CHARACTERIZATION OF FAK-TRAF2 MECHANISM INVOLVED IN THE REGULATION OF CELL SURVIVAL SIGNALING IN RELATION TO INFLAMMATORY BREAST CANCER

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DEDICATION

I dedicate this work to every cancer patient who suffered or still suffering from this disease, which we have to fight against making it a part of the medical history one day.

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ABSTRACT

Inflammatory breast cancer (IBC) is a highly aggressive cancer that lacks efficient targeted therapeutics. Cancer-associated inflammation has been implicated among drivers of aggressive cancer progression to metastasis. However, molecular mechanisms by which inflammation process contributes to metastasis signaling in IBC remain partially understood.

In this study, a comprehensive survey of publically available breast cancer genomic databases identified a predominant association between genes involved in inflammatory signaling (e.g. TRAFs, NF-κB, TNF-α) and breast cancer aggressiveness. As well, concomitant up-regulation of multiple genes involved in focal adhesion signaling (e.g. FAK, Paxillin) was seen in advanced stages. To further investigate these associations at the molecular levels, crosstalk between inflammation and focal adhesion signaling was investigated, focusing on TNF receptor-associated factor2 **(**TRAF2) and FAK. We used mouse embryonic fibroblast (MEF) cells proficient and deficient for TRAF2 and/or FAK, as well as a panel of human breast cancer cells including MDA-MB-231, SUM190PT and SUM149PT. Immunoprecipitation, immunofluorescence, and mapping studies demonstrated that TRAF2 physically interacts with FAK through its N-terminal portion. Moreover, a reporter-based luciferase assay as well as immunoblotting analysis revealed this interaction to modulate NF-κB activity regulating cell survival in part via promoting resistance to anoikis. In summary, our studies indicate that FAK-TRAF2 signaling regulates two crucial mechanisms associated with metastasis development.

RÉSUMÉ

Le Cancer du inflammatoire du sein (CIB) est un cancer très agressif qui manque de thérapies ciblées et efficaces. L'inflammation associée au cancer est reconnue comme un promoteur de la progression du cancer vers la métastase. Cependant, les mécanismes moléculaires par lesquels le processus d'inflammation contribue à la signalisation de la métastase restent partiellement compris.

Dans cette étude, une analyse exhaustive des bases de données génomiques disponibles publiquement a identifié une association prédominante entre les gènes impliqués dans la signalisation inflammatoire (par exemple TRAFs, NF-κB, TNF-α) et l' agressivité du cancer du sein. En outre, la régulation concomitante de plusieurs gènes impliqués dans la signalisation d'adhésion focale (FAK par exemple, Paxillin) a été observée dans les stades avancés. Pour approfondir ces associations au niveau moléculaire, a nous sommes intéresés au lien entre l'inflammation et la signalisation d'adhésion focale, en se concentrant sur TNF receptor-associated factor 2 (TRAF2) et FAK. Des fibroblastes embryonnaires (MEF) des cellules de souris compétent et déficient pour TRAF2 et / ou FAK, ainsi q'un panneau de cellules de cancer du sein humaines, y compris MDA-MB-231, SUM190PT et SUM149PT ont ètè vtilisé. L'immunoprécipitation, l'immunofluorescence ont démontré que FAK interagit physiquement avec par la partie N-terminale de TRAF2. De plus, un dosage à base de luciférase ainsi que l'analyse d'immuno-buvardage a révélé que cette interaction module l'activité de NF-κB et promeut la survie des cellules en partie par la promotion de la résistance à l'anoïkis. En résumé, nos études indiquent que la signalisation FAK-TRAF2 regule deux mécanismes cruciaux associés au développement de métastases.

PREFACE & CONTRIBUTION OF AUTHORS

This thesis is presented in the traditional format in accordance with the guidelines of the National Library of Canada. The data presented in this thesis is a combination of original work of the candidate (characterization of TRAF2 and FAK proteins in IBC cell lines and genomic analysis of publically available breast cancer databases) and work done in collaboration with Dr. Xu Bin and Dr. Ding Zhang Xiao (initially identified the TRAF2-FAK interaction in yeast two hybrid system and mammalian cells, respectively). Specifically, I performed the expression of TRAF2 in TRAF2-proficient and TRAF2-deficient cells, TRAF2 and FAK interaction and colocalization, and the impact of TRAF2-FAK interaction on cell survival.

All the research work of this thesis was performed at the Lady Davis Institute (LDI) in Montreal. The LDI is an independent non-profit and research center in Québec that is affiliated to McGill University.

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ABBREVIATIONS

AJCC	The American Joint Committee On Cancer
CCLE	Cancer Cell Line Encyclopedia
CHX	Cycloheximide
cIAP1	Cellular Inhibitor Of Apoptosis 1
CTC	Cancer Circulating Cells
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial- Mesenchymal Transition
ER	Estrogen Receptor
ErbB-2	Evian Erythroblastosis Oncogene B
ERK	Extracellular Receptor Kinase
FAK	Focal Adhesion Kinase
FAT	Focal Adhesion Targeting
FERM	Band 4.1-Ezrin-Radixin-Moesin
Fn-14	Fibroblast Growth Factor-Inducible 14
Graf	GTPase Regulator Associated With FAK
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
Her-2/neu	Human Epidermal Growth Factor Receptor 2
IBC	Inflammatory Breast Cancer
IDC	Immature Dendritic Cells
IKK	IκB Kinase
IκB	Inhibitor Of NF-κB
LABC	Locally Advanced Breast Cancer
MaCSC	Mammary Cancer Stem Cells
MAPK	Mitogen-Activated Protein Kinase

MEF	Mouse Embryonic Fibroblasts
MET	Mesenchymal- Epithelial Transition
MMP-9	Matrix Metalloproteinase-9
MMTV	Mouse Mammary Tumor Virus
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NIBC	Non-Inflammatory Breast Cancer
OS	Overall Survival
p130Cas	Crk-Associated Substrate
pCR	Pathological Complete Response
PFS	Progression-Free Survival
PH	Pleckstrin Homology
PTK2	Protein Tyrosine Kinase 2
RIP	Receptor-Interacting Protein
RIPK	Receptor-Interacting Protein Kinase
RT-PCR	Real Time PCR
SCID	Severe Combined Immunodeficiency Disease
SEER	Surveillance, Epidemiology, and End Results
SH3	Src Homology 3
Src	Steroid Receptor Coactivator
SYF	Deficient For Src, Yes and Fyn
TAK1	TGF-β Activated Kinase 1
TANK	TRAF Family Member-Associated NF-κB Activator
TCGA	The Cancer Genome Atlas
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
TNFRI	Tumor Necrosis Factor Receptor Type I
TNM	Tumor, Lymph Nodes, Metastasis
TRAF2	TNF Receptor-Associated Factor 2
TWEAK	TNF-Like Weak Inducer Of Apoptosis

CHAPTER 1: INTRODUCTION

Breast cancer is a prevalent disease and a major cause of cancer-related deaths worldwide. Genome-wide analyses revealed that breast cancer is a highly heterogeneous disease and encompasses diverse types and molecular subtypes with distinct biological and clinical manifestations, in particular in relation to metastasis incidence. For example, although all breast cancer types can progress to metastasis, including the most predominant luminal subtype, representative of the hormone-positive tumors, inflammatory, Her2-positive and the basal-like subtype breast cancers are notorious for their highest incidence of metastasis.

1. Overview Of Inflammatory Breast Cancer

1.1 History

Inflammatory breast cancer (IBC) is a rare and highly aggressive disease compared to other breast cancer types [3]. Sir Charles Bell was the first to describe the criteria of IBC in his book 'A System Of Operative Surgery' in 1816, as "when a purple color on the skin above the tumor accompanied by shooting pains, it is a very unpropitious beginning" [4]. In 1887, Thomas Bryant described the disease pathologically as it is characterized nonexclusively by the presence of tumor emboli in dermal lymphatic vessels that makes it easier to be misdiagnosed as bacterial infection or mastitis [5].

IBC is featured with the rapid onset of symptoms that was detailed as early as 1909 from an artistic point of Archibald Leitch in the Lancet including erythema, edema, "peau d'orange" (orange peel) appearance of breast skin conveying an image of minute pits spaced with about 0.25 inches from each other, and without an underlying palpable mass [3,6]. Later in 1924, Lee and Tannenbaum were the first to publish the term "inflammatory carcinoma of the breast" and they described some of the symptoms: "as the disease progresses, the skin becomes deep red or reddish-purple, and to the touch is brawny and infiltrated. The inflamed area presents a distinct raised periphery after the fashion of erysipelas. The examiner with his eyes closed … can distinguish the sharp contrast between normal and affected tissue" [7]. The disease was first categorized in 1938, into two clinical varieties "Primary" in which the inflammatory symptoms

are associated with the carcinogenesis in a normal breast tissue and "Secondary" refers to the recurrence of (non-inflammatory) breast carcinoma accompanied with inflammatory reactions [8]. Also, Saltzstien introduced the term of "clinically occult inflammatory carcinoma" in 1974 describing the histological tumor involvement in the breast dermal lymphatics of four patients [9]. A similar description of IBC was illustrated as "poussée évolutive" (rapidly progressing breast cancer) in a study carried out on 145 clinical cases in 1978 in Tunisia and followed by many publications shading the light on this disease [10]. According to this French system IBC is classified as PEV2 or PEV3 which are associated with the inflammatory signs involving less than half, and more than half of the breast respectively[11]. However, some recent investigators claim that these early-described cases may not be diagnosed sufficiently according to the present disease clinical criteria.

1.2 Epidemiology

IBC, usually affects patients at a young age and often manifests with poor prognosis ranging from locoregional to distant metastasis [12]. Although, it is a rare disease with an incidence rate of less than 6% in the US [13]; the data from Surveillance, Epidemiology, and End Results (SEER) program showed that its incidence rate has increased from 2 between 1988 and 1990 to 2.5 in the period between 1997 and 1999 (per 100,000 women- year) [14]. It is important to note that the IBC worldwide incidence reflects some racial disparities as it is estimated to represent less than 4% of all breast cancer types in individuals of Caucasian ancestry, whereas the reported percentage is much higher in patients of African ancestry. Additionally, some reports from North Africa and the Middle East showed the incidence of IBC to surpass 4% of all breast cancer cases registered in these regions [15].

The true IBC epidemiological distribution is partially known due to the lack of a large number of samples, and the variation of disease diagnostic criteria from region to region; even though, the incidence rate is generally higher in other countries than what's reported in the US. For example, in a retrospective study which reviewed 2,733 breast cancer patient records at Ege University in the period between January 1988 and May 2000 in Turkey, IBC was histologically confirmed in 142 of the patients of them with a rate of 5.2 percentage [16]. Another example is a case control study carried out in The Aga Khan University Medical Center, Pakistan on 40 breast cancer cases along with double controls for each case from 1992 to 1998, which confirmed that around

10% of all patients with breast cancer were IBC cases [17]. Also, the reports form Tunisia although contradicting each other; they are demonstrating a higher IBC incidence compared to the US without considering the diagnostic criteria variation. Mourali et al's epidemiological study at the Salah Azaiz Institute in 1980 reported that IBC constituted around 50% of all breast cancer cases between 1969 and 1974. Whereas the incidence rate dropped to 5-7% in another study carried out in the same institute which reassessed the IBC cases according to the currently applied disease criteria in 2008, where 419 breast cancer cases were analyzed in the period between 1975 and 1996 [18].

1.3 Clinical Picture

There was a huge debate for more than hundred years on the IBC disease case definition and diagnosis. Some scientists based their case identification on the inflammatory clinical appearance; and some of them used the pathologic features to confirm the clinical picture of the disease. While others were preferring the pathological appearance over what they observed clinically [19]. In the present clinical practice, the most commonly used system to characterize IBC is the TNM classification of the American Joint Committee on Cancer (AJCC) and according to it IBC is considered as a T4d N0-2, stage IIIb tumor [20]. It is a peculiar form of advanced breast cancer, characterized by a diffuse invasion of dermal lymphatic vessels by cancer cells obstructing them [21], which causes a rapid progression of symptoms including diffuse erythema and edema occupying at least one third of the breast, changes in skin color and texture to have the orange peel appearance (peau d'orange) not necessarily with an underlying palpable mass [3,22]. Although mimicking the inflammatory reaction, the vessels microscopic examination only shows carcinoma cells infiltrates [23].

1.4 Molecular Profiling

Compared to non-inflammatory breast cancer (NIBC), IBC is highly aggressive with higher metastatic potential in the brain, bones and soft tissues[24]. Hence and because it is a heterogeneous disease involving many interrelated signaling pathways, it is considered an excellent model to investigate the biology of metastasis, in particular in relation to interactions involving cancer cells, their microenvironment, and the host. Efforts from different research groups have contributed to define potential molecular signatures of IBC. In particular, microarray gene

expression profiling of IBC and non-IBC tissue samples revealed that IBC falls into all the classical six molecular subtypes including an estimated 19% of Luminal A, 19% of Luminal B, 17% of Basal type, 17% of Claudin-low, 22% of ErbB2 positive, and 5% of Normal breast-like. Generally, around 75% of IBCs belonged to aggressive subtypes that account for only 53% of the non-IBCs [25] (Figure 1).

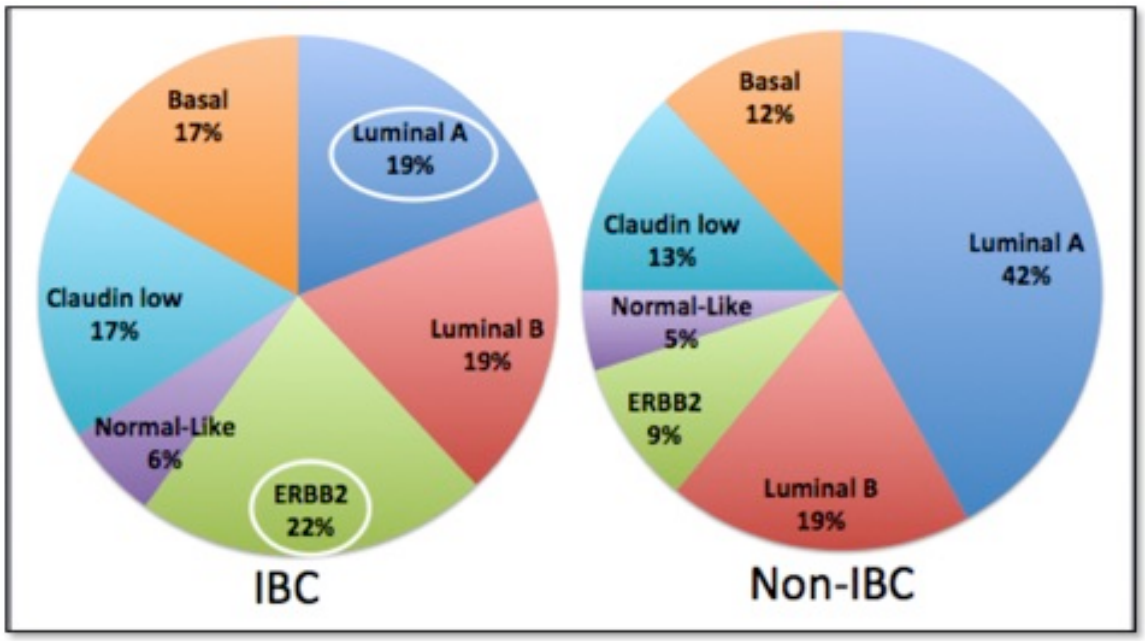

Figure 1: Molecular Classification Of IBC Versus NIBC Tumor Samples Based On Microarray Gene Expression Profiling.
Percentages determined from Bertucci et al. the breast, 2014 [25].

Correspondingly the molecular subtypes were shown to be associated with similar histopathological and clinical patterns described in the literature about common breast cancer profiling. Besides, the available studies generally based on very limited IBC sample cohorts and cell lines, suggesting that IBC cancer cells may be derived from the same cells- of-origin as non-IBC cancer cells. Moreover, these studies suggested that the inflammatory phenotype of the disease and its molecular subtype are biologically independent from each other[26]. Therefore, further categorization of the drivers behind the disease's inflammatory behavior is urgently needed.

2. Biology Of IBC And Current Knowledge Of The Contribution Of Inflammatory Signaling

2.1 Disease Models

In attempts to molecularly characterize the disease various models have been used. Up to present, there is no transgenic mouse model to represent IBC because of the disease heterogeneity. The existing used models to study the biology of the disease are categorized into two groups: in-vivo xenograft mice and in-vitro cell lines. Examples from each category will be briefly described in the following sections.

2.1.1 In-Vivo Models

Mary X is the first human transplantable IBC xenograft established in SCID/nude mice that grew exclusively within the murine lymphatic and blood vessels resembling its human counterpart [27]. It also demonstrated striking erythema of the overlying skin and at the molecular level it was negative for estrogen, progesterone, Her-2/neu hormonal receptors and positive for EGFR and p53 [27]. One of its interesting properties compared to the non-IBC xenografts is that it overexpressed E-cadherin and MUC1 consistent with what is presented in human IBC cases [27].

Shirakawa et al subsequently published the characteristics of their newly established xenograft model WIBC-9, further describing the impact of angiogenic and non-angiogenic pathways on the aggressive behavior of IBC [28]. Histologically it is characterized by an invasive ductal carcinoma with a hyper-vascular structure of solid nests and noticeable lymphatic permeation in the overlying dermis. Absence of endothelial cells, central necrosis, and fibrosis were detected in the central part of these solid nests**.** At the molecular level, WIBC-9 presented aneuploidy, *ErbB-2* gene amplification, and negative estrogen and progesterone receptors [28].

MDA-IBC-3 is the latest described mouse IBC xenograft that was generated in 2008 by using cells isolated from a primary IBC patient's pleural effusion [29]. The tumor cells were selected over nine serial transplantations of xenograft pieces into the mammary fat pad of SCID/Beige immunocompromised mice and tumor tissues were then digested and passaged *in vitro* in monolayer and 3D cultures, thereby generating a new IBC cell line [29]. This model grossly presents a skin erythema with loss of fur on the overlying skin [29]. Histologically, the tumor cells invade adjacent skin and muscle without an observable dermal lymphatic invasion or metastatic

disease [29], which may not entirely reflect the human disease. At the molecular level, the *in vitro* cell line generated from this model is negative for estrogen and progesterone receptors and positive for HER2, p53 and E-cadherin expression [29].

2.1.2 In-Vitro Models

Several IBC cell lines are available to be studied that were generated either from the primary tumor or from metastatic sites, examples include: KPL-4, SUM149PT, SUM190PT, and SUM1315MO2. However, after an application of 79-gene classifiers on a collection of seven IBC cell lines obtained through the World IBC Consortium, only three of them (SUM149, KPL4, and FC-IBC-01) were consistently classified as IBC specific [30]. As such, this significantly limits the tools to study the biology of this disease.

2.2 Immune Aspects Of IBC

Inflammatory Breast Cancer characterized by the tumor emboli broadly invading the lymphatic vessels, suggests that the patient's immune system is corrupted or that the tumor cells have acquired an ability to evade its surveillance [3]. Although the aggressiveness of this disease is well established, little is known about the behavior of IBC patient's immune system during the disease progression except that the IBC patients are immune competent, at least until the development of metastatic disease [3]. According to the most recent data, IBC patients with locally advanced disease are immunocompetent, as supported by their peripheral blood T-lymphocyte subsets normal distribution and function [3]. Whereas, the T-lymphocyte phenotype of metastatic IBC patients has been switched from Th1 to Th2 with a declined T-lymphocyte response profile, as demonstrated by a cytokine switch [3]. Also, Bertucci et al were able to demonstrate that IBC samples were enriched for both adaptive (CD8+ T-cell lymphocyte activation) and innate immunity genes. [31]. It has yet to be determined if the immune defects observed in metastatic IBC patients are due to the disease itself or due to the metastasis progression. Also these patients are likely to be more aggressively treated than the LABC patients, which might affect the immune system evaluation [3]. Therefore, detailed studies are still needed to clarify the inflammatory relation and its effect on the IBC patient's immune system at earlier stages of the disease.

2.3 Emerging Concepts (EMT, CSC) And IBC

Epithelial- mesenchymal transition (EMT) is an important physiological process in development, wound healing and stem cell behavior, and pathologically it contributes to fibrosis, cancer progression, cell invasion and metastasis [32]. Signaling pathways that are responding to external cues such as transforming growth factor-β (TGF-β) family signaling are controlling the gene expression required for reprogramming during EMT [32]. E-cadherin down- regulation balanced with N-cadherin (mesenchymal neural cadherin) overexpression, accepted as a 'cadherin switch' is an EMT hallmark [32]. Through which, the transformed cells are losing their association with epithelial cells and they develop an affinity for mesenchymal cells by homotypic N-cadherin interactions [32]. These types of interactions are generally weaker than homotypic E-cadherin ones facilitating cell migration and invasion [32]. It is likely that cancer epithelial cells undergo an EMT process prior to their leaking into the peripheral circulation as Cancer Circulating Cells (CTC) searching for a new niche. By their relative resistance to chemotherapy and radiotherapy, CTCs are suggested to mediate tumor metastasis by interacting with their new home that contains different cell types and ECM components [33]. They are considered as independent prognostic factors for progression-free survival (PFS) and overall survival (OS) in metastatic breast cancer patients [34].

Surprisingly, although IBC is characterized by its aggressive behavior, E- cadherin was shown to be overexpressed within the IBC tumor emboli and infiltrating tumor cells [30,35,36]. Additionally, TGF-β signaling pathway was exclusively attenuated in IBC samples by analyzing three different affymetrix gene expression datasets including 137 IBC and 252 NIBC patients' samples [30]. Therefore, scientists have suggested EMT to be far from being the main character promoting tumor cell migration and invasion in IBC [30]. However, these observations don't exclude the possibility of a switch into Mesenchymal- Epithelial Transition (MET). As it is well established that the EMT-MET flexible switch can occur within the primary tumor itself under the effect of miRNA promoting future metastasis [37]. Another indication that EMT might be the aggressiveness drive, few studies have detected CTCs in IBC patients' samples and discussed their impact to be used as IBC prognostic factors. For example, Pierga et al, 2015 has assessed CTC counts at four stages during a phase II clinical trial to explore the efficacy of combining neoadjuvant chemotherapy with bevacizumab and trastuzumab for the treatment of HER2-positive subset of patients [38]. Their study revealed CTC detection to predict a 3-year disease free survival [38]. In agreement with that, CTC counts were found to correlate with the patient's poor

prognosis in Mego et al's retrospective study, which included 147 newly diagnosed IBC patients [34]. Further investigations of the molecular events that govern the disease phenotype are urgently needed in order to understand the disease biology.

2.4 Major Inflammatory Signaling Pathways Involved In IBC

2.4.1 Cancer And Inflammation

The first connection between inflammation and cancer was reported back in 1863 when Rudolf Virchow noted leucocytes within neoplastic tissues [39]. He then suggested that these "lymphoreticular infiltrates" might be the origin of cancer in sites of chronic inflammation [39]. Recent epidemiological findings revived this concept which showed that chronic infections were key risk factors for several types of cancer [40]. They are connected to 15–20% of all cancer deaths; for example, hepatitis B virus (HBV) and hepatitis C virus (HCV) chronic infections are the foremost risk factors for hepatocellular carcinoma (HCC), and *Helicobacter pylori* infections are linked to gastric cancer [40]. Presently, the role of inflammation in tumorigenesis is typically accepted, where the inflammatory microenvironment is an important component of cancer malignancy that may be considered as the seventh hallmark of cancer [41].

2.4.2 NF-κB Signaling And IBC

The NF-κB pathway has an important and evolutionarily conserved role in regulating the immune system by adjusting the expression of inducer and effector transcription factors at different stages defining specific responses to external stimuli [42]. Though its effect is not limited to the immune response per se, it extends influencing gene expression events which impact cell survival, differentiation, and proliferation through transcriptional regulation [42]. The NF-κB transcription factor family include: Rel-A (p65), Rel-B, c-Rel, p50 (p105 precursor), p52 (p100 precursor), and Relish which are all retained in the cytoplasm as dimers bound to specific inhibitors (IκBs) before being activated by external stimuli (Figure 2) [40,43].

Once recognized by receptors and transmitted into the cell, the adaptor proteins initiate a signaling cascade and cause IκB kinase (IKK) activation [45]. This in turn phosphorylates the

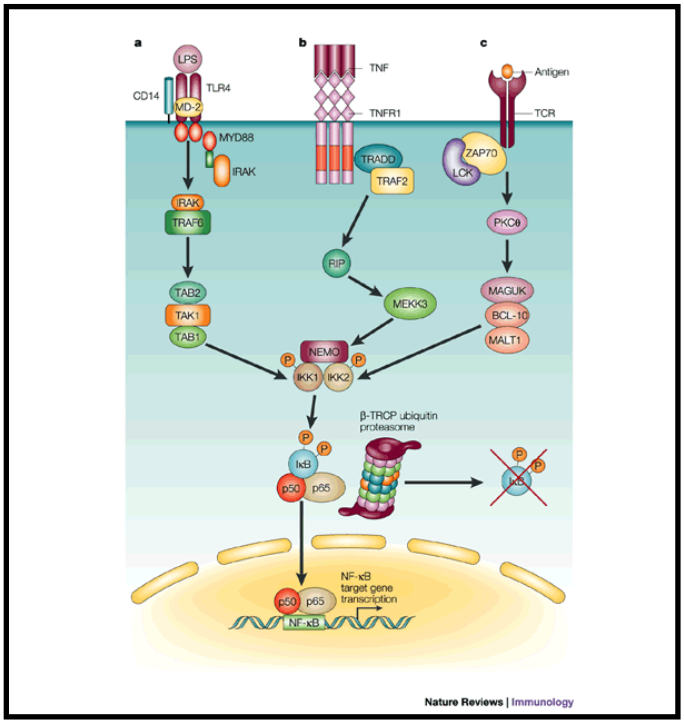

Figure 3: NF-kappa B Pathway Components And Regulation.
Adapted by permission from Li et al, 2002 [2].

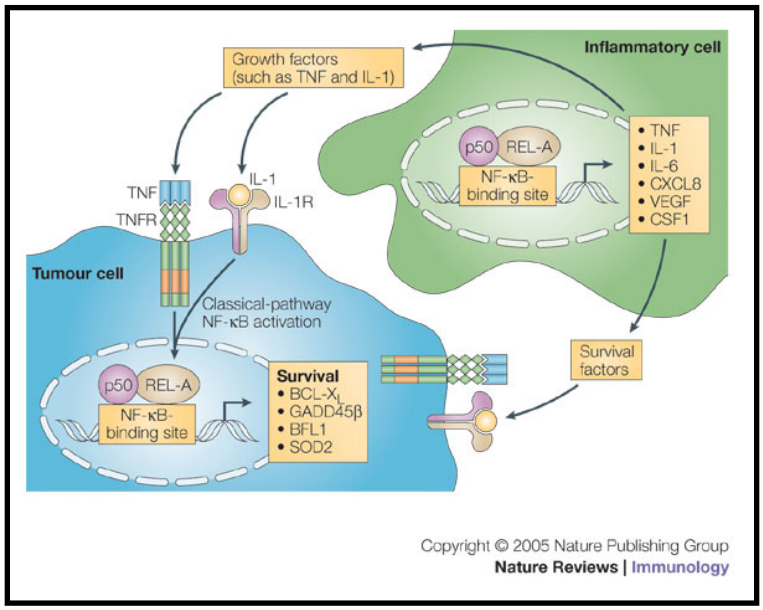

Figure 2: NF-kappa B Is The Link Between Inflammation And Cancer Progression.
Adapted by permission from Karin et al, 2005 [1].

inhibitory IκB subunit marking it by polyubiquitins for proteasomal degradation [45]. Thereby resulting in the release of the NF-κB dimers for nuclear translocation and activation of gene transcription via binding to target sequences [45].

The NF-κB pathway provides the molecular mechanism linking inflammation to cancer (Figure 3) [40]. It is an important factor enabling pre-neoplastic and malignant cells to resist the apoptotic tumor-surveillance mechanisms and it may also regulate tumor angiogenesis and invasiveness [40]. Among all pro-inflammatory signaling pathways, NF-κB is currently well accepted to be the most important component of the tumor-promoting machinery [40]. Many studies have shown the NF-κB pathway to be actively involved in IBC molecular signaling. Based on a previous in-depth cDNA microarray analysis, which identified a high number of NF-κB target genes to be highly expressed exclusively in IBC, Van Laere et al hypothesized the contribution of NF-κB to correlate with the aggressive phenotype of the disease [44]. Accordingly through Immunohistochemical, NF-κB DNA-binding, as well as quantitative RT-PCR studies they were able to validate this overexpression, which was confirmed for all eight NF-κB -target genes tested. Interestingly, these genes were significantly elevated in ER- compared to ER+ breast tumors [44]. Moreover, examination of the estrogen and EGFR receptor status on the NF-κB signaling demonstrated an inverse correlation noticed between the ER and NF-κB activation due to EGFR and/ or ErbB2 overexpression reflecting the MAPK signature [45]. After being considered a hallmark of IBC, the activation of NF-κB/ p65 was used by El-Shinawi et al to further study this disease in the context of the Human Cytomegalovirus (HCMV) infections [46]. First, they screened for anti-CMV IgG antibodies in the blood of 28 IBC patients versus 49 non-IBC invasive ductal carcinoma (IDC) cases [46]. Next, they performed a screening for HCMV-DNA in postsurgical breast tissues from non-IBC and IBC patients [46]. Moreover, they tested the potential of HCMV infection to modulate the expression NF-κB/ p65 [46]. As such they found that, IBC patients have a statistically significant increase in HCMV IgG antibody titers, compared to the non-IBC patients. Moreover, although viral DNA was detected in all cancer tissues IBC cancer tissues had significantly higher levels than in IDC tissues [46]. Furthermore, IBC patient tissues were characterized by containing various HCMV strains with enhanced NF-κB/ p65 signaling activation in comparison to NIBC specimens [46]. Therefore, a deeper understanding of this pathway's link to IBC aggressive phenotype is of a main interest and demand in order to find significant molecular targets advancing the therapeutic management of this disease.

2.4.3 The Case Of Focal Adhesion And TNF Signaling

Focal Adhesion Kinase (FAK) was linked to the NF-κB pathway activation and cytokine production back in 2003 when Funakoshi-Tago and her colleagues investigated this molecule from an inflammatory perspective [47]. FAK is a non-receptor cytoplasmic protein tyrosine kinase associated functionally to trans-membrane receptors and many nuclear protein complexes [48]. This protein is at the intersection of various signaling pathways modulated by integrin and growth factor receptors, capable of regulating different cellular functions through kinase-dependent and independent mechanisms [49]. This 125 kDa non-receptor protein kinase is encoded by the *PTK2* gene within the chromosomal region 8q24.3, and is (regulated) activated and repressed by NF-κB and p53 transcription factors respectively [48]. It is activated by a wide range of extracellular matrix (ECM) ligands that activates integrin such as fibronectin, as well as by a number of growth factors receptors [48] and cytokines [47]. It co-localizes with integrins at Focal Adhesions (FA), which are sites of contact between cells and ECM [50,51].

To understand FAK's biological functions, the molecular mechanisms by which it associates to different molecules must be appreciated. FAK is composed of a FERM (band 4.1-ezrin-radixin-moesin) domain in its N- terminus, followed by a central kinase region, Proline- rich domains, and a focal adhesion-targeting domain in the C-terminal [48]. FAK activation is best portrayed as that upon the cellular engagement of the extracellular matrix (ECM) proteins, integrin receptor is clustered and probably causing FAK molecule conformational changes leading to its release from auto-inhibited state. This triggers FAK auto-phosphorylation at Y397. Src-family kinases binding to this phosphorylated site, promotes phosphorylation of additional FAK tyrosines, including Y407, Y576, Y577, Y861, and Y925 and ends by an activated FAK-Src complex formation (Figure 4) [48]. FAK phosphorylation promotes multiple interactions with partners, including paxillin, p130Cas, Grb2, Grb7, Nck-2, the p85 subunit of phosphatidylinositol 3-kinase, phospholipase Cγ, MAPKs, and p120Rho-GAP [52] and members of the Rho GTPase family [53]. These multiple interactions mediate Focal Adhesion signaling and are required for cell-ECM interaction, survival, and cell migration among other mechanisms [69].

FAK is well documented to promote tumor progression and metastasis by affecting the cancer cells and their microenvironment, as well as being activated and overexpressed in many

advanced solid cancers [48]. According to large databases, about 37% of serous ovarian tumors and 26% of invasive breast cancers are presenting elevated FAK mRNA levels corresponding to patient's poor overall survival [48]. Several studies have provided insights on FAK's link to cancer; from a breast cancer point of view for instance, FAK activity was shown to increase MMP-9 expression, which facilitates matrix invasion by motile cells and consequently promotes spontaneous breast tumor metastasis in syngeneic and orthotopic mice models [48,54]. Correspondingly, the FAK deletion in PyMT mouse breast cancer model caused a decline in the activity of Src-mediated p130Cas and its ERK signaling [55]. Moreover, *Ex vivo* knockout and transient reconstitution experiments in the PyMT isolated cells demonstrated the importance of FAK activity in promoting tumor cells proliferation and anoikis resistance [56].

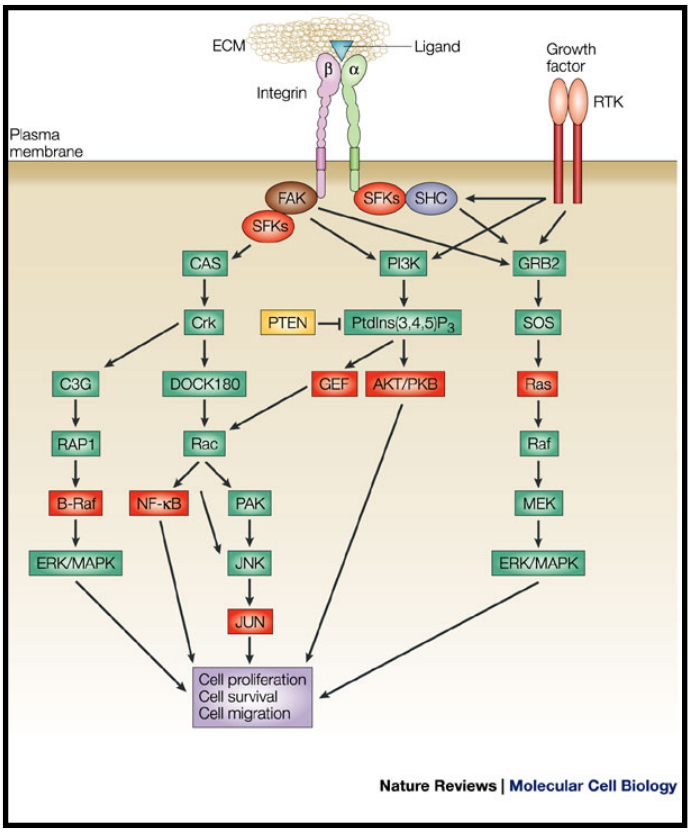

Figure 4: FAK Is Involved In Multiple Interrelated Signaling Pathways.
Adapted by permission from Guo et al, 2004 [2].

Other studies have demonstrated FAK's role in preserving the characteristics of cancer stem cells or progenitor cells via kinase-dependent and -independent mechanisms that in consequence facilitate tumor growth. One study where FAK was conditionally deleted in embryonic MMTV-PyMT mice, mammosphere number and size were significantly reduced together with the mammary cancer stem cells (MaCSC) surface markers [57]. Similarly, conditional FAK-Kinase dead knock-in model reduced MaCSC number and impaired the proliferation of luminal progenitors, without affecting FAK scaffolding functions needed for basal mammary stem cell self-renewal [49]. However, in another study FAK inhibition in basal-like breast carcinoma grafts such as murine 4T1 and human MDA-MB-231, prevented their orthotopic tumor growth and spontaneous metastasis [58].

FAK activity also contributes to another aspect of tumor progression namely the tumor microenvironment [48]. For example, the neutrophils capability to kill pathogens was abolished in myeloid-specific FAK-KO mice that further triggered accelerated spontaneous cell death [59]. Another study used a similar FAK-KO model studying the effect on macrophage function, which revealed impairment in the primary macrophages directional chemotaxis *in vitro* accompanied by decreased monocyte recruitment to inflammatory sites *in vivo* [60]. Presently several ongoing clinical trials are using small molecule FAK inhibitors that possess initial clinical activity in patients to decrease tumor growth and metastasis [48].

Further analysis of FAK's critical role in NF-κB pathway activation revealed the important role of TRAF2 in this pathway. [47]. Funakoshi-Tago et al paper looked at FAK's impact on TNF induced NF-κB activity using FAK proficient and deficient ($FAK^{+/+}$ and $FAK^{-/-}$) mouse embryonic fibroblast (MEF) cells [47]. Although the expression of the pathway components such as TNFRI, RIP, TRAF2, or IKK were unchanged in $FAK^{-/-}$ cells, the RIP molecule failed to recruit the IKK complex and caused NF-κB inactivation [47]. This observation led them to conclude that FAK is responsible to promote the physical association between RIP, TRAF2, and IKKγ proteins in the activation process [47].

2.4.4 TRAF Signalling

TRAF2 is a member of the adaptor proteins in the NF-κB pathway that function (after TNFR is activated) by forming multimeric complexes with multiple intracellular proteins including: CIAP1, RIPK, TANK and TAK1 to initiate a kinase cascade ending by NF-κB and c-Jun N-

terminal kinase activation [61]. Moreover, TRAF2 can be coupled to the Fn14 (fibroblast growth factor-inducible 14) receptor, a member of the TNF receptor family. Fn14 receptor is organized into a cysteine-rich extracellular domain and a short cytoplasmic region containing a TNF receptor-associated factor (TRAF)-binding motif [62]. The Fn14 receptor is activated by the ligand tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) [63] that binds to Fn14 to stimulate canonical and non-canonical NF-κB signaling pathways mediated by IκBα phosphorylation and p100 processing via TRAF molecules [64] and also stimulates MAPKs [65].

A role of TRAF2 in cancer has been suggested in several studies including Thomas et al's paper, which showed that TRAF2 is constitutively phosphorylated at Ser-55 in some cancer cell lines including: prostate, breast, lung cancers, melanoma, as well as Hodgkin's lymphoma cell lines. This phosphorylation was demonstrated to be responsible for the cancer cellular resistance towards stress- induced apoptosis [66]. In line with those observations, Blackwell et al, were able to demonstrate that TNF-α and/or cellular stress-induced TRAF2 phosphorylation at serine 11 is required for the expression of certain NF-κB target genes [67], consequently responsible for the increased basal NF-κB activity levels in certain human cancers [67]. Concurringly, when used with BRAF V600E mutant melanoma cell lines, TRAF2 was shown to facilitate the cells resistance to mitogen-activated protein kinase pathway inhibitors [68]. Bivona et al also identified TRAF2 as a substrate for the breast oncogene IKKε due to NF-κB activation [69]. A recurrent amplification at the focal chromosomal region 9q34 that encompasses TRAF2 locus was later identified in many human epithelial cancers including breast, lung, colorectal, gastric, melanoma, ovarian and esophageal cancer tissue samples and cell lines [61].Their findings were further validated by performing a parallel analysis with the Cancer Cell Line Encyclopedia (CCLE) and The Cancer Genome Atlas (TCGA) data sets for 9q34 copy number and transcript levels [61]. This lead them to suggest TRAF2 as an oncogene promoting NF-κB pathway activation particularly in human cancers harboring 9q34 amplification [61]. However, the role of the TRAF2 protein in promoting the inflammatory response in cancers has to be further elucidated.

CHAPTER 2: HYPOTHESIS AND RESEARCH AIMS

1. Hypothesis And Rationale

The fibroblast growth factor-inducible 14 receptor (Fn-14) and its ligand "tumor necrosis factor-like weak inducer of apoptosis" (TWEAK) are important regulators of inflammatory response via coupling cytokine to intracellular inflammatory and survival cell signaling, in particular activation of the NF-κB pathway [70]. Constitutive activation of NF-κB signaling is also seen in cells overexpressing the focal adhesion kinase (FAK) [71], a kinase and an adapter protein hyper-activated in many cancer types and established to play a role in cell migration and cell survival. Both FAK and Fn-14 are induced by components of the ECM [72], mechanical stress [73], and wound healing [74]. Fn-14 has no enzymatic activity but transmit signals through recruitment of the TRAF ring finger adapter proteins. In this study, we hypothesized that FAK and FN-14-TRAF2 cooperate in bridging inflammatory and cell invasion signaling to promote IBC cell survival and progression to metastasis.

2. Specific Aims

1. To investigate TRAF and FAK expression in breast cancer tissue databases.
2. To investigate FAK and TRAF2 interaction in genetically modified mouse embryonic fibroblast models and established inflammatory and non-inflammatory human breast cancer cell lines.
3. To investigate the impact of FAK-TRAF interaction on downstream mechanisms and on cell phenotypes.

CHAPTER 3: MATERIALS AND METHODS

Reagents And Antibodies

The mouse FAK plasmid was a kind gift provided by Dr. D. Schlaepfer [75]. The cDNA encoding FAK residues 231–1,538 and residues 2,281–3,378 were amplified by PCR from a human osteosarcoma cDNA library. Both fragments were subcloned into the BglII–ApaI sites of the pEGFP-N2 expression vector (CLONTECH Laboratories, Inc.). His-FAK was cloned from pBabe-GFP FAK-WT into pcDNA3.1/His A plasmid. pEBG-TRAF2 (GST) was from Addgene (plasmid 21586; initially from Dr. John M. Kyriakis [76]). NF-κB-Luc (reporter plasmid) and control CMVβ-galactosidase plasmid were kind gifts from Dr. Lin Rongtuan and Dr. John Hiscott [77].

Antibodies against Fn-14 (#4403) and TRAF2 (C192) were from Cell Signaling Technology, Inc. (Danvers, MA 01923); Antibody against FAK (clone 4.47, 05-537) was from Upstate (Millipore, Darmstadt, Germany); and FAK-pY397 were from Biosource International (Camarillo, CA); Antibody to Src (Clone GD11) was from Millipore (Billerica, MA); TRAF2 (sc-136999) and GST (Z-5, sc-459) antibodies and Protein A/G PLUS-Agarose (sc-2003) were from Santa Cruz Biotechnology (Santa Cruz, CA); Antibodies to GFP and GAPDH were from Roche Diagnostics (Mannheim, Germany) and Sigma-Aldrich (Mo 63103, USA), respectively; antibody to α-Tubulin was from Abcam Inc. (Cambridge, MA); Recombinant TWEAK and TNF-α were from R&D Systems, Inc. (Minneapolis, MN 55413 USA). Cycloheximide (C1988) and MG132 were from Sigma-Aldrich, Cayman Chemicals Company (Michigan 48108, USA) and Calbiochem (Millipore, MA, USA), respectively; Fibronectin was from Sigma-Aldrich; leupeptin and PMSF are from Bioshop Canada. Secondary antibodies for immunofluorescence were from Jackson ImmunoResearch (West Grove, PA).

Cell Culture

FAK deficient and proficient mouse embryonic fibroblast ($FAK^{-/-}$ or $FAK^{+/+}$ MEF) cells were provided kindly by Dr. D. Ilic (University of California, San Francisco, CA) [78], that were cultured in DMEM (Life Technologies) supplemented with 10% FBS, 1 mM sodium pyruvate, 1% (Vol./Vol.) nonessential amino acids, 100 μM 2-mercaptoethanol, and

penicillin/streptomycin. TRAF2 deficient and proficient mouse embryonic fibroblast ($TRAF2^{-/-}$ or $TRAF2^{+/+}$ MEF) cells were kind gifts from Dr. Tak W. Mak [79] (University of Toronto, Toronto, Ontario, CA), which were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acids and penicillin/streptomycin. MDA-MB-231 cells and HEK 293T cells were obtained from the American Type Culture Collection. The MDA-MB-231 cell variant MDA-231-M2 was established from a metastatic lung nodule induced in vivo by implantation of MDA-MB-231-ErbB2 cells into the mammary fat pad of SCID mice [80,81]. SYF and reconstituted SYF cells were from ATCC. All these cells were maintained in RPMI 1640 (Mediatech, Washington, DC) or DMEM supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin antibiotics. IBC cell lines: SUM149PT and SUM190PT were from Asterand maintained in HAM's F-12 medium supplemented with special components according to the manufacturer's instructions. Immortalized human mammary epithelial cells hTERT-HMEC-1 were purchased from ATCC and maintained in MEBM media along with all the additives obtained from Lonza. The breast adenocarcinoma cell lines MDA-231-AP2 and MDA-231-ErbB2 were described previously [80].

Knockdown Of FAK, TRAF2, And Fn-14 In Mouse And Human Cells

Mouse FAK siRNA (sc-35353) and TRAF2 siRNA (sc-36711) were from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were seeded into 6 wells plate, 24 hours later (about 60% confluent) followed by treatment with siRNA in final concentration of 100nM. 48hours later cells were used to analyze siRNA knockdown efficiency. For Stable Fn-14 knockdown on MDA231 cells were generated in a polycolonal population as described previously [80] Sequence GAGGGAGAATTTATTAATAAA targeting human Fn-14 gene 995-2015 was cloned as inverted repeats into pSuper-retro puromycin vector according to the manufacturer's instructions (Oligoengine).

Generation Of Cells Expressing Stable Or Transient siRNA

A specific 19-nt sequence spanning positions 466–484 of FAK human gene (GenBank/EMBL/DDBJ accession no. L10616) was cloned as inverted repeats into pSuper-retro puromycin vector according to the manufacturer's instructions (Oligoengine). Control retroviral

vector pRetro-Super puromycin alone or expressing FAK siRNA was transfected into Phoenix cells using Genejuice (Novagene). After 48 hours after transfection, the supernatant of Phoenix cells was filtered through a 0.45 μm filter and was used to infect target cell lines twice, 24 hours apart, in the presence of 8 μg/ml polybrene. 48 hours after infection, polyclonal populations were selected for resistance to 1 μg/ml puromycin for 2 weeks to generate stable siRNA-expressing cells and matched (bulk) controls. For transient siRNA oligos (Dharmacon Inc., Lafayette, CO) (100 nM) were incubated with DharmaFECT1 in Opti-MEM I Reduced-Serum Medium according to the manufacturer's instructions. After 5 hours of transfection, the cells were incubated with fresh complete medium at 37^{o}C. The cells were harvested 24 and 48 hours later for transfection efficiency analysis and experiments respectively.

Cell Proliferation Assay

Exponentially growing cells ($1x10^3$) were seeded in 96 well plates and incubated for 72 hours. Cell survival was evaluated by cell counting and using the MTT metabolic assay by removing culture media and replacing with fresh media containing 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dissolved in PBS. Following 3-4 hours incubation at 37^{o}C, the medium containing MTT was removed and 200 μL dimethyl sulfoxide (DMSO) was added to dissolve the insoluble reduced MTT formazan precipitate into a colored solution, followed by 25 μL of glycine/NaCl buffer (0.1 M glycine, 0.1 M NaCl pH 10.5). Once the formazan crystals dissolved, the absorbance was determined at 570 nm using a microplate reader. As yellow MTT is reduced to purple formazan in the mitochondria of living cells, the assay allows discrimination between viable and non-viable cells.

Western Blot Analysis

Western blotting analyses were carried out on total cell extracts from exponentially growing cells collected by scrapping into modified radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 20 mg/ml pepstatin A, 1 mM PMSF and Protease Inhibitor Cocktail (Roche) as described earlier [77]. Blots were detected using appropriate antibodies and signal detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence

(ECL) detection system. When indicated, membranes were subsequently stripped for re-probing. Immunoblots were quantified by using NIH image software.

Immunoprecipitation Assay

Cells were grown in 15-cm dishes (for FAK EMFs, TRAF2 EMFs endogenous immunoprecipitation) or 10-cm dishes (for HEK293T, overexpression proteins immunoprecipitation) to nearly confluent. After washing three times with PBS, cells were lysed with buffer N150 (20 mM sodium phosphate [pH 7.0], 0.15 M NaCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, plus Roche complete protease inhibitor) then sonicated on ice for 10 seconds per tube with Sonic Dismembrator (Fisher Scientific, Model 500). The slurry was centrifuged at 15,000 rpm for 30 minutes at 4°C and the soluble supernatant incubated with 100 μl Protein A/G PLUS-Agarose (sc-2003) three hours for pre-cleaning. The pre-cleaned supernatant was then transferred to new eppendorf tube and added indicated antibodies to rotate overnight at 4°C. After 30 minutes centrifuge at 15,000rpm, 20μl Protein A/G PLUS-Agarose was added to each tube rotate three hours at 4°C, followed by spinning down the tubes at 15,000 rpm for one minute. The agarose beads were then washed one time with N150 buffer and three times with N300 (the same as N150 except that the NaCl concentration was 0.3M) followed by SDS-PAGE GEL separation.

Immunofluorescence Microscopy

Immunofluorescence assay was performed as described previously [80]. Briefly, cells were seeded on coverslips and fixed with paraformaldehyde, blocked, then incubated with the following primary antibodies: TRAF2 polyclonal antibody (Cell Signaling Technology, Inc. C192; 1:100) and monoclonal anti-FAK (clone 4.47; Upstate; 1:200). Cells were next incubated with secondary antibodies conjugated to Cy2 or Texas Red (Jackson Immunoresearch Laboratories, West Grove, PA). After mounting with gelvatol medium (Airvol®205 polyvinyl alcohol, Air products and Chemicals, Inc., Allentown, PA), coverslips were analyzed using a fluorescent microscope (Axiophot; Carl Zeiss MicroImaging, Inc.).

Luciferase Assay For NF- κB Activity

The NF-κB activities were analyzed by luciferase assay as described earlier [81]. Briefly, MEFs were either treated by siRNA or reconstituted with its related plasmid. After seeding into 12-well plates (10^5 cells/well) followed by a transient transfection using LipofectAMINE (Invitrogen, Carlsbad, CA) with 500 ng/well of NF-κB-Luc (reporter plasmid) plus 250 ng/well of CMVβ-galactosidase plasmid (control for transfection efficiency) in serum-free medium according to manufacturer's instructions. After 5 hours of transfection, the cells were incubated with fresh serum-free medium with or without Tweak (10ng/ml, R&D systems). After 24 hours, the cells were rinsed with cold phosphate-buffered saline (PBS), and extracts were collected and assayed for luciferase activity as per Promega protocols by using a Lumat LB9507 luminometer (PerkinElmer Life Sciences, Boston, MA). Luciferase activity was normalized for β-gal activity.

Anoikis Assay

Quantification of anoikis carried out using the CytoSelect 96-well anoikis assay kit from Cell Biolabs, Inc. (San Diego, CA, USA). Briefly, 5×10^4 cells were seeded into each well of either control or pre-coated 96 well plates for 24 hours. After treatment, cell viability was determined by MTT (colorimetric) and Calcein AM (485nm/515nm, fluorimetric) detection. Anoikis propelled cell death was measured by Ethidium homodimer (EthD-1), which can only penetrate damaged cell membranes and fluoresce with a 40-fold enhancement upon binding ssDNA, dsDNA, RNA, oligonucleotides, and triplex DNA. Anoikis percentage indicated by relative immunofluorescence of EthD-1 (525nm/590nm) on anchorage resistant plate compared to its related Calcein AM on control plate.

Migration Assay

For the Scratch motility assay, cells were grown on coverslips were wounded by cell scraping with a micropipette tip. Cultures were washed and incubated in complete medium. Cells were incubated at 37^{o}C for different periods of time allowing migration toward the gap, they were fixed and then cell migration was quantified as previously described [80]. Cell migration was assayed using the qualitative wound-healing assay [68]. Each experiment was performed at least

three times and results were expressed as mean ± SD. Statistical significance was analyzed using student's *t*-test.

Genomic Expression Of FAK And TRAF2 In Breast Cancer Tissues

The University of California-Santa Cruz Cancer Genomics Browser database was used to analyze cancer expression and clinical data related to the *TRAF2* and *PTK2* genes expressions [82]. Gene Expression Omnibus (GEO) public functional genomics database was used to analyze the expression profiling of affymetrix human genome arrays included 48 samples (IBC and invasive non-IBC) in relation to *TRAF2* and *FAK* genes [83].

CHAPTER 4: RESULTS

1. FAK And TRAF2 Genes Are Overexpressed In Breast Cancer Cases

To investigate expression levels of Fn-14, TRAFs, and FAK in breast cancer we analyzed publically available TCGA breast cancer genomic database (University of California- Santa Cruz Cancer Genomics Browser database) for gene expression levels. Previous studies have observed overexpression of FAK [80], Fn-14 [84], and TRAF [85,86] in cancer but very limited information is available for IBC cases and co-overexpression of these markers has not been reported. Our TCGA analysis revealed that *TRAF2* gene is overexpressed in highly aggressive breast cancer subtypes such as triple negative tumors and is mostly absent in normal cases. Interestingly, *PTK2* (FAK) gene levels correlate with *TRAF2* gene expression levels supporting a possible cooperation, particularly in aggressive breast tumors (Figure 5). In IBC co-overexpression was seen in some cases; however, IBC (as noted earlier in introduction section) is a rare disease in North America and Western Europe and therefore the number of cases available for research is very limited and future studies using large cohorts are necessary to draw a firm conclusion (Figure 6).

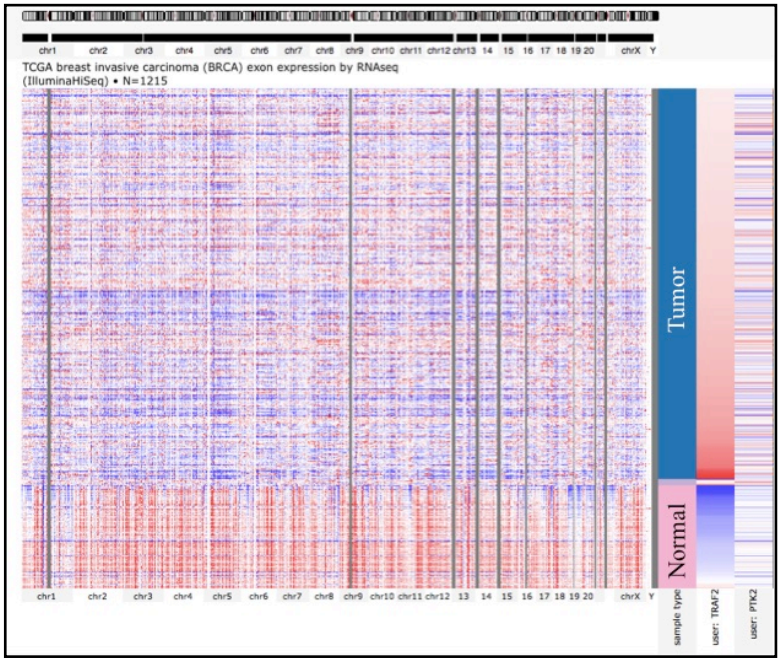

Figure 5: *TRAF2* And FAK (*PTK2*) Genes Signatures In 1215 Cases Samples (Normal Versus Invasive Breast Carcinoma) From The University Of California-Santa Cruz Cancer Genomics Browser.
Each column corresponds to a single sample, and each row corresponds to a biomolecular entity related to the current study. The genomic heat map was organized according to normal samples versus tumor samples. Red color indicates overexpression and blue color indicates low expression. *TRAF2* and *PTK2* genes are mostly overexpressed in tumor samples and absent from normal cases.

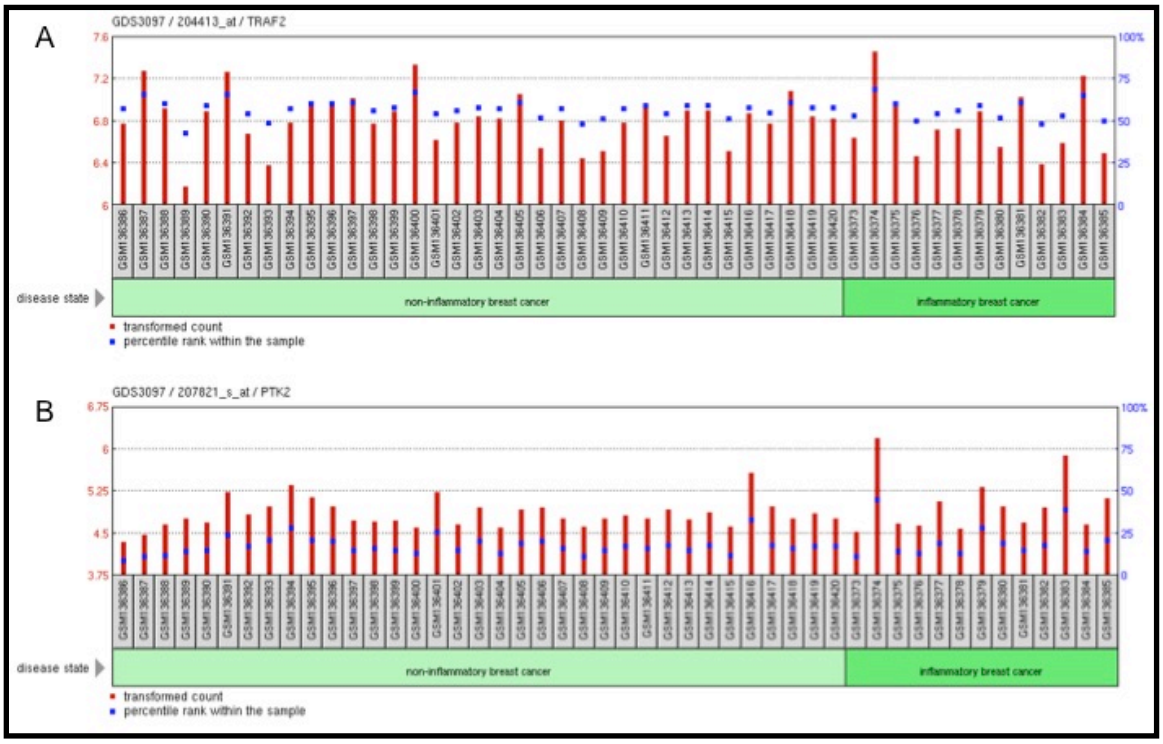

Figure 6: *TRAF2* And FAK (*PTK2*) Genes Signatures In Inflammatory Breast Cancer (IBC) And Non-IBC Patient Cases From GEO (Gene Expression Omnibus Dataset Browser).
Red bars represent the transformed count and blue dots represent gene expression percentile rank within each sample in an Analysis of TRAF2 (A) and PTK2 (B) gene expressions in patient tumor epithelia using affymetrix human genome array.

In IBC cells we investigated TRAF2 protein activity (P-TRAF at Ser11 residue) by Western blotting using a panel of breast cancer cell lines including two primary IBC primary cell lines (SUM149PT and SUM190PT), other non-IBC cells (MCF-7 and MDA-MB-231 overexpressing ErbB2 versus their controls), and normal immortalized human mammary epithelial cells (hTERT- HME1). The results revealed a robust overexpression of activated TRAF2 compared to non-IBC cell lines or normal epithelial cells (Figure 7). These data highlights previous immunohistochemistry studies focusing on non-phosphorylated TRAF to investigate clinical relevance of these proteins.

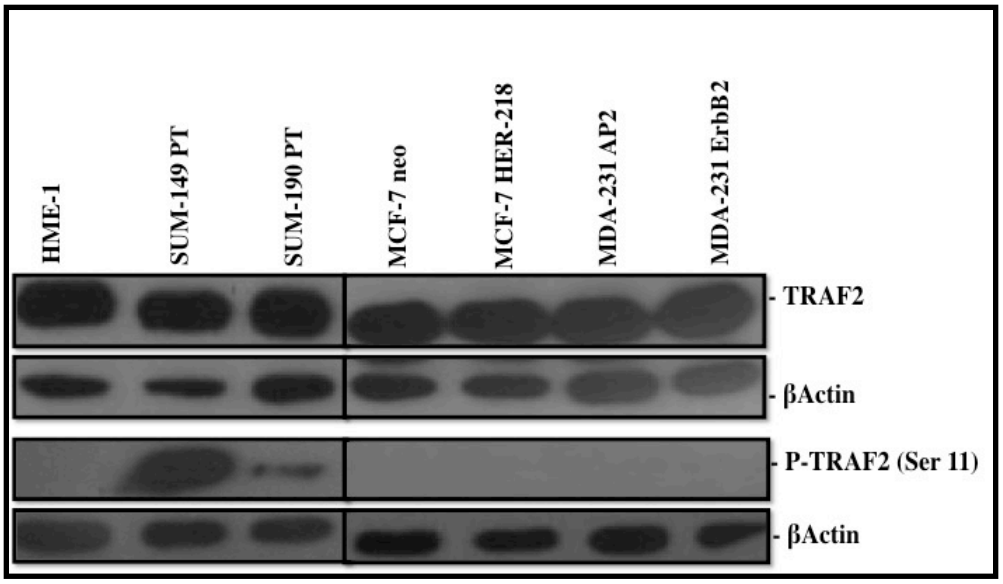

Figure 7: TRAF2 Protein Is Activated In IBC Cells.
Breast cancer cell lines were used including: SUM 149 PT, SUM 190 PT, MCF-7 neo, MCF-7 HER-218, MDA-MB-231 AP2, and MDA-MB-231 ErbB2, in addition to Immortalized human mammary epithelial cells (hTERT- HME1). Soluble lysates from all cells were immunoblotted with the indicated antibodies.

2. TRAF2 Interacts Physically With FAK

We examined the interaction between FAK and TRAF2 using $TRAF2^{+/+}$, and $TRAF2^{-/-}$ and $TRAF2^{-/-}$ reconstituted by GST-TRAF2 plasmid. We observed that FAK antibody was able to immunprecipitate endogenous TRAF2 in $TRAF2^{+/+}$ cells and TRAF2-reconstituted $TRAF2^{-/-}$ but not in $TRAF2^{-/-}$ cells (Figure 8A). To verify this interaction also exists in cancer cells, similar immunoprecipitation was carried out on MDA-MB-231 cell line and as expected Fn-14 interacted with TRAF2 in this cell line. Noticeable, Fn14 was unable to interact with FAK in our cell models (not shown). In a similar manner as for $TRAF2^{+/+}$ MEF cells, FAK immunoprecipitated with TRAF2 when using either FAK or TRAF2 antibody (Figure 8B). Immunofluorescence microscopy also confirmed the colocalization of FAK and TRAF2 in intracellular compartments with predominance in the cytosol and plasma cell protrusions (Figure 8C).

FAK comprises a highly conserved central catalytic domain flanked by large N- and C-terminal non-catalytic domains that contain N-terminal FERM region, proline-rich

residues with binding motifs for Src homology 3 (SH3) domain-containing proteins such as p130Cas (Crk-associated substrate), Graf (GTPase regulator associated with FAK), and Pleckstrin homology (PH) and SH3 domain containing Arf-GAP proteins, along with a focal adhesion-targeting (FAT) domain located in the C-terminus and which is critical for FAK recruitment to Focal Adhesions and for its association with paxillin and talin. To determine which domain contribute to the interaction with TRAF2 and unveil the potential function, we co-transfected GFP tagged FAK full length (WT), N-terminal (NT), C-terminal (CT) or phosphosite 397 mutant (P397) with GST-TRAF2 in HEK 293T cells, anti-GFP antibody was used to perform immunoprecipitate assay. Results show that FAK full-length and N-terminal, but not C-terminal and P397 could interact with TRAF2, which indicates that FAK N-terminal, particularly phosphosite 397 is contributed to the interaction between FAK and TRAF2 (Figure 8D). FAK activation triggers an autophosphorylation of FAK at Tyr397, which forms a binary complex with Src family kinases, promoting Src catalytic domain activation. Subsequently, Src bound to FAK Tyr397 site can phosphorylate additional FAK tyrosines, including Tyr407, Tyr576/Tyr577 in the kinase domain, Tyr861, and Tyr925. Since FAK Try397 is important to the interaction, so we used SYF (deficient for Src, Yes and Fyn) and Src-reconstituted (SYF + c-Src) cells to investigate if FAK activation is necessary for the interaction with TRAF2. The immunoprecipitation assay shows FAK can interact with TRAF2 in SYF + c-Src but not SYF cells, which indicates the interaction is Src dependent (Figure 8E).

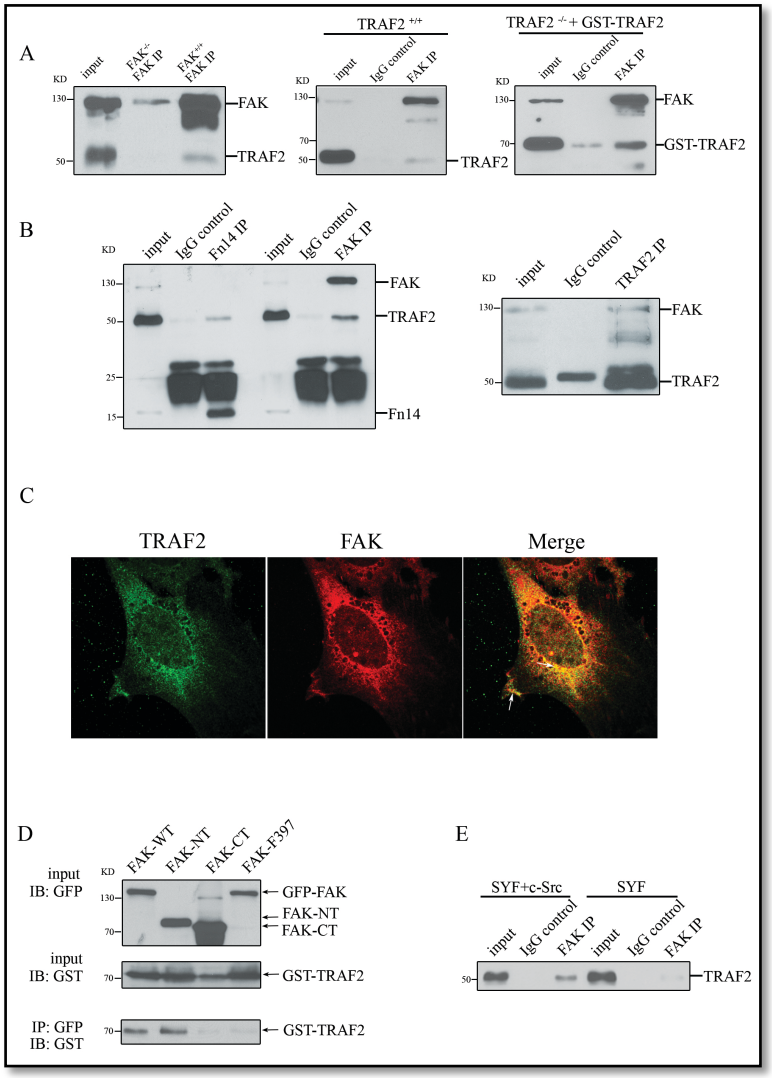

Figure 8: FAK Stabilizes Fn-14 Via Interaction With TRAF2.
A. FAK immunoprecipitation from cell lysates of $FAK^{+/+}$, $FAK^{-/-}$, $TRAF^{+/+}$, $TRAF2^{-/-}$, and GST-TRAF2 reconstituted $TRAF2^{-/-}$ cells reveals FAK to physically interact with both endogenous and exogenously expressed TRAF2. B. FAK immunoprecipitation from cell lysates of the breast cancer MDA 231 cells reveals endogenous FAK to interact with TRAF2 in human cells. Fn-14, which interacts with TRAF2 is shown as a control. C. Immunofluorescence microscopy confirmed FAK colocalization with TRAF2. $FAK^{+/+}$ cells grown on cover slides were fixed, incubated with TRAF2 and FAK antibodies, and then immunostained with secondary antibodies conjugated to Cy2 or Texas Red as described in Methods. Arrows show co-localization of FAK and TRAF2 in cytoplasm and cell protrusions. D. HEK293T cells co-transfected with GFP-tagged full length FAK or FAK mutants lacking the N-terminus (FAK-NT), the C-terminus (FAK-CT) or full length 397-phospho-mutant FAK, and GST-tagged TRAF2 for 48 hours. Total lysates were immunoprecipitated with GFP antibody and probed for GFP or GST (to determine GST-TRAF2) by immunoblotting. Input shows expression levels of GFP-FAK full length, N-terminus FAK, C-terminus FAK, FAK 397 phosphomutant (P397), and GST-TRAF2. E. Cell lysates from SYF (deficient for Src, Yes, and Fyn) and Src-reconstituted (SYF + c-Src) cells were used to immunoprecipitate FAK followed by immunoblotting for TRAF2.

3. FAK Regulates Fn-14 Stability In Isolated Cells

To understand crosstalk between Fn-14 and FAK signalling we investigated the impact of FAK on Fn-14 expression in FAK-deficient ($FAK^{-/-}$) and FAK-proficient ($FAK^{+/+}$) cells. As shown in Figure 9A, basal expression of Fn-14 is reduced in both non-stimulated and Tweak-stimulated $FAK^{-/-}$ cells compared to $FAK^{+/+}$ cells. Cell exposure to CHX for different time points revealed that Fn-14 is more stable in $FAK^{+/+}$ cells compared to $FAK^{-/-}$ cells (obvious at 30 minutes after CHX treatment where Fn-14 completely disappeared 2 hours after CHX treatment while in $FAK^{+/+}$ cells Fn-14 can still be seen even after this incubation time) (Figure 9A). To further establish if Fn-14 stability is FAK related, we reconstituted FAK in $FAK^{-/-}$ cells by expressing a gradient amounts of His-FAK plasmid that showed the Fn-14 basal expression can be restored in FAK reconstituted $FAK^{-/-}$ cells with expression levels correlated with the added plasmid amounts (Figure 9C). Similar result was observed in the MDA-MB-231 cell line when FAK was down regulated using siRNA (Figure 9D).

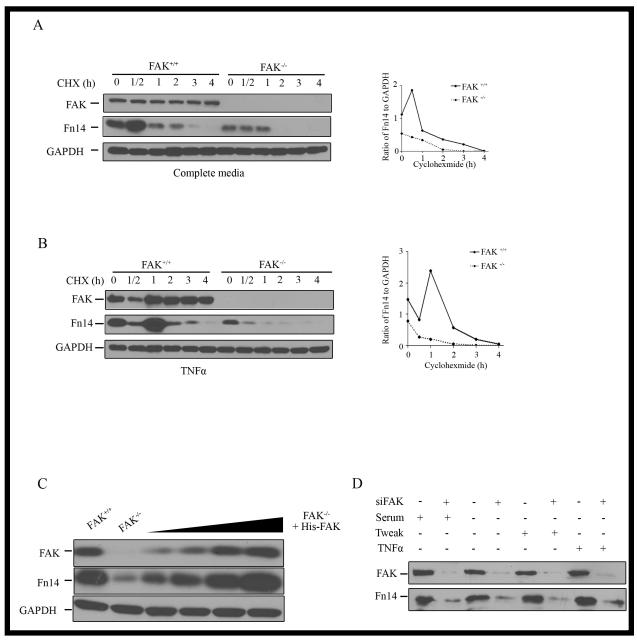

Figure 9: FAK Prevents Fn-14 Degradation.
A. FAK+/+ and FAK-/- MEF cells were cultured in complete media in the absence (0h) or presence of 50µg/ml cycloheximide (CHX) for the indicated time. Total cell lysates were analyzed for FAK and Fn-14 expression by western blotting. GAPDH is used as an internal control. The expression ratio of Fn-14 to GAPDH was indicated on the inserted graph. B. Serum free FAK+/+ and FAK-/- MEF cells stimulated with TNF-α (10ng/ml) in the absence (0h) or presence of 50µg/ml CHX for the indicated time. Total cell lysates were analyzed for FAK and Fn-14 expression by western blotting. GAPDH is used as an internal control. The expression ratio of Fn-14 to GAPDH was indicated on the inserted graph. C. Re-expression of His-FAK at increasing amounts (0.5, 1, 1.5, and 2µg) in FAK-/- cells rescues Fn-14 expression in these cells. D. In the human breast cancer MDA231 cell line, FAK knockdown by siRNA reduced Fn-14 expression under serum supplemented, serum free, or 4h Tweak or TNF-α stimulation.

4. FAK Regulation of Fn-14 Stability Is Proteasome-Dependent

Next we investigated the mechanism by which FAK regulates Fn-14 stability. $FAK^{+/+}$, $FAK^{-/-}$ and FAK-reconstituted $FAK^{-/-}$ cells were pre-treated with 20 μM of MG132 for 1 hour followed by treatment with 50 μg/ml CHX for up to 6 hours. In all these 3 cell types, MG132 blocks the degradation of Fn-14, which remains detectable in MG132-pretreated cells and exposed to CHX for up to 6 hours (Figure 10 A). In contrast to MG132, exposure of cells to Calpain inhibitor ALLN (100μM), PMSF (200μM) or leupeptin (200μg/ml) protease inhibitors followed by 50μg/ml CHX for up to 4 hours had no impact on preventing Fn-14 degradation in $FAK^{+/+}$ versus $FAK^{-/-}$ cells (Figure 10 B). This data supports that Fn-14 degradation is a proteasome-dependent.

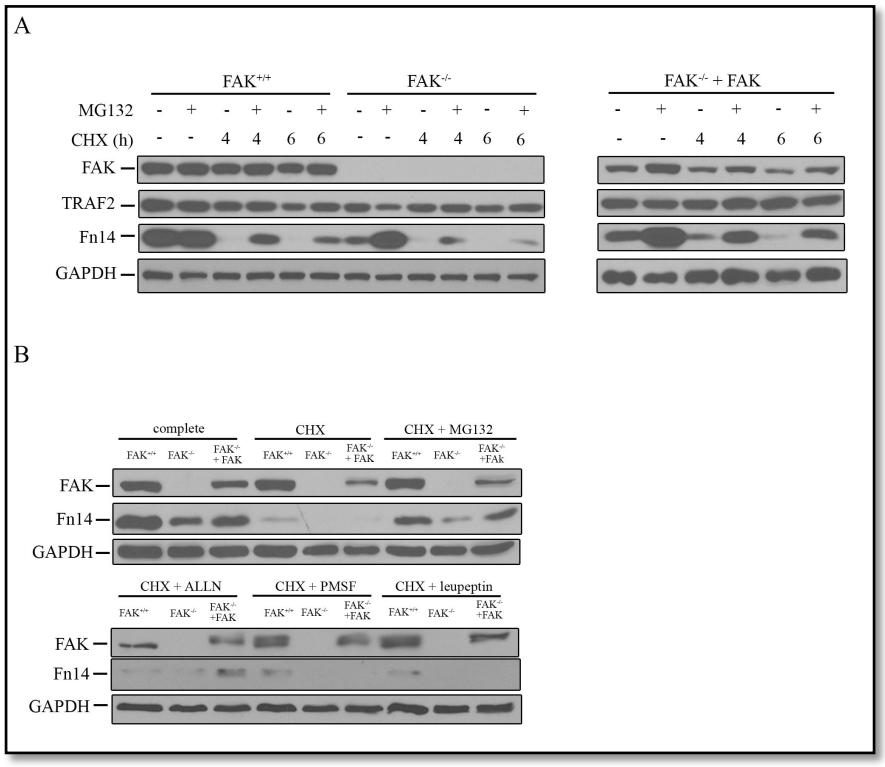

Figure 10: FAK Stabilizes Fn-14 Via A Proteasome-Dependent Mechanism.
A. $FAK^{+/+}$ and $FAK^{-/-}$ cells (left panel), and $FAK^{-/-}$ cells rescued by His-FAK (right panel) were pre-treated with 20μM MG132 for 1 h, followed by treatment with 50μg/ml CHX for 4 or 6 h. Total cell lysates were used to analyse FAK, Fn-14 and TRAF2 expression by western blotting. GAPDH is used as an internal control. B. $FAK^{+/+}$ and $FAK^{-/-}$ cells, and $FAK^{-/-}$ cells rescued by His-FAK were pre-treated for one hour with MG132 (20μM), ALLN (100μM), PMSF (200μM), or leupeptin (200μg/ml) followed by treatment with CHX for 4 h. Cell lysates were used to determine Fn-14 expression by western blotting.

5. Loss Of FAK And Fn-14 Following Tweak Stimulation Attenuates NF-κB

NF-κB signalling pathway plays a key role in cancer proliferation, metastasis. Multiple cytokines secreted due to NF-κB activation can promote cancer cells survival and anti-apoptosis. It is well known TRAF2 plays a key role in NF-κB activation, FAK also contributes NF-κB activation by PI3K/Akt signaling pathway. The activation of NF-κB caused by Tweak is a response of series upstream signal transductions lead to p-IκB activation commanding IκB degradation, which lead to NF-κB activation. Here we used MDA-MB-231 cells to examine changes in NF-κB activities due to FAK and Fn-14 loss by analyzing p-IκBα and IκBα. Tweak treatment for indicated time points (Figure 11 A), accompanied by FAK siRNA knockdown can lead to less active p-IκBα, which causes IκBα to be more stable. In the case of Fn-14, we generated stable knockdown cells for Fn-14 using constructed retroviral shRNA. When treated with Tweak, these cells didn't show a significant increase in p-IκBα expression compared to their controls. Similarly, that caused IκBα to be more stable (Figure 11 B). These results indicate that both FAK and Fn-14 are critical components of tweak induced NF-κB activation.

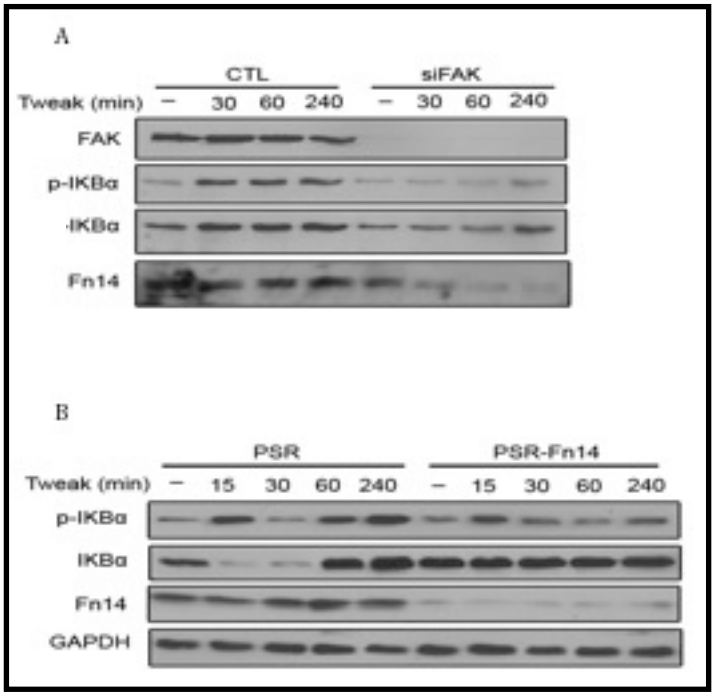

Figure 11: Loss Of FAK And Fn-14 Following Tweak Stimulation Attenuates NF-κB Activity.
FAK or Fn-14 expression was downregulated in MDA-MB-231 cells using FAK siRNA (A) or Fn-14 shRNA (B), respectively. Control cells were transfected with matched empty expression vectors alone. Cells were then treated with Tweak (10ng/ml) at the indicated time, and phospho-IκBα (p-IκBα) and IκBα levels were determined by immunoblotting.

6. FAK And TRAF2 Cooperates For The Regulation Of NF-κB Promoter Activity Measured By The Luciferase Assay

Since FAK interact with TRAF2, we hypothesise that TRAF2 may contribute to the process where FAK promotes the NF-κB activation by Tweak simulation. To evaluate this, we subsequently examined NF-κB activities by luciferase activity assay in TRAF2 and FAK MEF cells. We reconstituted a series of cell lines as indicated in Figure 12, after Tweak (10ng/ml) treatment, luciferase activities were measured and graphed in bars represent fold changes compared to the controls. We found that FAK deficiency can attenuate NF-κB significantly, especially when combined with TRAF2 knockdown ($FAK^{-/-}$ + siTRAF2) that serve cooperatively. TRAF2 deficient cells also show reduced NF-κB activity compared with matched TRAF2 proficient cells, which also show the most attenuation when combined with FAK knockdown ($TRAF2^{-/-}$ + siFAK). These results suggest FAK and TRAF2 cooperation to serve NF-κB activation.

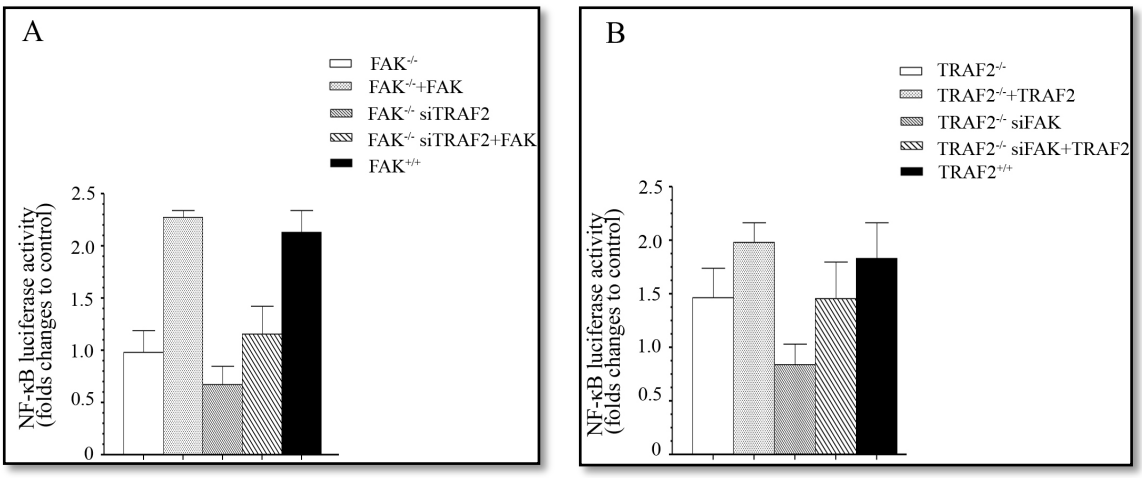

Figure 12: FAK And TRAF2 Cooperates For The Regulation Of NF-κB Promoter Activity Measured By The Luciferase Assay.
FAK (A) or TRAF2 (B) deficient cells and proficient cells where TRAF2 or FAK are down regulated respectively by siRNA, then exposed to Tweak (10ng/ml). The luciferase reporter assay was performed as described in Materials and Methods. The NF-κB activities compared to controls represented as folds change (mean ± SD).

7. FAK And TRAF2 Cooperates To Promote Cell Survival

To determine the function of FAK and TRAF2 in promoting cell death downstream of active NF-κB pathway, we used TRAF2 and FAK MEFs to siRNA down-regulate FAK or TRAF2 respectively, followed by 48 hours stimulation with TNF-α (10 ng/ml). Cells were then

trypsinized and stained by trypan blue. Using hemocytometer, viable cells were counted and the numbers were presented in bar graphs (Figure 13). We found that FAK or TRAF2 deficient MEF showed more cell death triggered by TNF-α. Moreover, TRAF2 knockdown in $FAK^{-/-}$ and FAK knockdown in $TRAF2^{-/-}$ increased TNF-α cellular sensitization. Microscopically, nonviable cells were floating and rounded in shape, with obvious DNA fragment ladders and nuclear segments, which are the typical phenotypes of apoptosis (data not shown).

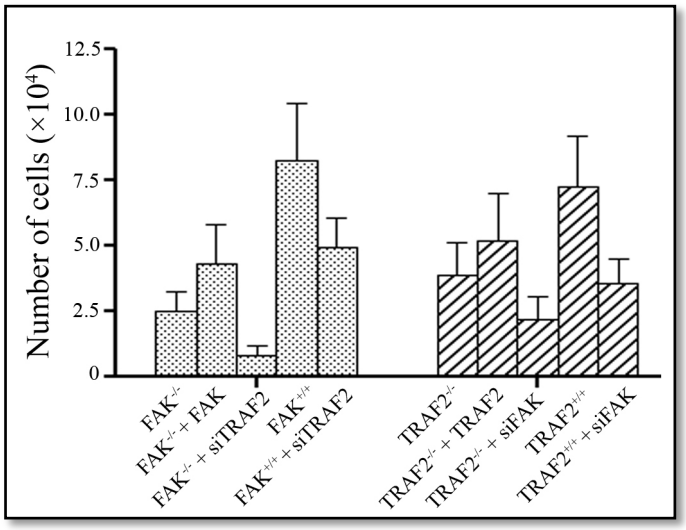

Figure 13: FAK And TRAF2 Cooperate To Promote Cell Survival.

FAK or TRAF2 deficient and proficient mouse embryonic fibroblast (MEF) cells were transiently knockdown by siRNA as indicated. Cells were seeded at 10×10^4 /well into 6 well plates. After serum free staving for 4 h, cells were then treated with 10ng/ml TNFα. 48 h later cells were stained with trypan blue and viable cells counted using haemocytometer.

8. FAK Promotes Cell Survival Via Anti-Anoikis Mechanism

Anoikis is a process of apoptosis in anchorage dependent cells triggered by detachment from ECM [87]. FAK plays a critical role in the formation and maintenance of focal adhesion complexes and endothelial anoikis prevention [88]. To evaluate the cooperative role of FAK and TRAF2 serving the anti-anoikis mechanism, we down-regulated TRAF2 in FAK MEFs and FAK in TRAF2 MEFs respectively. The cells were seeded on control or pre-coated anchorage resistant 96 well plates for 24 hours, and then cells were kept as controls or stimulated with 10 ng/ml Tweak for 24 hours. Cell viability was determined by MTT (colorimetric) and Calcein detection.

Anoikis propelled cell death was measured as described earlier in the methods section. Figure 14 represent percentages of cells undergoing anoikis indicated by relative immunofluorescence of EthD-1 on anchorage resistant plates compare to its related Calcein AM on control plates (*, $P<0.05$). As expected, $FAK^{-/-}$ has a higher percentage of anoikis than $FAK^{+/+}$ in both serum free media (Figure 14 A) and Tweak stimulation media (Figure 14B). FAK knockdown also leaded to a significant percentage of anoikis. Similarly, when tweak stimulated TRAF2 deficient cells showed significant higher percentage of anoikis compared to TRAF2 proficient cells. Also, when TRAF2 is down, more cells undergo anoikis, and with Tweak treatment, the increase is significant (Figure 14 B). In TRAF2 and FAK deficient cells, when combined with down regulation of FAK or TRAF2 respectively, cells have significant increase of anoikis, which indicate FAK and TRAF2 may cooperate in anti-anoikis process.

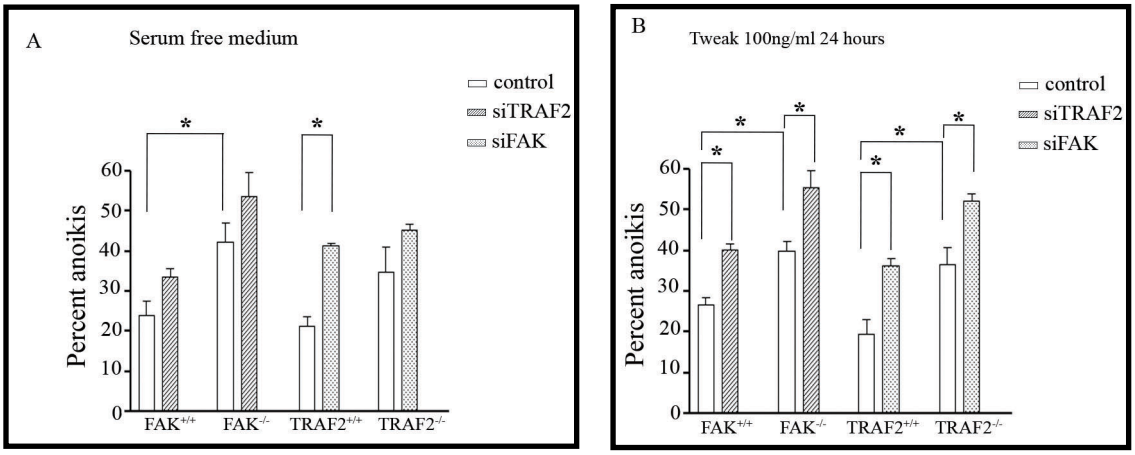

Figure 14: FAK Promotes Cell Survival Via Anti-Anoikis Mechanism.
FAK and TRAF2 were down regulated by siRNA in TRAF2 or FAK EMFs respectively. Cells were seeded into control or anchorage resistant 96 well plates for 24 hours. Cells were kept un-stimulated or stimulated with Tweak (10ng/ml). Anoikis propelled cell death was measured as described in Methods. Percentage of cells undergoing anoikis indicated by relative immunofluorescence of EthD-1 on anchorage resistant plates compare to its related Calcein AM on control plates (*, $P<0.05$).

CHAPTER 5: DISCUSSION

Inflammation-associated signaling has a broad implication for the regulation of tissue homeostasis, wound healing, and immune response. In cancer, pro-inflammatory signaling impacts at various levels of the carcinogenic process from cancer development to tumor progression to metastasis, as well as treatment failure leading to recurrences [89]. In general, inflammatory cancers including IBC are more aggressive and often have the worst prognosis [90]. In our research, we focused on signaling cross talks involving the focal adhesion kinase (FAK) and Fn-14 receptor.

FAK activation involves conformational changes leading to its release from auto-inhibited state triggering its auto-phosphorylation at Tyr397 residue. This activation recruits and forms a binary complex with Src family kinases leading to Src catalytic activation [75]. Src bound to FAK Tyr397 site subsequently promotes phosphorylation of additional FAK tyrosine residues, including Tyr407, Tyr576, Tyr577, Tyr861, and Tyr925. FAK phosphorylation promotes multiple interactions with other partner proteins, including paxillin, p130Cas, Grb2, Grb7, Nck-2, the p85 subunit of phosphatidylinositol 3-kinase, phospholipase Cγ, MAPKs, and p120Rho-GAP [52] and members of the Rho GTPase family [55]. These multiple interactions mediate FA signaling and are required for cell-ECM interaction, survival, and cell migration among other mechanisms [91].

Fn-14, a type I trans-membrane protein, is activated by its ligand Tweak (Tumor necrosis factor-like weak inducer of apoptosis). Fn-14 has no enzymatic activity but propagate its signaling via interaction with members of the TRAF adaptor protein family with TRAF2 being the major adaptor partner. This in turn stimulates multiple signal transduction pathways including activation of NF-κB pathway known to regulate multiple mechanisms, including inflammatory response cell proliferation, and cell migration [70]. Previous studies including ones from our laboratory have reported FAK [80] and Fn-14 [64-67] to be overexpressed in subsets of solid tumor types, including advanced stages of breast cancer. In our study, analysis of publically available genomic databases further revealed co-overexpression of FAK and TRAF2, the major adaptor protein for Fn-14, in breast

cancer compared to normal tissue (these proteins are weakly expressed in normal epithelial tissues) and within breast cancer distinct subtypes in particularly triple negative breast cancer. In IBC, we also observed a co-overxpression in many cases; however, a meaningful comparison between IBC and non-IBC was not possible due to the limited number of IBC cases available because of the disease rarity. Interestingly, we found TRAF2 to be highly expressed in all breast cancer cell lines used in our study as well as the normal breast cells whereas only inflammatory breast cancer cell-lines express the active (phosphorylated) form of TRAF2 protein.

Using a panel of genetically modified mouse embryonic cells lacking FAK, TRAF2, or both, and their matched reconstituted cells, as well as human cancer cell lines we identified a physical and functional interaction between FAK and TRAF2. Specifically, our results indicate that TRAF2 co-localizes with FAK and interacts with its N-terminal portion; this was demonstrated both in MEF cells and MDA-MB-231 breast cancer cell line. Furthermore, we have also observed that this interaction is also Src-dependent since it is significantly reduced in the Src-deficient cells SYF (negative for Src, Yes, and Fyn) but not in the Src-reconstituted cells. As mentioned earlier in the introductory background, FAK activation promote an intermolecular conformational changes that induce auto-phosphorylation at tyrosine 397 residue and create docking sites for Src kinases, which in turn promotes additional phosphorylations on FAK molecule. Noticeable, Fn-14 has been shown to be induced following Src activation and acts as a positive regulator of Src-driven cell invasion in Cheng et al study 2014 which included non–small cell lung cancer cell lines [84]. Therefore, Src acts as a dual regulator of both FAK and Fn-14.

Further phenotypic studies revealed that FAK-TRAF interaction impact on NF-κB activity, cell survival and anoikis. The later is a form of cell death triggered by the loss of cell contact with extracellular matrix and there is mounting evidence that resistance to anoikis contributes to metastasis development [91]. We also noticed that the presence or absence of TRAF2 did not affect cell migration (investigated using wound healing assay,

data not shown) arguing for a primary function of FAK-TRAF axis in the regulation of cell survival.

In addition, our study revealed a complex regulation of Fn-14-TRAF2 stability by FAK. Using MEF cells we have shown that following Fn-14 activation by Tweak, TRAF2 degradation is caused in TRAF2 proficient cells, in support with the literature [80]. We consistently observed that the stability of Fn-14 to be considerably reduced in cells lacking FAK and this could be rescued by restoration of FAK in these cells (Figure 9). As well, we demonstrated that FAK stabilizes Fn-14 via a proteasome-dependent mechanism as Fn-14 stability was up regulated when cells were exposed to the proteasomal inhibitor MG132 but not other proteases such as PMSF, leupeptin, and ALLN. Interestingly, loss or presence of FAK greatly impact on NF-κB activity in a TRAF2-dependent manner, supporting a contribution of this regulatory mechanism to the observed cooperation between TRAF2 and FAK in regulating NF-κB activity cell survival and anoikis. A well-studied function for TRAF proteins is to regulate ubiquitin-dependent degradation of nuclear NF-κB -inducing kinase (NIK), a mechanism involving the E3 ubiquitin ligase cIAP (cIAP1 or cIAP2) [92]. This aspect has not been addressed in my thesis but is under investigation in our laboratory.

In summary, our results provide insights into a novel pro-survival cooperative signaling involving FAK and TRAF proteins in MEF and breast cancer cells. . The observation that activated TRAF2 is highly expressed in IBC cells opens-up the rationale for further studies using larger cohort of IBC cells and tissues, Given the current status of clinical trial using FAK inhibitors and revealing utility and limitation of these inhibitors [48] as potential therapeutics for cancer, our results would support that dual targeting of both FAK and TRAF2 should be considered for the development of more effective therapeutics.

CHAPTER 6: CONCLUSION

In this thesis we target inflammatory breast cancer (IBC), as it is a highly aggressive type of breast cancer that is characterized by its distinctive inflammatory symptoms but under-investigated compared to other breast cancer types. While the incidence of this disease is rare in North America, it is reported to be higher in other regions of the world and in particular North African and Middle East. Therefore, IBC represents a relevant model to study cancer-associated inflammation, which is an important hallmark of cancer aggressiveness and progression to metastasis.

Many comparative genomic hybridization studies of IBC versus non-IBC have identified predominant changes in a wide spectrum of suspected molecular targets that are predominately overexpressed or suppressed in IBC exclusively. And by using the publically available breast cancer genomic databases, such as TCGA we have observed predominant associations between inflammatory signaling genes such as TRAFs, NF-κB, TNF-α and breast cancer aggressiveness. Which are well correlating with simultaneous up-regulation of multiple genes involved in focal adhesion signaling such as FAK and Paxillin. Throughout this project, we aimed to further investigate these associations at the molecular level. We have used a panel of mouse embryonic fibroblast (MEF) cell lines proficient and deficient for TRAF2 and/ or FAK as well as human breast cancer cell lines established from IBC (SUM149PT and SUM190PT) to investigate the crosstalk between inflammation and focal adhesion signaling, focusing on TRAF2 and FAK. By doing immunoprecipitation studies on MEF cells as well as breast cancer cells we were able to confirm a direct interaction between TRAF2 and FAK proteins. A colocalization was also observed using immunofluorescence microscopy between the two proteins in intracellular compartments with predominance in the cytosol and plasma cell protrusions. This interaction was then mapped by doing co-transfection and immunoprecipitation experiments using different versions of FAK protein (WT, NT, CT and P397) to reveal the FAK N-terminal is the main domain in this interaction, particularly phosphosite 397 and TRAF2. We additionally confirmed the FAK activation necessity to this Src dependent interaction by using SYF cells in immunoprecipitation studies.

Working on our original hypothesis of FAK and FN-14-TRAF2 cooperation to bridge inflammatory reactions and breast cancer progression to metastasis, we gave evidences that FAK by an interaction with TRAF2 regulates the Fn-14 stability via a proteasome-dependent mechanism.

Next, we looked at this interaction downstream effect to provide proofs that the two proteins cooperate to regulate NF-κB promoter activity, which greatly impacts on the whole pathway promoting cell survival and resistance to cell anoikis, a process believed to act among drivers of cancer progression and metastasis.

Certainly, further studies are needed to expand this work to deeply understand the basic for FAK-induced regulation of Fn-14-TRAF2 protein turnover and their relevance to inflammatory response in IBC cell and cases. As well, investigation of other TRAF members and partners, particularly the ubiquitin system is necessary to dissect the TRAF-FAK network and establish the implication to IBC cell invasion and identify potential molecular markers to be exploited for prognosis and/or therapeutic intervention.

APPENDIX: OTHER CONTRIBUTIONS

Cao YH, Krikor B, Alkailani M, and Alaoui-Jamali MA. Tumor-derived exosomes in pro-metastasis signaling: A multidirectional host-tumor cell communication with therapeutic implications (book chapter) for a Springer book edition on cell-cell communication in cancer, 2015 (in press)

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