resistance. 100 μg/ml of hygromycin was added into the medium, and multiple monoclones were picked up after at least one weeks culturing.

The expression of both STAT3 constitutively active and STAT3 dominant negative mutants were controlled by 2 μg/ml of dexamethasone. Expression was determined by immunoblotting for STAT3 and the tags after 24 hr exposure to dexamethasone. Expressing clones were tested.

2.6 RNA INTERFERENCE

RNA interference (RNAi) is a process in which double-stranded RNA triggers the degradation of a homologous mRNA [247]. RNA interference is a technique that introduces exogenous, double-stranded RNAs (dsRNAs) which are complimentary to known mRNA's into a cell to specifically destroy that particular mRNA, thereby diminishing or abolishing gene expression and translation. I use the siRNAs specifically against human and mouse STAT3 to block the expression of the STAT3 gene in the target cell lines, which as a double confirmation of STAT3 antisense oligonucleotides. The siRNAs of human and mouse STAT3 and control siRNA were designed and ordered from the website of IDT. (http://www.idtdna.com) The sequence for human STAT3 siRNA is 5'-AUCCUGAAGG UGCUGCUCCTT-3'; the sequence for mouse STAT3 siRNA is 5'-UGCAUUCUCCUU GGCUCUUGAGGGUU-3' and the sequence for control eGFP siRNA is 5'-GACCCGCG CCGAGGUGAAGTT-3'. Cells for RNA interference experiment were grown in an antibiotic-free medium before transfection. For a 6-well plate, 4 μl of siRNA (20 μM) was incubated with 100 μl OptiMEM (GIBCO) per well (2ml medium) for 5 minutes, at the same time mix 4 μl of Lipofectamine 2000 (Invitrogen) with 100μl OptiMEM, then mix the siRNA and Lipofectamine dilution and incubate at room

temperature for 20 minutes before adding into the culturing cells. Incubate the cells at 37˚C for at least 4 hours, change fresh antibiotic-free medium and let the cells recover for another 12 to 24 hours [246].

2.7 NUCLEAR EXTRACT PREPARATION AND EMSA

Electrophoretic mobility shift assay (EMSA) is used for studying the binding between protein and specific DNA sequences. It can separate different types of complexes, such as monomer and dimer. These complexes can be recognized by transcription factor-specific antibodies that retard their mobility in gels for further identification.

For STAT3 analysis by EMSA [248], confluent cells were harvested and washed with ice cold 1X PBS, with the pellets resuspended in 5-fold buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 1 mM NaF; 0.5 mM DTT; 0.2 mM PMSF; 1 μg/μl profilin; 5 μg/μl aprotinin; 2 μg/μl leupeptin). After incubating on ice for 15 minutes, the nuclei were centrifuged at 4℃ for 10 seconds to obtain the nuclear-localized active transcription factors. Pellets were resuspended in 5-fold volumes (20μl ~ 100μl) cold buffer C (20 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 420 mM NaCl; 10 mM NaF; 1 mM Na_3VO_4; 25% glycerol; 0.2 mM EDTA; 0.5 mM DTT; 0.2 mM PMSF; 1 μg/μl profilin; 5 μg/μl aprotinin; 2 μg/μl leupeptin), incubated on ice for 30 minutes and microcentrifuged for 2 minutes at the maximum speed. Aliquots were stored at -80℃. For EMSA, 10 μg of total extractions were used for each experimental point. EMSA was performed using a γ-^{32}P labeled double strand oligonucleotide probe m67 (sense: 5'-GAT TTC CCG TAA ATC AT-3') that binds STAT3 and STAT1 proteins [249]. Protein-DNA complexes were resolved by non-denaturing PAGE gel and detected by autoradiography. Anti-STAT3

antibodies (Upstate Biotechnology, Waltham, MA) were used for super shift assay and a 50-fold excess of unlabeled m67 probe was used in cold competition assay.

2.8 IMMUNOBLOT

Protein expression was determined by immunoblotting. Confluent cells were washed with ice cold 1 X PBS twice followed by treated with lyses buffer (100mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol and 5% B-mercapto-ethanol). Sample proteins were denatured at 100℃ for 5 minutes in water bath before loading to the gel. After electrophoresis, the proteins were transferred to an Immobilon-P membrane. Membranes were blocked by 1% BSA. After blocking, membranes were incubated with a primary antibody: mouse anti STAT3 (Zymed); mouse anti GAPDH (abCam); mouse anti Flag (Stratagene); rabbit anti phosphor STAT3 (Cell Signaling) and rabbit anti HA-tag (Cell Signaling) at 4℃ for over night or at room temperature for 1 hour. The membranes were washed twice with 0.5 % Tween-20 (TBST) before incubated with secondary antibodies (Goat anti-mouse Ig or Goat anti-rabbit Ig, Biosource) [250].

2.9 WOUND HEALING ASSAY

Wound healing assay was used *in vitro* to assess cell motility in two dimensions. I used this assay to study the migration of both fibroblasts and human prostate tumor cells under the treatment of EGF and/or STAT3 inhibitors. Cells were plated on a 12-well plate and grown to confluence in their regular medium. Confluent cells were quiesced in 1% dialyzed FBS for 24 hours before each experiment. A rubber policeman was used to create a denuded area. Cells were washed twice with PBS and treated with or without specific effectors for 24 hours. Photographs

were taken at hour 0 and hour 24, and the distance traveled was determined by subtracting the values obtained at hour 0 from hour 24. Mitomycin C (0.5 μg/ml) was added to the medium to prevent the confounding issue of cell proliferation [251].

2.10 INVASION ASSAY

Invasive potential was determined in vitro by transmigration of an ECM [252]. Matrigel invasion chamber plates were purchased from Becton Dickinson/Biocoat (Bedford, MA). The Matrigel was used to mimic the environmental extracellular matrix. The upper surface of the matrix was challenged with 1.5 X 10^4 cells, a number derived from empirical experimentation. Cells were kept in serum-free medium containing 1% BSA for the first 24 h and then replaced with only serum-free medium for the remaining 24 h; the lower chamber contained medium containing 10% serum for the entire assay. Enumeration of the cells that invaded through the matrix over a 48-h period was accomplished by visually counting cells on the bottom of the filter, as per routine procedures, after any un-invaded cells were removed from the top of the filter with a cotton swab. In all of the cases, individual experiments were performed in duplicate chambers.

2.11 ANNEXIN V STAINING FOR APOPTOSIS ASSAY

The Annexin V staining is based on the observation that soon after apoptosis initiation, the membrane phosphatidyl-serine was translocated from the inner face of the plasma membrane to be exposed on the cell surface. Once on the cell surface, the phosphatidyl-serine can easily be detected by staining with a fluorescent conjugate of Annexin V, which is a protein that has high affinity for it. Annexin V-Cy3 kit (BioVision) was used in the apoptosis assay. Cells were

washed with 1 X PBS and then digested by Tripsin-EDTA. The pellets of cells were washed again by 1 X PBS twice and re-suspended in 500µl of 1 X Binding Buffer come with the kit. After incubation at room temperature for 5 minutes, Annexin V was added into the cells at a ratio of 1:100. The mixture was incubated in darkness for 10 minutes at room temperature and ready for counting fraction of Annexin V-staining cells under a fluorescence microscope [253].

2.12 PROLIFERATION ASSAY

To study the viability and the toxicity of STAT3 inhibitors to the cell, the cells proliferation assay was performed by direct cell enumeration. A Z-series Coulter Counter (Coulter Corp) was used for the counting. Cells were counted by the Coulter Counter before being passed into new culture plates, after the treatment and growth for 48 or 72 hours to allow for one or two rounds of replication, cells were digested by Tripsin-EDTA (GIBCO) and then re-suspended in the original medium. The cells were diluted for 100 times in Isoton Ⅱ solution (Beckman) before counting [250].

2.13 STATISTICAL ANALYSES

All data from migration, proliferation, invasion and apoptosis staining were analyzed and expressed as mean ± S.E.M. Evaluation of statistical significance was performed by use of T-test. A value of $P < 0.05$ was considered significant.

3.0 STAT3 IS REQUIRED BUT NOT SUFFICIENT FOR EGFR MEDIATED MIGRATION AND INVASION OF FIBROBLAST AND HUMAN PROSTATE CARCINOMA CELLS

3.1 STAT3 ANTISENSE DECREASES EXPRESSION OF STAT3 IN NR6WT CELLS

EGFR signaling activates STAT proteins in fibroblasts [187] and a variety of carcinoma cells [254] [255]. As our lab has previously shown, the prostate carcinoma cells present autocrine EGFR signaling. I determined whether this also invoked STAT3 activation. To study this issue, I used the motility model system, murine NR6WT fibroblasts, to examine EGF-triggered events as autocrine signaling is absent in these cells in contrast to prostate carcinoma cells which present autocrine EGFR-activating signaling loops [92], and thus the role of EGFR signaling can be cleanly parsed. The two human prostate cancer cell lines, DU145WT and PC3, present autocrine activation of endogenous and exogenous EGFR, as is the norm for prostate carcinoma cells. However, to study the function of EGFR signaling in these cells, I tested our prostate cells under conditions that minimize autocrine activation [187].

Initially to parse STAT3 mediated responses from EGFR even in the fibroblasts, I downregulated the protein expression by using antisense oligonucleotides. This intervention reduced whole cell levels of STAT3 significantly in NR6WT fibroblasts (Fig 3-1).

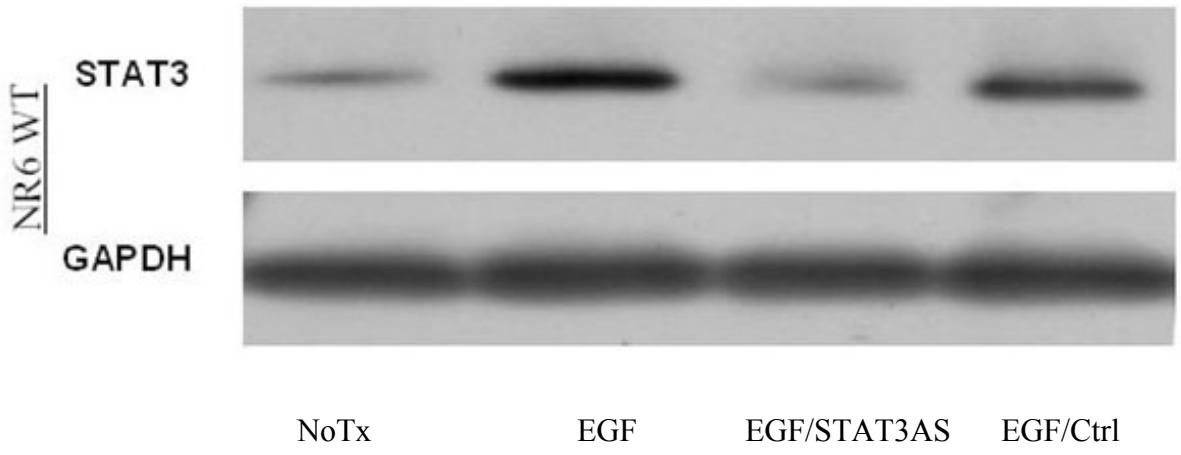


Figure 3-1. EGF increase the expression of STAT3 in NR6WT cells and it can be inhibited by STAT3 antisense.

In murine fibroblasts, NR6WT cells, EGF exposure increased the expression of STAT3 at protein level, and this increase was inhibited by STAT3 antisense. Cells were quiesced in 0.5% FBS medium for 24 hours before the treatment. After quiescing, the NR6WT cells were treated with 1 nM EGF and/or 10 μM of oligonucleotides (STAT3 antisense or non-specific control) for over night. NoTx: non-treatment control; EGF: 1 nM human EGF; EGF/STAT3AS: 1 nM human EGF plus 10 μM mouse STAT3 antisense oligonucleotides; EGF/Ctrl: 1 nM human EGF plus 10 μM non-specific oligonucleotides. The treated cells were lysised in 2X loading buffer and denatured at 100 ℃n water bath for 5 minutes. Lysates were loaded to 10% acrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibody against mouse STAT3. After being exposed, the membrane was stripped and immunoblotted with antibody against GAPDH as a loading control.

3.2 STAT3 ANTISENSE DECREASES DNA BINDING ACTIVITY OF STAT3 IN NR6WT CELLS

The signaling pathway of STAT3 is a complicated process, cytoplasm STAT3 must be dimerized to get activated, and the performing of its transcriptional regulation function need the binding of STAT3 to its target DNA sequence. I used the EMSA assay to check the activity of STAT3 after STAT3 antisense treatment. A valuable aspect of EMSA is that is can separate different types of complexes, such as monomer and dimer, and it is easier to see the bound complex. In murine NR6WT fibroblasts cells, EGF exposure increased EMSA detection of the STAT3 band, which was negated upon addition of an antibody to mouse STAT3 (Fig 3-2). A competing binding of 50X concentration of cold nucleotide probe completely eliminated the band, showing the specificity of the binding of probe DNA and STAT3.

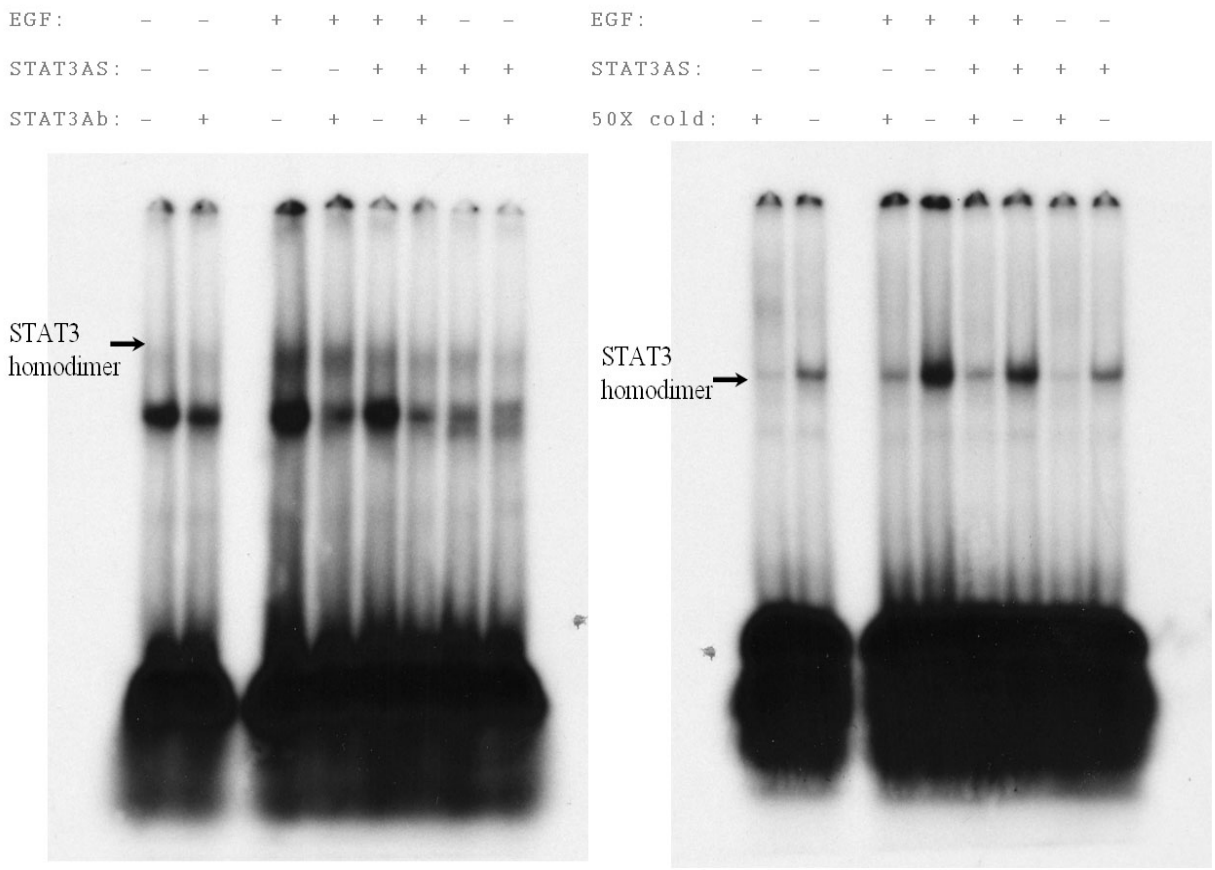


Figure 3-2. Electrophoretic mobility shift assay (EMSA) detects EGF-dependent STAT3 activity in NR6WT fibroblasts cells.

EMSA analysis of STAT3 DNA-binding activity in nuclear extracts prepared from NR6WT mouse fibroblast cells using the γ-P^{32} labeled m67 probe. Negation of STAT3 shifting with the STAT3 antibody (STAT3Ab) shows the specificity of the STAT3 protein and cold competition (50X cold) demonstrates the specificity of the m67 probe. Cells were treated with 1nM EGF, 1nM EGF plus 10uM STAT3 antisense (STAT3AS) or 10uM STAT3 antisense alone (cells were treated over night, which is 14-18hrs). Lower concentrations of cold competition assays (10X and 5X) were also performed and showed the similar trending results (data not shown).

3.3 STAT3 ANTISENSE DECREASES EXPRESSION OF STAT3 IN DU145WT CELLS

Now that I could downregulate STAT3 activity in the NR6WT model cell, I changed my focus to the human prostate tumor cells, DU145WT cell. The DU145WT cells have a TGF-α/EGFR autocrine loop, which implies its independence or insensitivity of extraneous EGF stimulation. To overcome that disadvantage, a significant higher concentration of EGF and shorter treatment were applied on the DU145WT cells. The result showed that the STAT3 antisense oligonucleotide reduced STAT3 levels in the DU145WT cells while no significant inhibition happened in the non-specific oligonucleotides control (Fig 3-3).

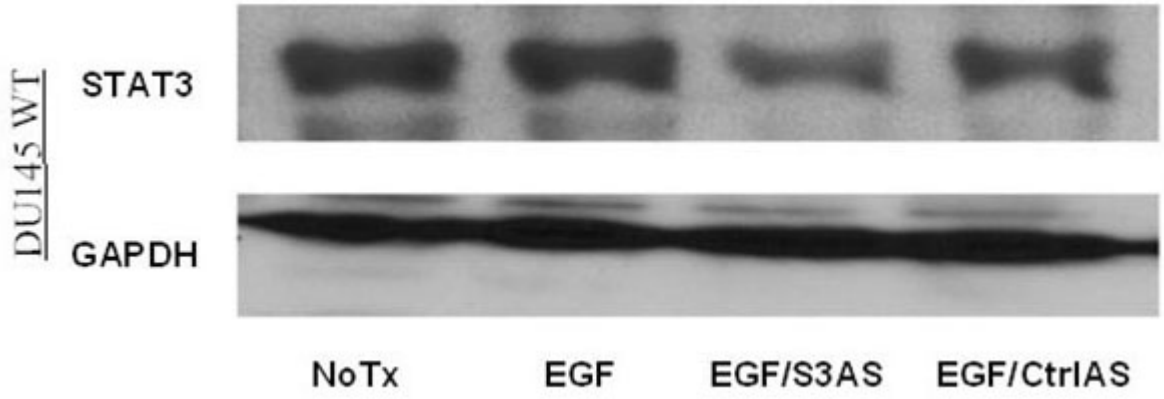


Figure 3-3. EGF increase the expression of STAT3 in DU145WT cells and it can be inhibited by STAT3 antisense.

DU145WT cells were quiesced in 0.5% FBS medium for 24 hours before the treatment. After quiescing, the DU145WT cells were treated with 10 μM of oligonucleotides (STAT3 antisense or non-specific control – S3AS or CtrlAS, respectively) for over night followed by and/or 10 nM EGF for 15 minutes. NoTx: non-treatment control; EGF: 10 nM human EGF; EGF/STAT3AS: 10 nM human EGF plus 10 μM human STAT3 antisense oligonucleotides; EGF/Ctrl: 10 nM human EGF plus 10 μM non-specific oligonucleotides. The treated cells were lysised in 2 X loading buffer and denatured at 100^{o}C in water bath for 5 minutes. Lysates were

loaded to 10% acrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibody against mouse STAT3. After being exposed, the membrane was stripped and immunoblotted with antibody against GAPDH as a loading control.

3.4 STAT3 ANTISENSE DECREASES DNA BINDING ACTIVITY OF STAT3 IN DU145WT CELLS

To check the activity of STAT3 after STAT3 antisense treatment, we performed the EMSA assay. In human prostate tumor cell, DU145WT cells, EGF exposure increased EMSA detection of the STAT3 band, which was up-shifted upon addition of an antibody to murine STAT3 (Fig 3-4). A competing binding of 50X concentration of cold nucleotide probe eliminated the band, showing the specificity of the binding of probe DNA and STAT3.

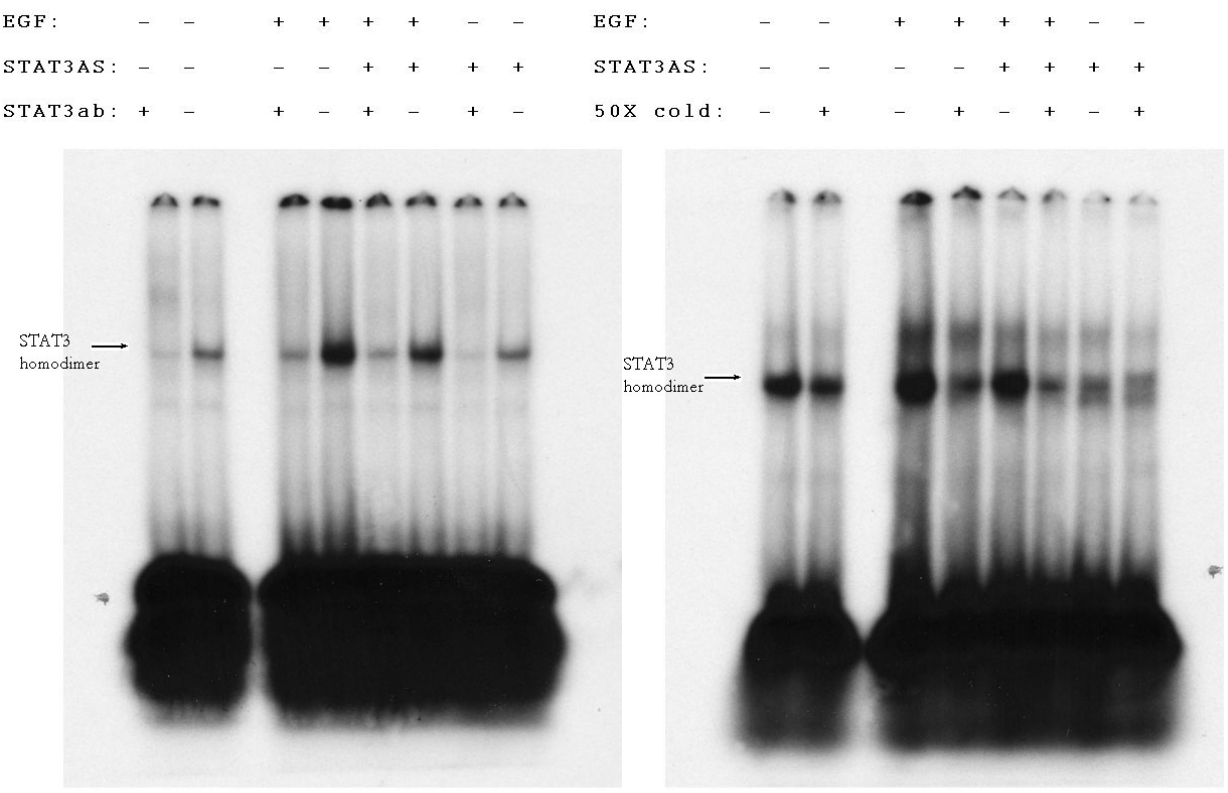


Figure 3-4. Electrophoretic mobility shift assay (EMSA) detects EGF-dependent STAT3 activity in DU145WT human prostate tumor cells.

EMSA analysis of STAT3 DNA-binding activity in nuclear extracts prepared from DU145WT human prostate tumor cells using the γ-P^{32} labeled m67 probe. Supershift with the STAT3 antibody (STAT3ab) shows the specificity of the STAT3 protein and cold competition (50X cold) demonstrates the specificity of the m67 probe. Cells were treated with 10nM EGF, 10nM EGF plus 10uM STAT3 antisense or 10uM STAT3 antisense alone (STAT3AS) (cells were treated 14-18hr). Lower concentrations of cold competition assays (10X and 5X) were also performed and showed the similar trending results (data not shown).

3.5 STAT3 SIRNA DECREASES EXPRESSION OF STAT3 IN DU145WT CELLS

Thought the antisense interventions worked, I sought a second, confirmatory way to downregulate STAT3 signaling. RNA interference (RNAi) is a process in which double-stranded RNA triggers the degradation of a homologous mRNA [Bass, 2003]. Today, RNA interference is a technique that introduces exogenous, double-stranded RNAs (dsRNAs) which are complimentary to known mRNA's into a cell to specifically destroy that particular mRNA, thereby diminishing or abolishing gene expression and translation., I used RNA interference to further confirm the blocking effect got from STAT3 antisense nucleotide. I downregulated the protein using siRNA directed against STAT3 in DU145WT cells (Fig 3-5).

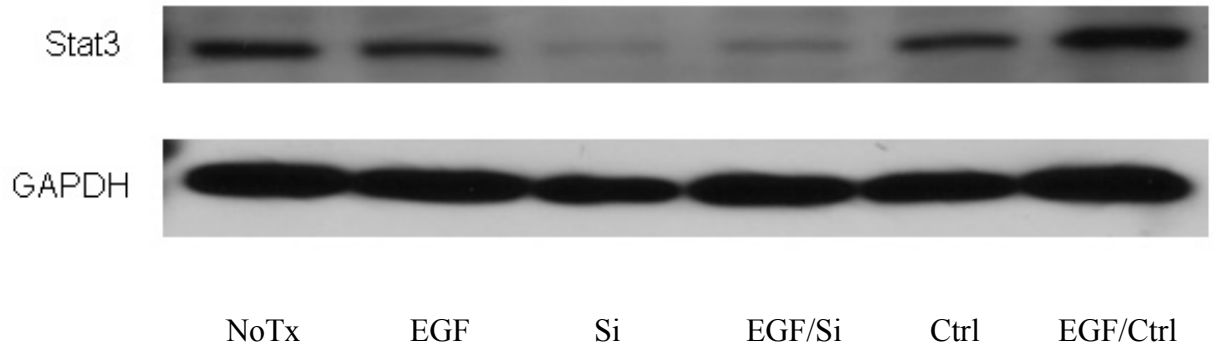


Figure 3-5. EGF-mediated increase of STAT3 expression and activity is suppressed by STAT3 siRNA in DU145WT cells.

EGF-mediated increase of STAT3 expression and activity is suppressed by STAT3 siRNA (S3Si) in DU145WT cells, but not by a non-target siRNA (against eGFP)(CtrlSi). DU145WT cells were grown in an antibiotic-free medium before being transfected by siRNA (20 μM) with Lipofectamine 2000 to a final concentration of 100 pM. After been transfected and recovered, DU145WT cells were quiesced in 0.5% FBS medium for 24 hours before the treatment. After quiescing, the DU145WT cells were treated with 10 nM EGF for 15 minutes. NoTx:

non-treatment control; EGF: 10 nM human EGF; Si: 100 pM of STAT3 siRNA transfection; ESi: 10 nM human EGF plus 100 pM of human STAT3 siRNA; Ctrl: 100 pM of eGFP siRNA as non-specific control; EGF/Ctrl: 10 nM human EGF plus 00 pM of eGFP siRNA. The treated cells were lysised in 2 X loading buffer and denatured at 100℃ in water bath for 5 minutes. Lysates were loaded to 10% acrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibody against human STAT3. After being exposed, the membrane was stripped and immunoblotted with antibody against GAPDH as a loading control.

3.6 STAT3 SIRNA DECREASES EXPRESSION OF STAT3 IN PC3 CELLS

To inhibit the STAT3 protein expression in a second human prostate cell line, PC3, I also used RNA interference. As with DU145WT cells, PC3 is a metastasis human prostate tumor cell, the difference between them is that PC3 is separated from bone metastases [237], not from brain as DU145 cells [238]. I also downregulated the STAT3 protein using siRNA directed against STAT3 mRNA sequence in PC3 cells (Fig 3-6).

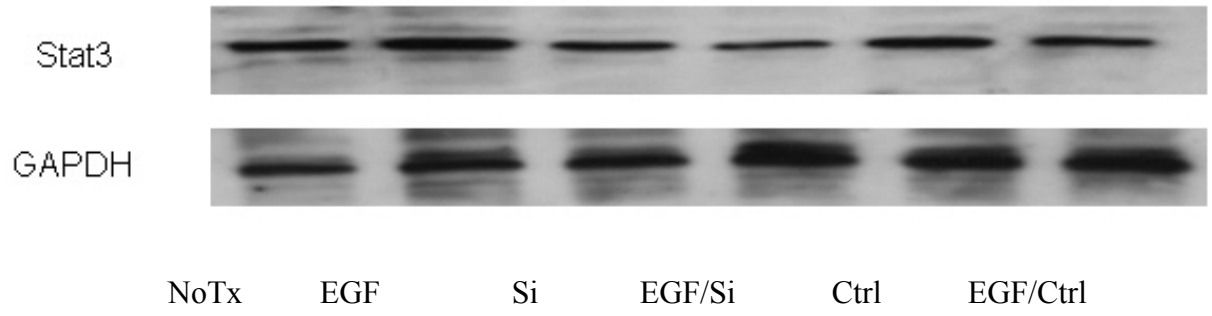


Figure 3-6. EGF-mediated increase of STAT3 expression and activity is suppressed by STAT3 siRNA in PC3 cells.

EGF-mediated increase of STAT3 expression and activity is suppressed by STAT3 siRNA (S3Si) in PC3 cells, but not by a nontarget siRNA (against eGFP)(CtrlSi). PC3 cells were grown in an antibiotic-free medium before being transfected by siRNA (20 μM) with Lipofectamine 2000 to a final concentration of 100 pM. After been transfected and recovered, PC3 cells were quiesced in 0.5% FBS medium for 24 hours before the treatment. After quiescing, the PC3 cells were treated with 10 nM EGF for 15 minutes. NoTx: non-treatment control; EGF: 10 nM human EGF; S3Si: 100 pM of STAT3 siRNA transfection; ES3Si: 10 nM human EGF plus 100 pM of human STAT3 siRNA; CtrlSi: 100 pM of eGFP siRNA as non-specific control; EGF/CtrlSi: 10 nM human EGF plus 100 pM of eGFP siRNA. The treated cells were lysised in 2 X loading buffer and denatured at 100^{o}C in water bath for 5 minutes. Lysates were loaded to 10% acrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibody against human STAT3. After being exposed, the membrane was stripped and immunoblotted with antibody against GAPDH as a loading control.

3.7 STAT3 SIRNA DECREASES DNA BINDING ACTIVITY OF STAT3 IN DU145WT CELLS

Inhibition of STAT3 protein expression of STAT3 siRNA was demonstrated by immunoblotting. Now the next question is whether the DNA-binding activity of STAT3 protein is also been suppressed by STAT3 siRNA. To get the answer, I also performed EMSA assay on STAT3 siRNA transfected DU145WT cells. The result is as same as in the previous STAT3 antisense experiments. EGF significantly increases STAT3 binding activity while it can be reversed by STAT3 siRNA (Fig 3-7).

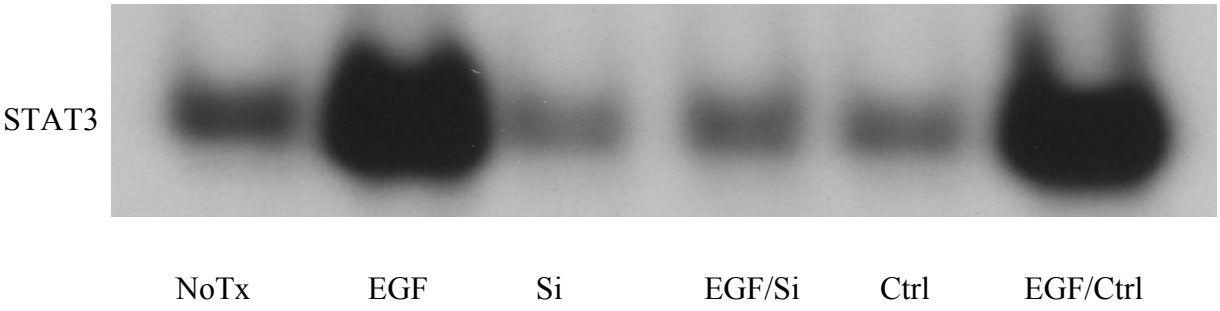


Figure 3-7. The STAT3 siRNA negated EGF induced STAT3 activity in DU145WT prostate tumor cells.

EMSA analysis of STAT3 DNA-binding activity in nuclear extracts prepared from DU145WT human prostate tumor cells using the γ-P^{32} labeled m67 probe. Cells were treated with 10nM EGF, 10nM EGF plus 100 pM STAT3 siRNA or 100 pM STAT3 antisense alone. A non-specific siRNA (eGFP siRNA) was used as control. NoTx: non-treatment control; EGF: 10 nM human EGF; S3Si: 100 pM of STAT3 siRNA transfection; ES3Si: 10 nM human EGF plus 100 pM of human STAT3 siRNA; CtrlSi: 100 pM of eGFP siRNA as non-specific control; EGF/CtrlSi: 10 nM human EGF plus 100 pM of eGFP siRNA.

3.8 STAT3 ANTISENSE INHIBITS EGF INDUCED MIGRATION IN NR6WT CELLS

Now that I could reproducibly limit STAT3 functioning, I asked what this did to EGFR-mediated cell migration. EGFR signaling drives motility in fibroblasts and carcinoma cells [256] and invasion of human prostate carcinoma cells [187] [257]. As such, we asked whether STAT3 might be critical for sustained motility and invasion in response to EGFR signaling. To quantitate cell motility, an in vitro wound healing assay was performed and demonstrated that the addition of STAT3 antisense oligonucleotides and the EGFR signaling pathway inhibitor PD153035 greatly decreased the migration distance of NR6WT fibroblast cell. The extent of migration inhibition was similar to that achieved by blocking EGFR kinase activity with the selective agent PD153035. Interestingly, the level of migration achieved with STAT3 down regulation, and with PD153035 was below that of basal motility, strongly suggesting that either EGFR autocrine signaling via STAT3 or basal STAT3 was contributing to this cell behavior, as has been suggested earlier for EGFR autocrine activation [187].

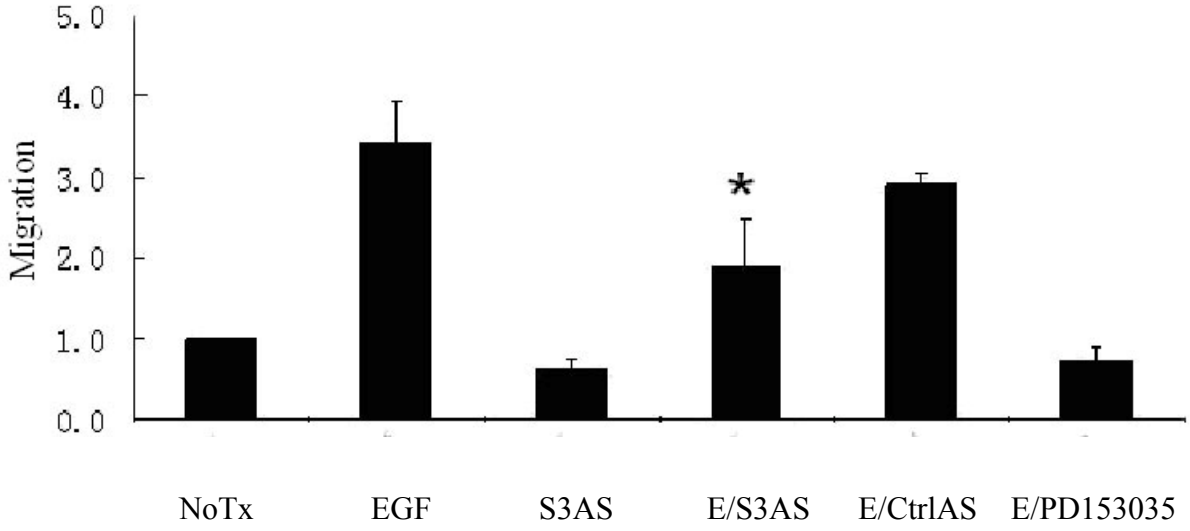


Figure 3-8. STAT3 is required for EGF receptor mediated cell migration in NR6WT fibroblast cells.

*The migration distance of NR6WT fibroblast cells after treated with 1nM EGF for 24hr was significantly greater and this increase was suppressed by 10 μM STAT3 antisense (E/S3AS) but not scrambled non-specific oligonucleotides (CtrlAS). Additionally, EGF-induced motility was determined in the presence of the EGFR ATP binding inhibitor PD153035. * $P < 0.01$ compared to EGF treatment. The migration distance (on the Y axis) is normalized to diluent alone (NoTx) within each experiment. For each experiment, no treatment was normalized as 1-fold. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate and normalized to no treatment. NoTx: non-treatment; EGF: 1 nM human EGF; S3AS: 10 μM mouse STAT3 antisense oligonucleotides; E/S3: 1 nM human EGF plus 10 μM mouse STAT3 antisense oligonucleotides; E/Ctrl: 1 nM human EGF plus 10 μM non-specific oligonucleotides; E/PD153035: 1 nM human EGF with 500 nM PD153035. All the cells were grown in a medium with 0.5 μg/ml Mitomycin C to inhibit proliferation.*

3.9 STAT3 ANTISENSE INHIBITS EGF INDUCED MIGRATION IN DU145WT CELLS

Based on the inhbition of motility noted in the fibroblasts, I proceeded to ask whether STAT3 might be critical for sustained motility and invasion in response to EGFR signaling in the prostate carcinoma cells. STAT3 downregulation greatly decreased the migration distance of DU145WT prostate cancer cell as well as in NR6WT fibroblast cell. The results showed that the migration distance of DU145WT cells after treated with 10 nM EGF for 24hr was significantly greater and this increase was suppressed by 10 μM STAT3 antisense (S3AS) but not scrambled non-specific oligonucleotides (Ctrl). Additionally, EGF-induced motility was determined in the presence of the EGFR inhibitor PD153035 (Fig 3-9).

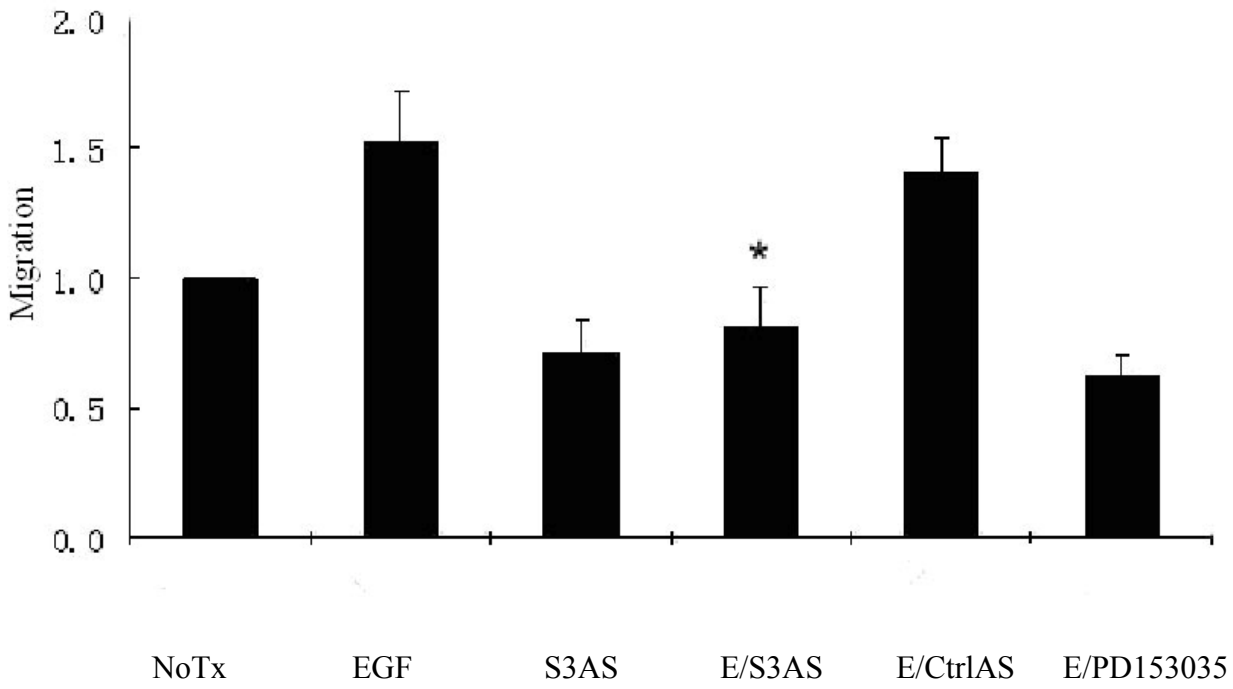


Figure 3-9. STAT3 is required for EGF receptor mediated cell migration in human prostate tumor cells.

The results showed that the migration distance of DU145WT cells after treated with 10 nM EGF for 24hr was significantly greater and this increase was suppressed by 10 μM STAT3 antisense (S3AS) but not scrambled non-specific oligonucleotides (Ctrl). Additionally, EGF-induced motility was determined in the presence of the EGFR inhibitor PD153035. $P < 0.01$ compared to EGF treatment. The migration distance (on the Y axis) is normalized to diluent alone (NoTx) within each experiment. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate and normalized to no treatment. NoTx: non-treatment; EGF: 1 nM human EGF; S3AS: 10 μM mouse STAT3 antisense oligonucleotides; E/S3: 1 nM human EGF plus 10 μM mouse STAT3 antisense oligonucleotides; E/Ctrl: 1 nM human EGF plus 10 μM non-specific oligonucleotides; E/PD153035: 1 nM human EGF with 500 nM PD153035. All the cells were grown in a medium with 0.5 μg/ml Mitomycin C to inhibit proliferation.*

3.10 STAT3 SIRNA INHIBITS EGF INDUCED MIGRATION IN DU145WT AND PC3 HUMAN PROSTATE TUMOR CELLS

Since the STAT3 siRNA inhibit STAT3 expression and its DNA binding activity as well as STAT3 antisense oligonucleotides, it is reasonable to posit that STAT3 siRNA also inhibits the EGF induced motility as the later. This would further confirm the specificity of the antisense approach to that of disruption of STAT3 function. To study cell motility, an in vitro wound healing assay was performed and demonstrated that the addition of human STAT3 siRNA greatly decreased the migration distance of DU145WT prostate cancer cell (Fig 3-10).

The same experiment was performed in another aggressive, androgen-independent human prostate tumor cell line, the PC3 cells. This was done to show that the results were not unique to a single cell line. To study cell motility, an in vitro wound healing assay was performed and demonstrated that the addition of human STAT3 siRNA greatly decreased the migration distance of PC3 prostate cancer cell (Fig 3-10).

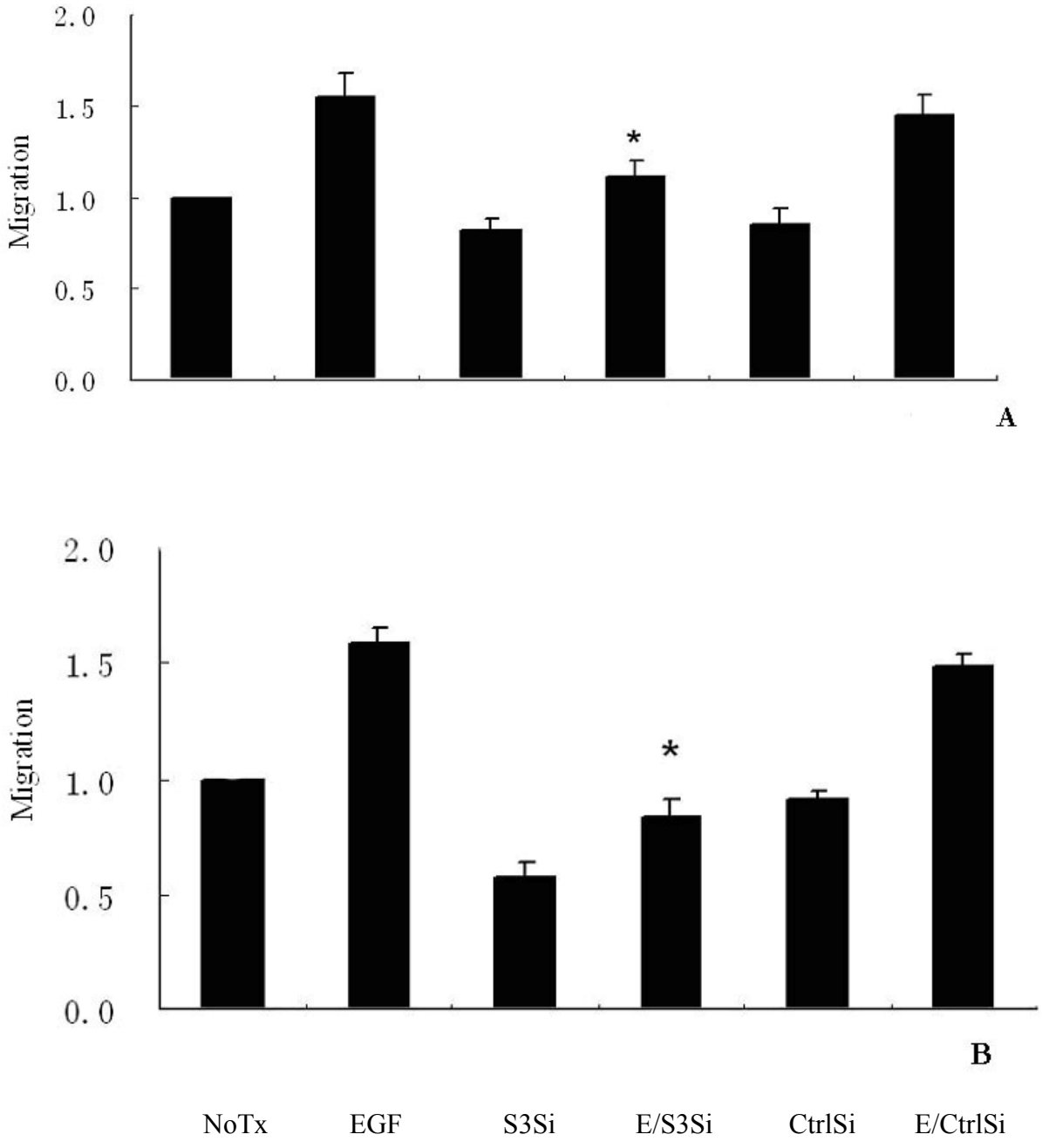


Figure 3-10. STAT3 is required for EGF receptor mediated cell migration in human prostate tumor cells.

The migration distance of DU145WT (A) and PC3 (B) prostate tumor cells after treatment with 10 nM EGF for 24hr was significantly greater and these increases were

*suppressed by STAT3 siRNA (S3Si) but not siRNA against eGFP (CtrlSi). Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate. * P < 0.01 compared to EGF treatment. The migration distance (on the Y axis) is normalized to diluent alone (NoTx) within each experiment. NoTx: non-treatment control; EGF: 10 nM human EGF; Si: 100 pM of STAT3 siRNA transfection; ESi: 10 nM human EGF plus 100 pM of human STAT3 siRNA; Ctrl: 100 pM of eGFP siRNA as non-specific control; EGF/Ctrl: 10 nM human EGF plus 100 pM of eGFP siRNA. All the cells were grown in a medium with 0.5 μg/ml Mitomycin C to inhibit proliferation.*

3.11 STAT3 ANTISENSE INHIBITS EGF INDUCED INVASION IN DU145WT

As motility is a key component to tumor cell invasion [1], we determined whether this inhibition affected tumor cell invasion by examining the transmigration of a Matrigel barrier. Antisense oligonucleotides directed against STAT3 limited DU145WT tumor cell transmigration to below the levels noted in the absence of added EGF.

This extent of inhibition is expected as both DU145WT and PC3 cells express EGFR ligands [258], which generate autocrine stimulatory signals in the physical confines of a matrix, and Matrigel contains competent levels of EGFR ligands [259]. That this is due to EGFR signaling is shown by blockade using the selective inhibitor PD153035. This inhibitor reduced not only EGF-enhanced invasiveness but also the invasiveness in the absence of exogenous EGF. Thus, during transmigration of Matrigel the cells are in an active EGFR signaling mode. The results showed that STAT3 antisense (S3AS) treated DU145WT cells have a much lower invasion rate than those cells treated by EGF (Figure 3-11).

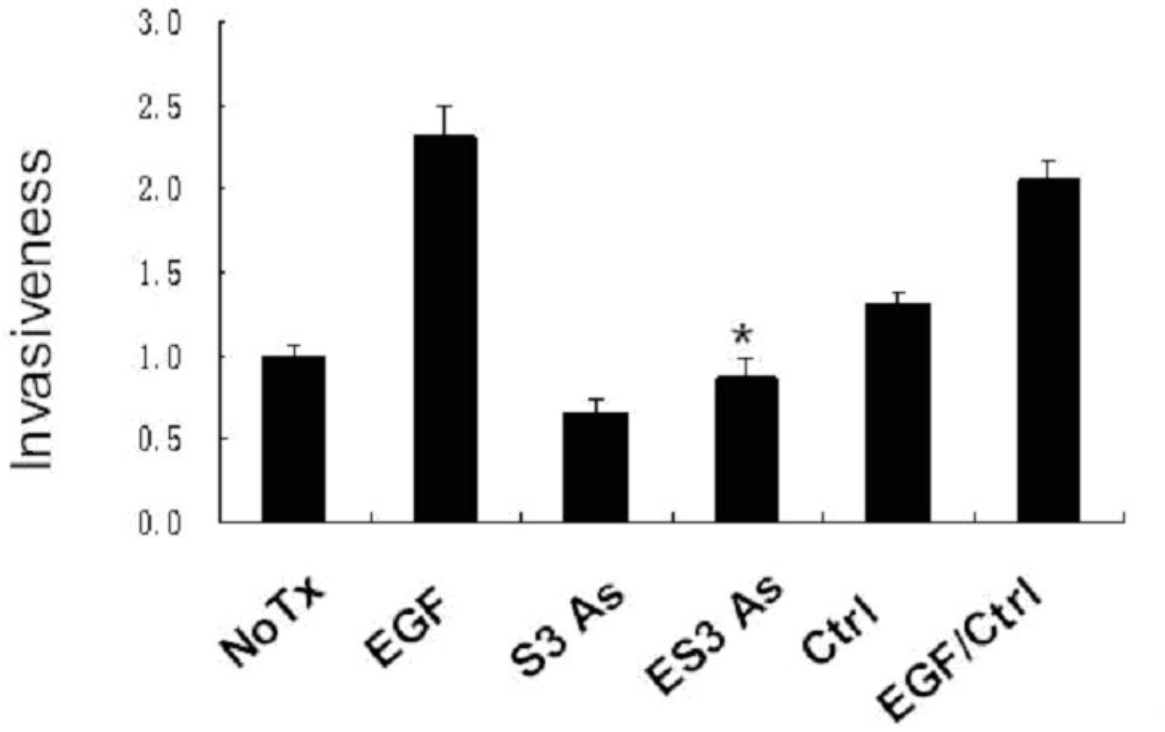


Figure 3-11. STAT3 is critical for EGFR mediated prostate tumor cell invasiveness

*STAT3 antisense (S3AS) treated DU145WT cells have a much lower invasion rate than those cells treated by EGF while non-specific control oligonucleotides had no effect on the invasion. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate. * P < 0.05 compared to EGF treatment. The invasiveness as determined by cell number transmigrated through the Matrigel (on the Y axis) is normalized to diluent alone (NoTx) within each experiment. NoTx: non-treatment; EGF: 10 nM human EGF; S3AS: 10 μM human STAT3 antisense oligonucleotides; E/S3: 10 nM human EGF plus 10 μM human STAT3 antisense oligonucleotides; CtrlAS: 10 μM non-specific oligonucleotides; E/Ctrl: 10 nM human EGF plus 10 μM non-specific oligonucleotides.*

3.12 STAT3 SIRNA INHIBITS EGF INDUCED INVASION IN DU145WT AND PC3 HUMAN PROSTATE CANCER CELLS

Since the STAT3 siRNA inhibit EGFR mediated migration as well as STAT3 antisense oligonucleotides, it is reasonable to posit that STAT3 siRNA also inhibits the EGFR induced invasion as the later. To study the invasion of human prostate cancer cells, examining the transmigration of a Matrigel barrier was performed. The results showed that STAT3 antisense (S3AS) treated DU145WT and PC3 cells have a much lower invasion rate than those cells treated by EGF (Figure 3-12).

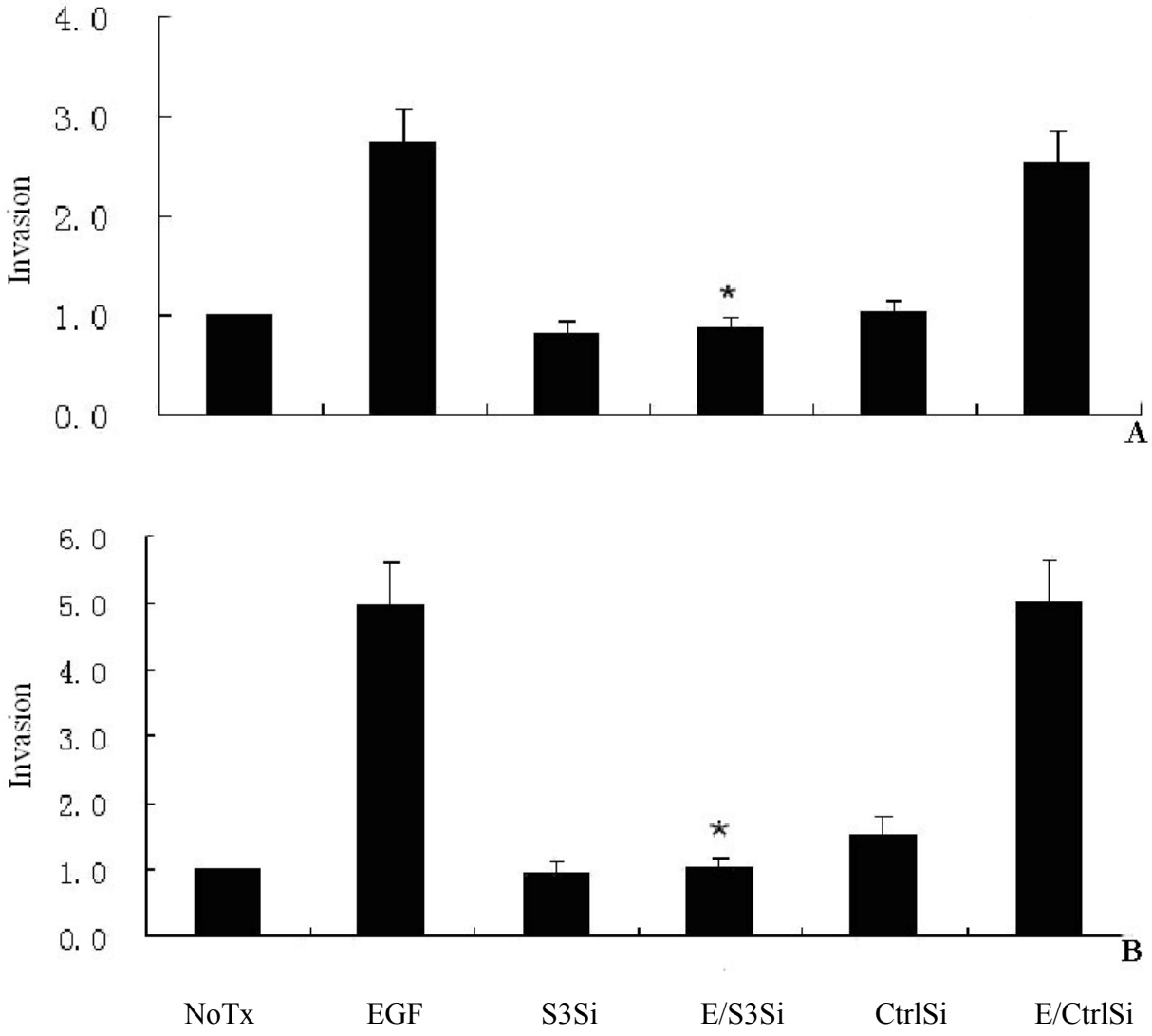


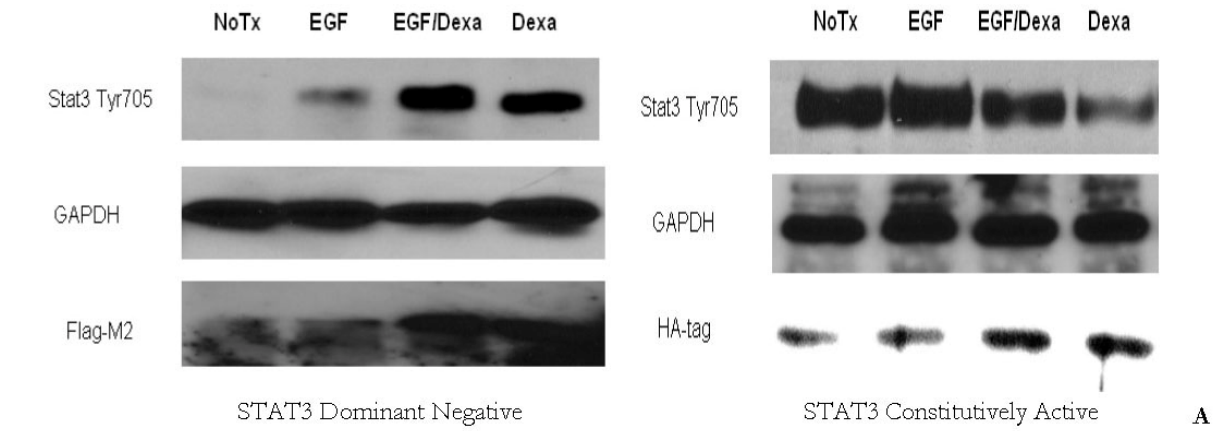
Figure 3-12. STAT3 is critical for EGFR mediated prostate tumor cell invasiveness.

*STAT3 siRNA (S3Si) inhibited EGFR mediated invasion of DU145WT (A) and PC3 (B) prostate tumor cells, whereas eGFP-directed siRNA (CtrlSi) did not. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate. * $P < 0.01$ compared to EGF treatment. The invasiveness as determined by cell number transmigrated through the Matrigel (on the Y axis) is normalized to diluent alone (NoTx) within each experiment. NoTx: non-treatment control; EGF: 10 nM human EGF; Si: 100 pM of STAT3 siRNA transfection; E/Si: 10 nM human EGF plus 100*

pM of human STAT3 siRNA; Ctrl: 100 pM of eGFP siRNA as non-specific control; EGF/Ctrl: 10 nM human EGF plus 100 pM of eGFP siRNA.

3.13 STAT3 IS NOT SUFFICIENT FOR MIGRATION IN NR6WT CELLS

The foregoing data demonstrate that STAT3 activity is required for increased cell migration and invasion upon EGFR signaling. Up regulation of STAT3 levels in various cancers [206] [254] [255] might even suggest this functions as an 'oncogenes' or 'tumor progression' gene. Thus, we asked whether upregulation was sufficient to drive these behaviors. A constitutively active (CA) STAT3 mutant failed [260] to drive motility of NR6WT cells in the absence EGF (Figure 3-13). A dominant negative (DN) STAT3 construct did block EGF-induced motility, as expected. The STAT3 constructs were induced by dexamethasone to avoid adaptation or cellular modifications during prolonged selection; the dexamethasone by itself had only a small negative effect on migration that was minimal compared to the effects of either EGF or the constructs. These data are consistent with STAT3 being required, but not sufficient, for transcriptional activation of either replacement proteins or modulation of the proteome at the transcriptional to enable a locomotive state.



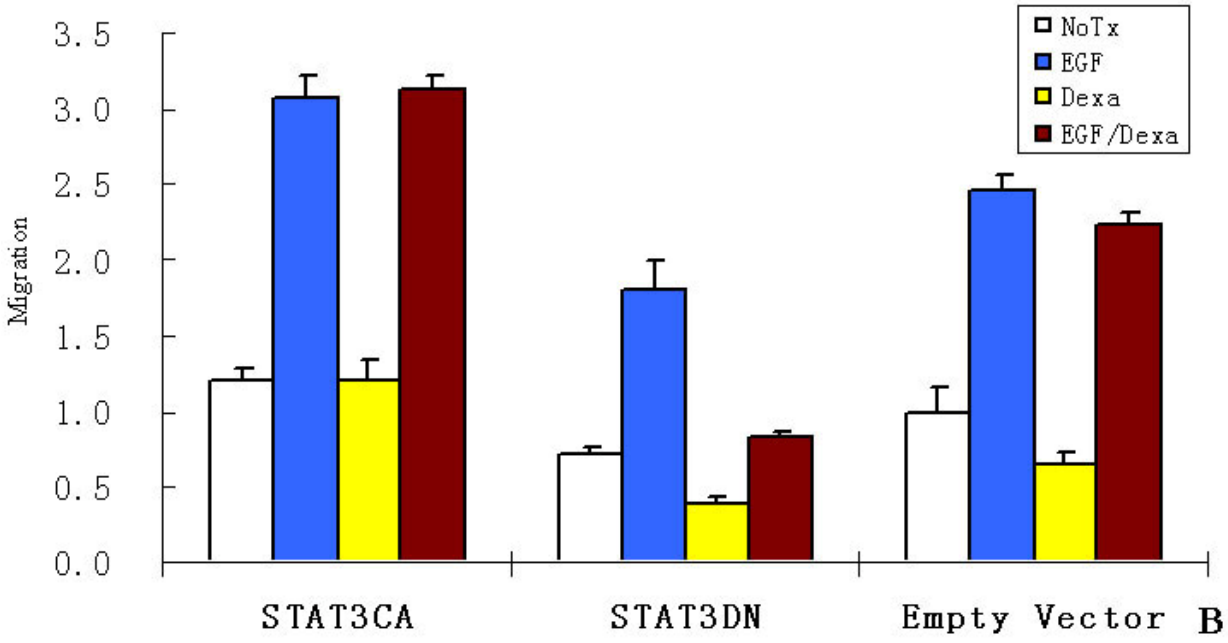


Figure 3-13. STAT3 is not sufficient for EGFR-mediated migration in NR6WT fibroblasts.

(A) Immunoblotting demonstrates expression of STAT3 dominant negative and constitutively active mutants in NR6WT cells upon transcriptional up regulation by dexamethasone. Cells were quiesced in 0.5% FBS medium for 24 hours before the treatment. After quiescing, the NR6WT cells were treated with 1 nM EGF and/or 2 μg/ml Dexamethasone for over night. The treated cells were lysised in 2X loading buffer and denatured at 100 ℃ in

*water bath for 5 minutes. Lysates were loaded to 10% acrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibody against phosphorylated STAT3 (STAT3 Tyr705) and the special expression tag on the STAT3 mutants (For STAT3 dominant negative, HA-tag; for STAT3 constitutively active, Flag-M2). Shown are representative blots of at least three experiments each. NoTx: non-treatment; EGF: 1 nM human EGF; EGF/Dexa: 1 nM human EGF with 2 μg/ml Dexamethasone; Dexa: 2 μg/ml Dexamethasone. After being exposed, the membrane was stripped and immunoblotted with antibody against GAPDH as a loading control. (B) The STAT3 constitutively active mutant did not drive motility of NR6WT cells in the absence or presence EGF, while the dominant-negative construct blocked both basal and EGF-induced motility, which indicates STAT3 is critical but not sufficient for the motility. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate. * $P < 0.01$ compared to EGF treatment. The migration distance (on the Y axis) is normalized to diluent alone of the empty vector (NoTx) within each experiment. NoTx: non-Treatment; EGF: 1 nM human EGF; EGF/Dexa: 1 nM human EGF with 2 μg/ml Dexamethasone; Dexa: 2 μg/ml Dexamethasone.*

3.14 STAT3 ANTISENSE DOES NOT INHIBIT EGFR MEDIATED PROLIFERATION IN NR6WT FIBROBLAST CELLS

High expression of STAT3 is found correlated with tumor cells proliferation in many human cancers [261] [262] [263]. If STAT3 blockade or downregulation limited proliferation, this would confound longer term migration and invasion assessments. In the above, the time period

and manipulations precluded proliferation from substantively contributing to migration and invasion, but in vivo, the situation would be reversed.

I also expected a decrease of NR6WT proliferation after the treatment of STAT3 antisense oligonucleotides. Surprisingly, no significant change was found in the *in vitro* proliferation assay between NR6WT cells treated with EGF and EGF plus STAT3 antisense oligonucleotides (Fig 3-14).

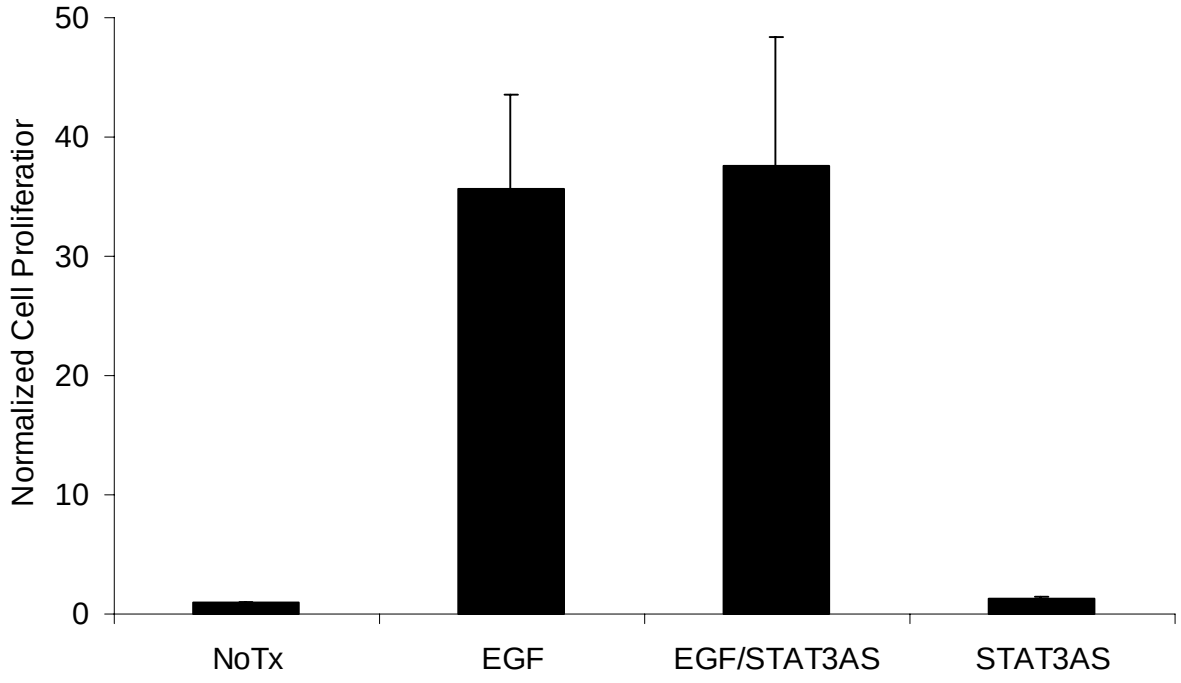


Figure 3-14. STAT3 antisense does not inhibit EGFR mediated proliferation in NR6WT cells.

EGF plus STAT3 antisense treated NR6WT cells have no statistically significant change on proliferation with those cells treated by EGF alone. The cell proliferation (on the Y axis) is normalized to diluent alone (NoTx) within each experiment. NoTx: non-treatment control; EGF:

1 nM human EGF; EGF/STAT3AS: 1 nM human EGF plus 10 μM mouse STAT3 antisense oligonucleotides; STAT3AS: 10 μM mouse STAT3 antisense oligonucleotides. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate.

3.15 STAT3 ANTISENSE DOES NOT INHIBIT EGFR MEDIATED PROLIFERATION IN DU145WT CELLS

Cancer cells present unique signaling pathways and dependencies. As such we examined the effect of STAT3 downregulation on the prostate carcinoma cells. It has been reported that many carcinoma cells undergo apoptosis or proliferation arrest in the face of STAT3 downregulation [246] [264] [265]. As such I expected a decrease of DU145WT proliferation after the treatment of STAT3 antisense oligonucleotides. Surprisingly, again, no significant change was found in the *in vitro* proliferation assay between DU145WT cells treated with EGF and EGF plus STAT3 antisense oligonucleotides (Fig 3-17). Similar experiments were performed with STAT3 siRNA to block the STAT3 expression instead of STAT3 antisense and got the same results (Data not shown).

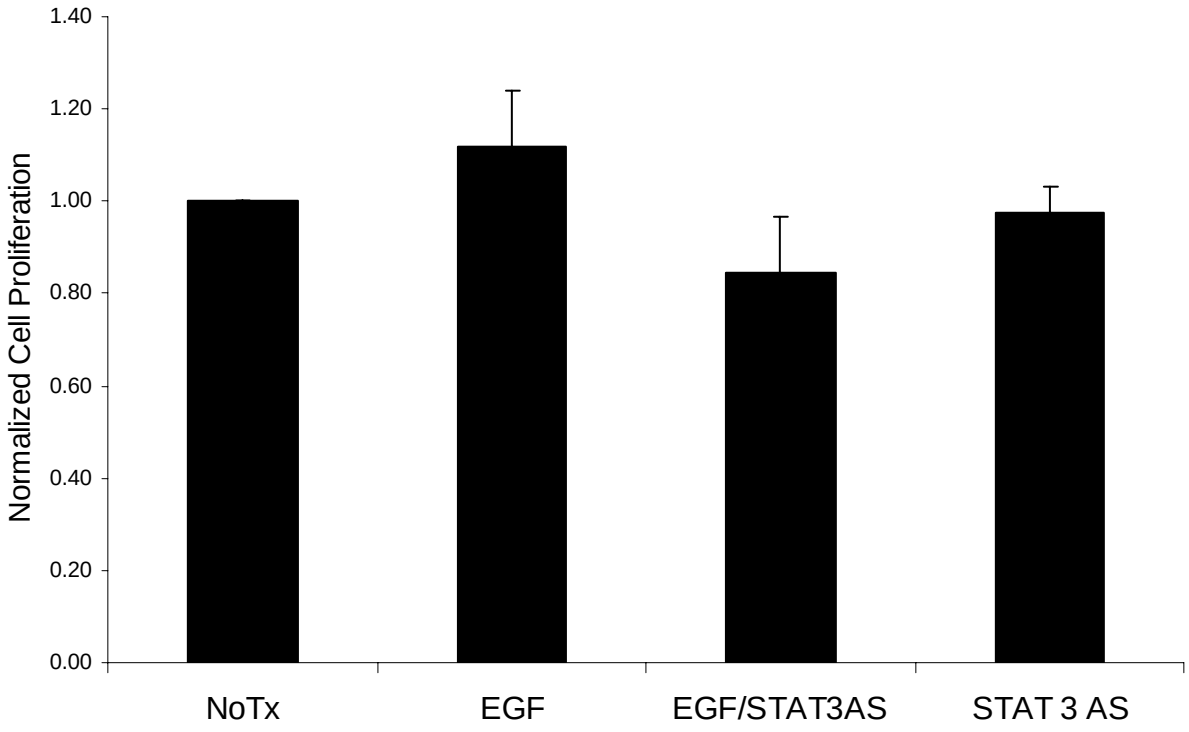


Figure 3-15. STAT3 antisense does not inhibit EGFR mediated proliferation in DU145WT cells.

EGF plus STAT3 antisense treated DU145WT cells have no statistically significant change on proliferation with those cells treated by EGF alone. The cell proliferation (on the Y axis) is normalized to diluent alone (NoTx) within each experiment. NoTx: non-treatment control; EGF: 1 nM human EGF; EGF/STAT3AS: 1 nM human EGF plus 10 μM human STAT3 antisense oligonucleotides; STAT3AS: 10 μM human STAT3 antisense oligonucleotides. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate.

4.0 STAT3 IS REQUIRED IN CASPASE 3 INDUCED HUMAN PROSTATE CARCIMONA CELL APOPTOSIS

4.1 MICROARRAY DATA SHOWS SIGNIFICANT CHANGES OF SOME PROTEINS' TRANSCRIPTION LEVELS AFTER EGF AND/OR STAT3 ANTISENSE TREATMENT IN NR6WT CELLS

To further probe the role of the EGF/STAT3 pathway, an initial microarray analysis was performed on NR6WT cells, thus avoiding variable amounts of autocrine signalling, to determine transcripts that were altered by EGF in a STAT3-dependent manner. Interestingly, these did not include the constituents of the well known epigenetic cascades but rather extracellular matrix or cell cytoskeleton components. It is possible that the enzymatic epigenetic effects are regulated by the other EGFR-induced transcription cascades, and that STAT3 primarily functions to provide the motors and pathways for motility. Of note, we did not detect either collagenase-1 (MMP-1) or stromelysin-2 (MMP-10) that was seen to be up regulated in T24 bladder carcinoma cells [147]; this discrepancy is likely due to our examining the non-transformed NR6WT fibroblasts presenting a more limited set of transcription changes, though the two changes noted in the fibroblasts also were found in the cancer cells

EGF/NoTx Expression Ratio	EGF + STAT3AS/NoTX Expression Ratio	Genbank Number	Name
			EGF up STAT3AS down
1.514	0.823	3883001	myotubularin related protein 1
1.518	1.027	3600099	ATP-dependent metalloprotease FtsH1
1.605	0.918	348968	Procollagen, type XVIII, alpha 1
1.608	1.086	4160555	kinesin-related mitotic motor protein
1.782	1.255	2460054	acetylcholinesterase-associated collagen
2.115	1.058	487140	disintegrin-related protein
2.194	0.923	193565	Selectin, platelet
2.392	1.290	1171097	Procollagen, type XVIII, alpha 1
2.734	0.885	193559	endothelial ligand for L-selectin
3.165	0.824	1568624	Laminin, alpha 4
3.283	1.310	192663	alpha-1 type II collagen
3.409	1.443	4159992	endomucin mRNA
3.478	1.277	2465567	A disintegrin and metalloprotease domain (ADAM) 7
3.820	1.052	3929109	Kinesin heavy chain member 5C
4.992	1.189	4587237	T-cadherin
5.201	0.908	2995448	Midline 1
7.539	1.411	1666650	Cyritestin 1
			EGF down STAT3AS up
0.209	1.378	2160436	Procollagen, type XV
0.236	1.231	1401050	Dishevelled 2, dsh homolog
0.331	0.841	3219171	collagen a1(V)
0.349	0.867	1617401	ena/VASP
0.380	0.937	2104494	Dynactin 1
0.413	0.754	220589	Lysyl oxidase
0.442	0.989	1313903	collapsin-1
0.474	0.796	595918	Capping protein alpha 2
0.492	0.908	2282607	A disintegrin and metalloprotease domain
0.523	1.093	396816	BM-90/fibulin extracellular matrix glyroprotein
0.633	1.002	5771423	delta-sarcoglycan
0.648	1.203	53991	T-complex protein 10a
0.651	1.454	348971	Desmin
0.664	1.453	537490	Integrin alpha V
0.669	1.118	1742912	Rab 11
0.670	0.982	497774	Fascin 1
0.690	1.120	193223	Focal adhesion kinase

Table 4 Microarray in NR6WT cells shows some proteins' mRNA expression level changed significantly after EGF treatment and went back after blocking the STAT3 by antisense nucleotide.

The NR6WT cells were quiesced in 0.5% FBS MEMα media for 24 hours before treatment with 1nM of EGF and/or 10μM of mouse STAT3 antisense nucleotide (5'-GTT CCA CTG AGC CAT CCT GC-3') for another 24 hours. RNA from the treated cells was then purified. and prepared for microarray analysis. Affymetrix U74AV2 murine genome array chip was choosen for the microarray. 5 μg of purified total RNA were added into a 20 μl first strand reaction with 200 U of SuperScript II (Invitrogen) and 1 μg T7 primer in 1X first strand buffer (Invitrogen) followed by a 42°C incubation for 1 hour. Second strand synthesis was performed followed by purification according to the manufacturer's protocol (Affymetrix). The purified cDNA was amplified to produce 70-120 μg of biotin labeled cRNA.

Murine U74Av2 GeneChip probe arrays were pre-hybridized in 200μl of the hybridization cocktail (containing 10 μg of labeled cRNA) according to the manufacturer's protocol. 15 μg of labeled cRNA were fragmentedand and resuspended.

The raw data from microarray were normalized with the Affymetrix Microarray suite 5.0, based on the housekeeping gene expression profile. Expression values were adjusted to the intensity of the expression value of the 100 housekeeping genes.

4.2 EGF INHIBITS THE EXPRESSION OF VASP IN NR6WT CELLS, WHICH CAN BE REVERSED BY STAT3 SIRNA

Previous research demonstrated VASP as a negative regulator of fibroblast cell motility [168]. We also noticed significant decrease of VASP mRNA expression after EGF treatment in

NR6WT cells. I used immunoblot to examine if the protein level of VASP also changed. The results showed that 1 nM EGF suppressed VASP protein expression in NR6WT cells 30 minutes after treatment and continues for about 24 hours. (Data not shown) The inhibition of VASP expression was reversed by STAT3 siRNA, which implicated STAT3 may play a role in EGF mediated VASP expression (Fig 4-1).

Caspase 3 is a well known protein that plays a central role in effecting apoptosis. We also found dramatically change of Caspase 3 mRNA after EGF and STAT3 antisense stimulation in microarray screening. Preliminary data shows decreased Caspase 3 protein expression after EGF stimulation. The inhibition of Caspase 3 expression was reversed by STAT3 siRNA, which implicated STAT3 may play a role in EGF mediated Caspase 3 expression and apoptosis inhibition (Fig 4-1).

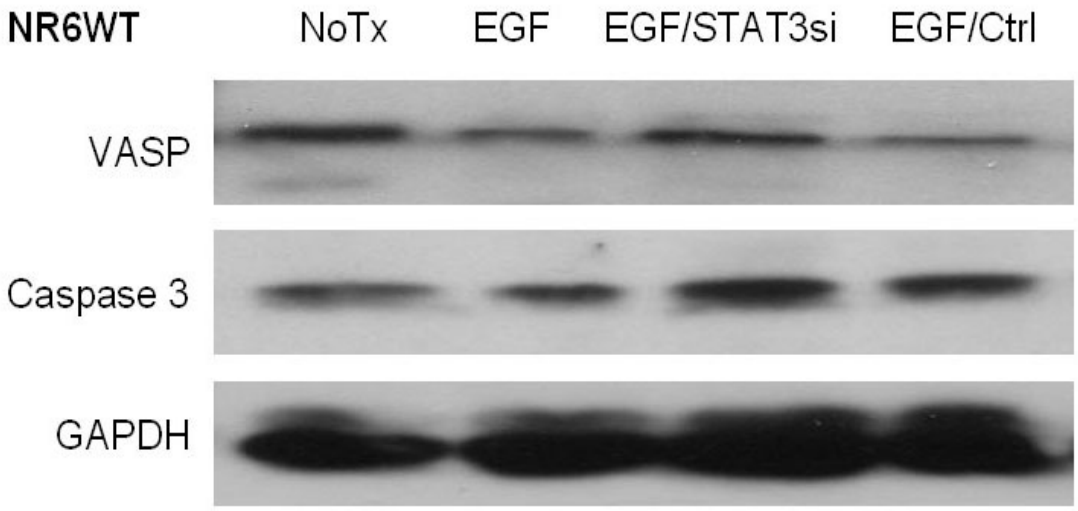


Figure 4-1. EGF inhibits the expression of VASP and Caspase-3 in NR6WT cells, which can be reversed by STAT 3 siRNA.

In murine fibroblasts, NR6WT cells, EGF exposure decreased the expression of VASP and Caspase-3 at protein level, and this decrease was reversed by STAT3 siRNA. The NR6WT

cells were transfected with 20 nM of STAT3 siRNA or non-specific control (eGFP siRNA) for at least 4 hours and recovered in fresh antibiotics-free medium for 24 hours. After that, cells were quiesced in 0.5% FBS medium for another 24 hours before the addition of EGF. NoTx: non-treatment control; EGF: 1 nM human EGF; EGF/STAT3si: 1 nM human EGF plus 20 nM mouse STAT3 siRNA; EGF/Ctrl: 1 nM human EGF plus 20 nM eGFP siRNA. The treated cells were lysised in 2X loading buffer and denatured at 100℃ in water bath for 5 minutes. Lysates were loaded to 10% acrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies against mouse VASP and Caspase-3. After being exposed, the membrane was stripped and immunoblotted with antibody against GAPDH as a loading control.

4.3 EGF INHIBITS THE EXPRESSION OF VASP AND CASPASE 3 IN DU145WT CELLS AND CAN BE REVERSED BY STAT3 SIRNA

As mentioned above, VASP had been shown to be a negative regulator of fibroblast cell motility [168]. We also found significant decrease of VASP mRNA expression after EGF treatment in NR6WT cells and confirmed it by immunoblot. The inhibition of VASP and Caspase-3 expression by EGF and reversion by STAT3 siRNA also was observed in DU145 human prostate tumor cells, which implicated STAT3 may plays a role in EGF mediated VASP and Caspase 3 expression and the tumor cell resistance to apoptosis (Fig 4-2). Similar results were observed in PC3 cells (data not shown).

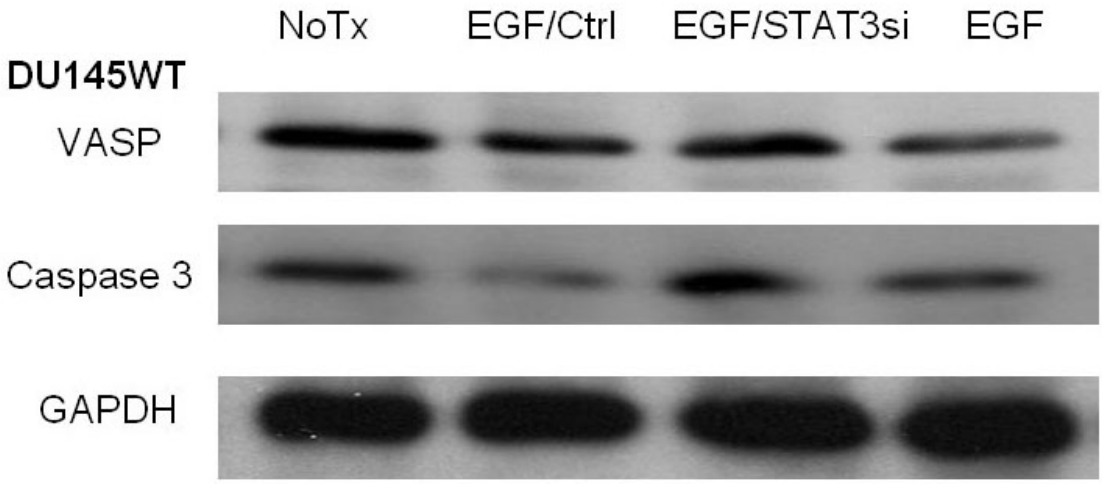


Figure 4-2. EGF inhibits the expression of VASP and Caspase-3 in DU145WT cells, which can be reversed by STAT 3 siRNA.

In DU145WT human prostate cancer cells, EGF exposure decreased the expression of VASP and Caspase-3 at protein level, and this decrease was reversed by STAT3 siRNA. The DU145WT cells were transfected with 40 nM of STAT3 siRNA or non-specific control (eGFP siRNA) for at least 4 hours and recovered in fresh antibiotics-free medium for 24 hours. After that, cells were quiesced in 0.5% FBS medium for another 24 hours before the addition of EGF. NoTx: non-treatment control; EGF: 10 nM human EGF; EGF/STAT3si: 10 nM human EGF plus 40 nM human STAT3 siRNA; EGF/Ctrl: 10 nM human EGF plus 40 nM eGFP siRNA. The treated cells were lysised in 2X loading buffer and denatured at 100℃ in water bath for 5 minutes. Lysates were loaded to 10% acrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies against VASP and Caspase-3. After being exposed, the membrane was stripped and immunoblotted with antibody against GAPDH as a loading control.

4.4 STAT3 IS REQUIRED FOR RESISTANCE TO TNF-A INDUCED APOPTOSIS OF DU145WT CELLS

Since Caspase 3 plays a critical role in apoptosis and we found some relationship between EGF, STAT3 and Caspase 3 expression, it is quite reasonable to querying if the first 2 proteins also involved in Caspase 3 mediated apoptosis. Most recent research indicated that EGF protects prostate cancer cells from apoptosis [266], which put the role that EGFR mediated signalling pathway plays in apoptosis under focus. An Annexin-V staining assay for apoptosis induced by TNF-α was performed. Result shows EGF protect DU145WT human prostate tumor cells from apoptosis, and the protection can be reversed by STAT3 siRNA, which indicated a major role STAT3 maybe plays in Caspase 3 mediated apoptosis (Fig 4-4). I did not see major cell death in the experiment, but still sensitivity to the TNF-α. A similar apoptosis assay that induced by cisplatin instead of TNF-α was also performed and showed the same result. (Data not shown)

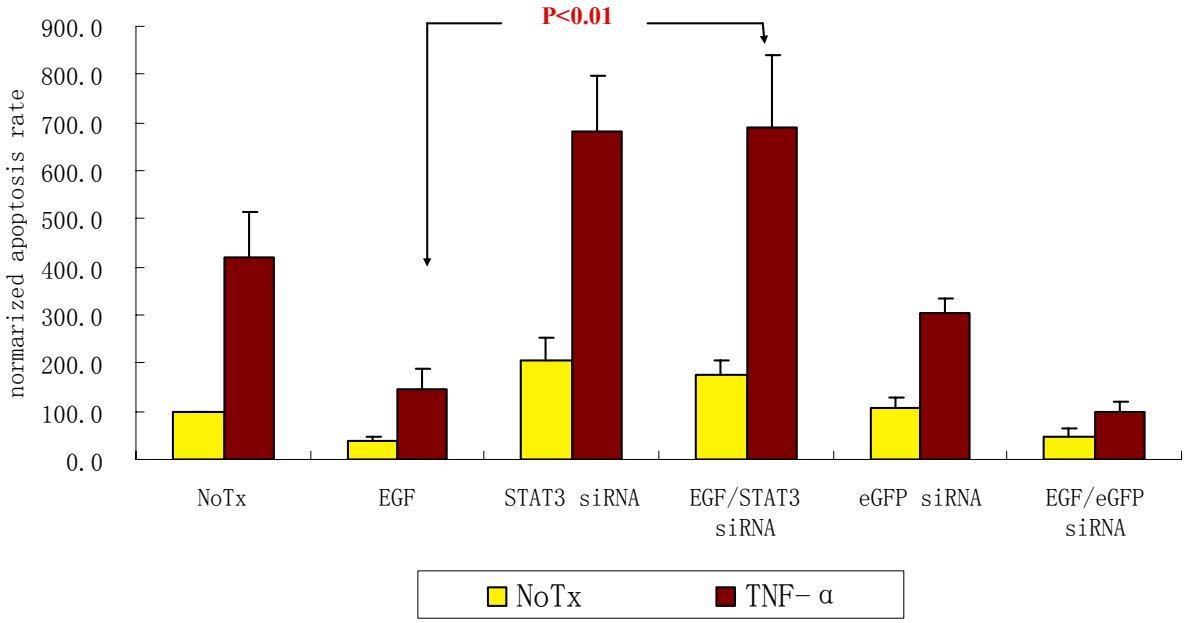


Figure 4-3. Annexin-V staining data for apoptosis in DU145WT prostate tumor cells with the treatment of EGF and STAT3 siRNA

*EGF protects apoptosis induced by TNF-α in DU145WT cells, while the inhibition of apoptosis can be reversed by STAT3 siRNA. The cells were transfected by human STAT3 siRNA and followed by a 6 hours treatment with 30 ng/ml TNF-α and/or 2 nM EGF. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate. * P < 0.01 compared to EGF treatment. The migration distance (on the Y axis) is normalized to diluents alone of the NoTx within each experiment. NoTx: non-Treatment; EGF: 2 nM human EGF; STAT3si: 40 nM human STAT3 siRNA; EGF/STAT3si: 2 nM human EGF with 40 nM human STAT3 siRNA; eGFPsi:40 nM eGFP control siRNA; EGF/eGFPSi: 2 nM human EGF with 40 nM control eGFP siRNA; TNF-α: 30 ng/ml human TNF-α.*

4.5 STAT3 IS REQUIRED FOR RESISTANCE TO TNF-A INDUCED APOPTOSIS IN PC3 CELLS

The same Annexin-V staining assay for apoptosis is also performed in another human prostate cancer cell line, the PC3 cells. Result shows EGF protect PC3 human prostate tumor cells from TNF-α induced apoptosis, and the protection can be reversed by STAT3 siRNA, which is the same as what I got from DU145WT cells. (Fig 4-5) I did not see major cell death in the experiment, but still sensitivity to the TNF-α. A similar apoptosis assay, which induced by cisplatin instead of TNF-α, was also performed and showed the same result. (Data not shown)

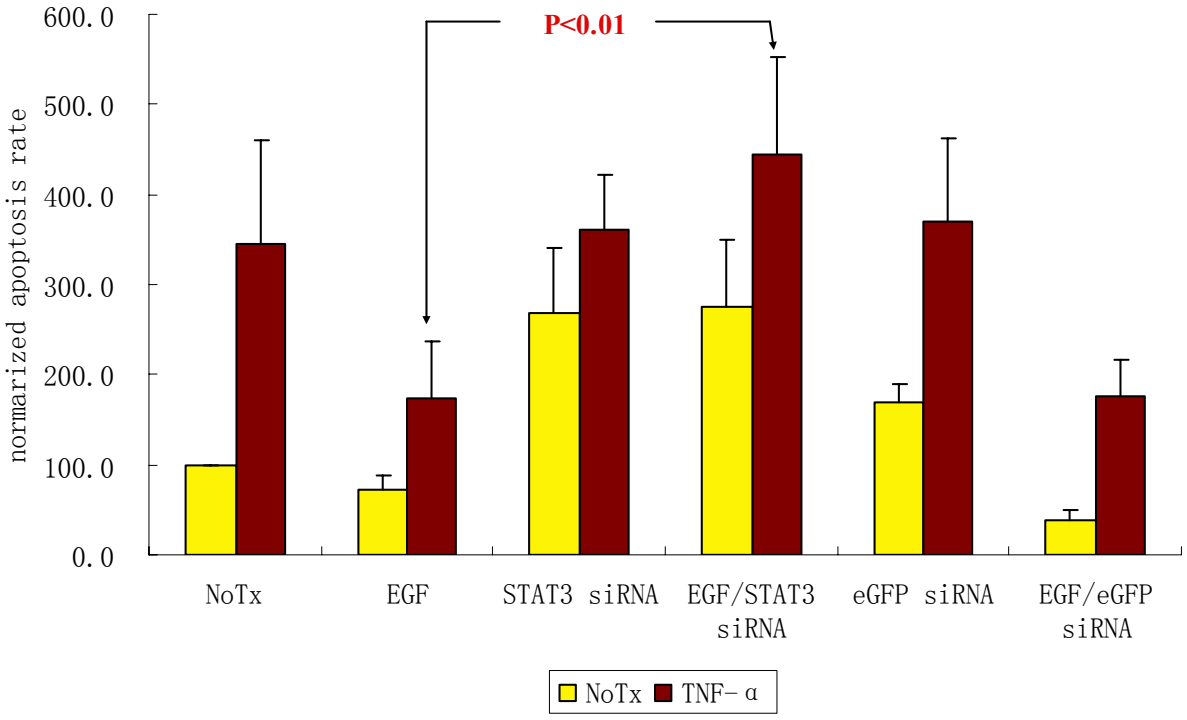


Figure 4-4. Annexin-V staining data for apoptosis in PC3 prostate tumor cells with the treatment of EGF and STAT3 siRNA.

The EGF induced suppression of apoptosis was turn over by STAT3 siRNA in PC3 human prostate tumor cells. The cells were transfected by human STAT3 siRNA and followed by a 6

*hours treatment with 30 ng/ml TNF-α and/or 2 nM EGF. Shown is mean ± s.e.m. for at least 3 experiments each performed in triplicate. * P < 0.01 compared to EGF treatment. The migration distance (on the Y axis) is normalized to diluents alone of the NoTx within each experiment. NoTx: non-Treatment; EGF: 2 nM human EGF; STAT3si: 40 nM human STAT3 siRNA; EGF/STAT3si: 2 nM human EGF with 40 nM human STAT3 siRNA; eGFPsi:40 nM eGFP control siRNA; EGF/eGFPsi: 2 nM human EGF with 40 nM control eGFP siRNA; TNF-α: 30 ng/ml human TNF-α.*

5.0 DISCUSSION CHAPTER

Carcinogenesis is a multi-step process, in which cancer cells disseminate from a localized primary tumor mass to both invade local tissue and metastasize to distant organs. It has been well known that tumor invasiveness is a distinct character of tumor progression involving induced motility in addition to dysregulated proliferation. Since there is no catholicon for the current anti-cancer treatments, disrupting or at least inhibiting the tumor invasiveness would greatly ameliorate the morbidity and mortality. In this study, we investigated whether activated STAT3 protein is involved in the EGF receptor mediated migration in fibroblasts and human prostate cancer cells and the invasiveness of the latter. EGF increased STAT3 activity along with cell motility and invasiveness of these cells. Decreased expression and activation of STAT3 by treatment with STAT3 antisense oligo nucleotides or siRNA or in the presence of a dominant negative mutant of STAT3 abrogated migration and invasion. The results together, indicated that STAT3 could be a targeted critical element in the EGF- mediated cell migration and invasion of prostate carcinoma cells.

Epigenetic events, in addition to transcriptome changes signaled through STAT3, also contribute to tumor cell motility and invasion. Comparing the migration of NR6WT cells expressing constitutively activated STAT3 to those expressing the STAT3 dominant negative mutants, it is interesting to find that constitutively activated STAT3 alone does not increase cell motility, nor does it increase EGF-stimulated migration. This result suggests that STAT3 is not sufficient for cell migration but that it provides for end effectors that require additional signals for either activation or full phenotypic expression. EGF stimulation of a cell results in the activation of multiple pathways that lead to transcriptional control including Src, PLC- ɣ , PI3-K, Ras/MAPK and JAK/STAT [38]. These pathways are often functionally interlinked [267]. Thus,

the need for de novo transcription and translation that one notes during growth factor induced motility [268] [269], can be accomplished by a number of downstream transcription factors but requires STAT3 in addition to the previously determined PLCγ pathway [270]. Recently, it has been noted that EGFR signalling promotes progression of T24 bladder carcinoma cells via STAT3 upregulation of transcription, including that of matrix metaloproteinases [271].

To further probe the role of the EGF/STAT3 pathway, an initial microarray analysis was performed on NR6WT cells, thus avoiding variable amounts of autocrine signalling, to determine transcripts that were altered by EGF in a STAT3-dependent manner. (The subset of these transcripts that are classified as related motility and cell adhesion are provided in the Supplemental Table). Interestingly, these did not include the constituents of the well known epigenetic cascades but rather extracellular matrix or cell cytoskeleton components. It is possible that the enzymatic epigenetic effects are regulated by the other EGFR-induced transcription cascades, and that STAT3 primarily functions to provide the motors and pathways for motility. Of note, we did not detect either collagenase-1 (MMP-1) or stromelysin-2 (MMP-10) that was seen to be upregulated in T24 bladder carcinoma cells [271]; this discrepancy is likely due to our examining the non-transformed NR6WT fibroblasts presenting a more limited set of transcription changes, though the two changes noted in the fibroblasts also were found in the cancer cells. Obviously, these early hints are under active further investigation as these questions lie beyond the scope of the current communication.

Recent studies suggest that aberrant STAT3 signalling may play an important role in the carcinogenesis of prostate cancer. Previous data from other groups shows higher phospho-STAT3 expression in prostate tumor tissues than in adjacent normal tissues [272] [273], and that this correlates with invasiveness [252]. We also found increased expression of STAT3

protein in prostate tumor tissues compared to normal tissue from the same patient (data not shown, as similar data have been previously published). It is quite reasonable that the high expressed pSTAT3 in prostate tumor tissues is from the increased expression of STAT3 as well as activation by autocrine growth factor signalling [274]. Our study indicating that STAT3 is critical for invasion of prostate cancer cells DU145WT and PC3, secondary to increased cell motility is consistent with these findings and provide for a mechanism by which phosphorulated STAT exerts its effects.

Contrary to other studies [275] [276], we didn't find decreased tumor cell numbers upon STAT3 down regulation; this may be due to the incomplete nature of such interventions (though the other studies suffered from similar non-quantitative abrogation), high levels of autocrine EGFR signalling overcoming this limitation via other pathways, or likely the shorter time course of our experiments. However, in accord with these other studies, when we overexpressed the dominant-negative STAT3 construct in DU145WT cells, we noted a high degree of apoptosis in these cells (for the experiments in Figure 19, we achieved a lower, non-apoptotic level of DN STAT3); our failure to established stable clones expressing this dominant-negative construct likely relates to this inhibition of proliferation and/or increased apoptosis. In addition, when we stress the cells using apoptosis inducers the blockade of STAT3 does increase tumor cell apoptosis. As such, we feel that this discrepancy is more likely a quantitative rather than a qualitative effect.

VASP is known as a negative regulator of fibroblast motility [80]. The result from microarray indicated significant change of VASP mRNA after the treatment of EGF and STAT 3 antisense (Table 4). Immunoblot on NR6WT fibroblast cells showed the expression of VASP protein is also inhibited by EGF. The inhibition can be observed 30 minutes after the EGF

treatment and last for about 24 hours. After adding STAT 3 antisense or STAT 3 siRNA, the expression of VASP goes back to normal level. This result supports the previous finding, as EGF suppressed VASP expression, it is quite reasonable to understand that EGF induced fibroblast cell motility can be turned over by blocking the expression of STAT 3, which is needed for VASP expression. Further experiments also showed that EGF can suppress VASP expression in DU145WT and PC3 human prostate tumor cells, though not as significant as in NR6WT fibroblast cells. The reason could be high autocrine level of EGF in those cancer cells.

6.0 CONCLUSIONS AND SPECULATION

6.1 CONCLUSIONS

Growth factor-induced migration is a rate-limiting step in tumor invasiveness. The molecules that regulate this cellular behavior would represent novel targets for limiting tumor cell progression. Epidermal growth factor (EGF) receptor (EGFR)-mediated motility, present in both autocrine and paracrine modes in prostate carcinomas, requires *de novo* transcription to persist over times greater than a few hours. Therefore, we sought to define specific signalling pathways that directly alter cellular transcription. STAT3 (Signal Transducer and Activator of Transcription 3) is activated, as determined by electrophoretic motility shift assays, by EGFR in DU145 and PC3 human prostate carcinoma cells in addition to the motility model NR6 fibroblast cell line. Downregulation of STAT3 activity by antisense oligonucleotides or siRNA or expression of a dominant-negative construct limited cell motility as determined by an *in vitro* wound healing assay and invasiveness through a extracellular matrix barrier. The expression of constitutively activated STAT3 did not increase the migration, which indicates that STAT3 is necessary but not sufficient for EGFR-mediated migration. These findings suggest that STAT3 signalling may be a new target for limiting prostate tumor cell invasion.

The inhibition of STAT3 expression does not decrease the basal or EGFR mediated proliferation of cells studied in this project, NR6WT murine fibroblasts and DU145WT and PC3 human prostate tumor cells. These results contrasted with many of the previous research from other groups suggesting a positive regulation of tumor cell proliferation from EGF and STAT3 [186] [261] [277] [278] [279]. On the other hand, other publications demonstrated that EGF

suppresses tumor growth [280] [281], or has different results between tumor cells *in vivo* and *in vitro* [282]. The most likely explanations for my proliferation data is that these cell lines either do not require STAT3 for in vitro proliferation (possibly by the presence of redundant inductive pathways) or the level of STAT3 inhibition attained is insufficient for proliferation and survival suppression. We prefer this second explanation as the apoptosis studies suggest that lacking STAT3 predisposes to cell death. This latter explanation also would suggest that motility is the more sensitive to transcriptional perturbations, in line with the laboratory's earlier findings [100].

In a microarray analysis of what transcription units are altered by EGF in a STAT3-dependent manner in NR6WT fibroblast cells, we found that the expression of motility-limiting VASP protein and the apoptosis nexus Caspase-3 were both down regulated upon EGF exposure. The microarray data were confirmed by preliminary immunoblot experiments.

As recent study shows, Ena/VASP proteins antagonize capping protein to inhibit actin polymerization at barbed ends *in vitro* [172], which suggested that Ena/VASP proteins associate with actin filaments at or near the barbed end and protect them from being capped by capping protein. The depletion of Ena/VASP causes the lamellipodia to protrude more slowly but to persist longer, yielding a faster moving cell [153]. Immunoblot on NR6WT fibroblast cells showed the expression of VASP protein is inhibited by EGF 30 minutes after the EGF treatment and last for about 24 hours. After adding STAT 3 antisense or STAT 3 siRNA, the expression of VASP goes back to normal level. This result supports the previous finding in microarray. Since EGF suppresses VASP expression, it is quite reasonable to ask in future studies if STAT3 plays a role in the VASP regulated-aspects of cell motility.

Further experiments showed that EGF could suppress VASP expression in DU145WT and PC3 human prostate tumor cells, though not as significantly as in NR6WT fibroblast cells. The reason could be high autocrine level of EGF in those cancer cells.

Caspase-3 plays an essential role in the process of apoptosis as an executor of the caspase cascade. Recent publications demonstrated decreased caspase-3 level in various human tumor cells, which suggests the dire pathology of losing its effect on apoptosis; overcoming the inhibition of caspase-3-induced apoptosis in tumor cells and have little toxicity on normal cells [259]. My preliminary data from microarray and immunoblot sheds some light on the relationship between EGF/STAT 3 pathway and caspase-3. In sum, EGF can inhibit the activation and expression of caspase-3, which save the cells from apoptosis, while STAT 3 siRNA overcomes the effect and make the cells vulnerable to apoptosis inducer. More experiments to focus on the EGF/STAT 3/caspase-3 apoptosis regulation pathway are ongoing.

As a conclusion, the model that emerges is one in which STAT3 signaling downstream from EGFR is required for persistent cell motility and invasion, and that partial abrogation of this pathway hinders this tumor progression. Only more extensive abrogation of STAT3 signalling compromises carcinoma cell survival and proliferation. Thus, STAT3 inhibition, even if suboptimal, would slow tumor progression.

6.2 SPECULATION

There are still many questions about the EGF/STAT3 signalling pathway and its branches in human prostate cancer. One of the most crucial questions is the role STAT3 plays in the prostate tumor *in vivo*. Previous data showed aberrant STAT3 expression in many cancers

including prostate, as described in the introduction, and STAT3 activation is required for the proliferation of many cancer cell lines; though my in vitro proliferation data did not find such a connection in DU145WT and PC3 prostate tumor cells. Considering the complexity of signal transduction pathways *in vivo*, it is too early to say that STAT3 does not related to prostate tumor growth. At least, an in vivo assessment is necessary for determining this discrepancy.

Early embryonic death in STAT3 knockout mice indicated that STAT3 is essential for early embryonic development [283]. Unforunately, this also excludes the possibility of adult $STAT3^{-/-}$ mice in which to induce de novo prostate tumor formation; crossbreeding with the TRAMP mice would be an example. The attempt of getting STAT3 dominant negative mutants in DU145WT prostate cancer cells was also performed as in NR6WT fibroblasts. Unfortunately, there were no stable clones survived from transfection and the following selection. The most likely explanation is that STAT3 is critical to the survival or viability of this cell line, and that the dominant negative was more efficient at downregulating STAT3 function than siRNA or antisense; these were only partially abrogating at best. A second possibility is that the STAT3 DN also inhibited the functioning of other STAT isoforms through heterodimerization. The high-level background expression of STAT3 dominant negative even in the absence of dexamethasone may also contribute to this fatal result. To overcome the toxicity, I am planning an alternatively induced STAT3 mutant to avoid the highest levels of STAT3 signaling blockade.

The lethality of STAT3 dominant negative expressing DU145WT human prostate cancer cells also attracts my attention to the cell death. Combined with previous microarray data, an EGF-STAT3-Caspase-3 signalling pathway emerged. Caspase-3 is a pivotal executor of apoptosis and the crosslink of the two main apoptosis pathways. Both Caspase-8 activated by the death receptor pathway and Caspase-9 activated by the mitochondria pathway will cleave

pro-Caspase-3 to then activate the downstream proteins to finish the program cell death. I used TNF-α to stimulate and activate the death receptor pathway of apoptosis, which can be suppressed by EGF and recovered by STAT3 abrogation by siRNA. Cisplatin is another apoptosis inducer, which initiates the mitochondria pathway by binding to the DNA of target cells [284]. It was also used in the experiment and obtained similar data. Those results suggested a critical role for STAT3 in apoptosis. Many other experiments are needed to confirm that EGF/STAT3 signaling pathway is involved in Caspase-3 regulation. For example, the measurement of Caspase-3 activity on its substrate with or without STAT3, is an essential complement for previous microarray and immunoblot data. I also need some other method like flow assay to check the cell apoptosis besides Annexing-V staining.

The next step of studying STAT3 signaling in apoptosis would be the define of the whole signal transduction pathway. The intersection of STAT3 target genes and apoptosis related genes offers me a promising lode to mine. A well known such protein is BCL-XL [208]: increased expression of BCL-XL, an anti-apoptotic BCL-2 family gene, is dependent on constitutively activated STAT3.

Since the STAT3 is a transcription factor, questions will be asked on how STAT3 regulates the expression of those genes involved in apoptosis. From previous publications, it looks like STAT3 performs its mission by activating the expression of anti-apoptosis genes. Is the activation from direct STAT3 binding on the target DNA sequence, or from indirect mechanisms? Is the STAT3 activation also suppress some pro-apoptosis genes, such as Caspase 3 herein? This was one of the unexpected aspects of the microarray, that STAT3 signaling appeared to repress gene transcription. As STAT3 is generally considered a transcription induced and not repressor, the question is whether this repression is direct by STAT3 or secondary to

STAT3 induction of a transcription repressor. The answer of those questions will be found in a more detailed study on the molecular level.

While my findings reported herein suggest a molecular basis for the STAT3 dependence of EGFR-mediated prostate tumor progression, many more questions have been raised than answered. I hope that others can find value in addressing these issues.

7.0 ABBREVIATIONS

ABL	Abelson Leukemia Protein
Arp	Actin-related protein
ATP	Adenosine triphosphate
BAD	Bcl Associated Death promoter
BAX	BCL-2 Associated X protein
BCL-2	B-Cell Lymphoma 2
DAG	Diacylglycerol
DFF	DNA fragmentation factor
DHT	Dihydrotestosterone
EGF	Epidermal Growth Factor
eGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EMSA	Electrophoretic Mobility Shift Assay
Ena	Enabled
ERK	Extracellular Signal-Regulated Kinase
EVH	Ena-VASP-Homology
EVL	Ena-VASP like
FBS	Fetal Bovine Sera
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HGF	Hepatocyte Growth Factor
IFN	Interferon
IHC	Immunohistochemistry

IL-6	Interleukin-6
IP_3	Inositol 1,4,5-triphosphate
JAK	Jauns Kinase
MAPK	Mitogen-Activated Protein Kinase
Mena	Mammalian Enabled
PARP	Poly ADP-Ribose Polymerase
PCR	Polymerase Chain Reaction
PIP_2	Phosphatidyl Inositol 3,4-bisphosphate
PKC	Protein Kinase C
PLC-γ	Phospholipase C-γ
PSA	Prostate Specific Antigen
RNAi	RNA Interference
SH2	SRC Homology 2
STAT	Signal Transducer and Activator of Transcription
TAD	Transcriptional Activation Domain
TGF-α	Transformation Growth Factor-α
TKI	Tyrosine Kinase Inhibitor
TNF	Tumor Necrosis Factor
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular Endothelial Growth Factor
XIAP	X-Chromosome-Linked Inhibitor Of Apoptosis Protein
ZBP	Zipcode Binding Protein

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