FRA-1 LEVEL IN AGGRESSIVE CANCER CELL LINES UNDER SERUM STARVED STATE AND ITS IMPACT ON AN AUTOCRINE LOOP

By

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ABSTRACT

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Fos related antigen 1 (Fra-1) is a component of the dimeric AP-1 transcription factor that plays an important role in both cell cycle regulation and cancer initiation and progression. Fra-1 is highly increased in invasive types of breast cancer, e.g. MDA-MB-231 cells, when compared to the noninvasive types. Furthermore, Fra-1 in normal fibroblasts show a strictly regulated pattern of expression during G0 to G1 transition in response to growth factors.

My thesis research focused on the significance of the excessively high levels of Fra-1 in serum starved MDA-MB-231 cells, and how this phenomenon is maintained in absence of any external stimuli? To answer this question, we characterized the pattern of expression of Fra-1 and other AP-1 family members as a function of cell cycle in invasive breast cancer cell lines, and compared them to non-invasive and normal cells. One of the major results we observed was the excessive levels of Fra-1 produced by MDA-MB-231 under serum starvation and its maintenance all-through the cell cycle. Also, we identified a role for Fra-1 in keeping MDA-MB -231 cells growing, albeit very slowly.

Additionally, we explored different properties of Fra-1 in MDA-MB-231. Our work showed that Fra-1 is increased in this cell line due to both increased expression and stability. In addition, we found Fra-1 in MDA-MB-231 cells to be

both nuclear and cytoplasmic in distribution which was contrary to what was found before in normal cells where Fra-1 is solely nuclear.

Furthermore, we utilized A-Fos, a dominant negative form of Fra-1, to test the role of AP-1 in maintaining the oncogenic and metastatic properties of MDA-MB-231 cells. We found that A-Fos completely suppressed the ability of MDA-MB-231 cells to grow on agar. In addition, it attenuated the migration of these cells.

A further major finding of our work demonstrated an ability of MDA-MB-231 cells to secrete in their medium some factors(s) that can induceFra-1, as tested in MCF10A maintained in serum free medium. We found that both the MEK/ERK and MLK mediated pathways are involved in this process. These secreted factors were found to have a significant role in controlling migration and proliferation of neighboring cells, an effect that was mediated through Fra-1. Furthermore, by inserting A-Fos in MDA-MB-231 cells we were able to show that the control of such secretion of these substances is mediated through Fra-1. The ability of these factors to self-control MDA-MB-231 cells is yet to be tested.

Lastly, we extended these results to invasive cancers from other tissue origins, e.g. prostate and colon cancers, suggesting a universal role of Fra-1 in invasive cancers.

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KEY TO ABBREVIATIONS

AP-1	Activating Protein-1
CDKs	Cyclin Dependent Kinases
СМ	Conditioned Medium
DN	Dominant Negative
Dox	Doxycycline
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinases
Fos	FBJ murine osteosarcoma oncogene
Fra-1	Fos Related Antigen-1
Hrs	Hours
JNK	C-Jun N-Terminal Kinase
MAPK	Mitogen Activated Protein Kinase
MEK	Mapk/Erk Kinase
MLK3	Mixed Linkage Kinase 3
NCD	Nocodazole
PKC	Protein Kinase C
ТАМ	Tumor Associated Macrophages
TGFβ	Transforming Growth Factor B
TNBC	Triple Negative Breast Cancer
TRE	TPA Responsive Element

CHAPTER ONE

REVIEW OF LITERATURE

Breast cancer is the third most common cause of death in women in the united states with 40,000 deaths per year (Siegel et al., 2012). One of the hallmarks of cancer is the ability of the cancer cells to migrate and seed in organs other than the organ of origin (Hanahan and Weinberg, 2000), a phenomenon called metastasis. Metastasis is the leading cause of death from cancer comprising about 90% of the total deaths among all cancers(Gupta and Massague, 2006). Metastatic cancer cells develop different cell markers and surface profiles from the primary tumor cells as they differ in their gene expression pattern and their pattern of mutation. Although some successful specific inhibitors of metastatic cell growth have been produced, problems have shown up in long term treatment (ref: erbb2). Thus, further investigation is crucially required in order to highlight the specific markers of metastatic cancers.

The most aggressive subtype of breast cancer is the so-called triple negative breast cancer (TNBC). These cells do not express the estrogen (ER), progesterone (PR) and Her2 receptors. TNBC cells represent 20-25% of the breast cancer cases (Howlader et al.). The main problem of this type of breast cancer is the lack of targeted therapy. Despite a strong initial response to chemotherapy, TNBC shows the highest rate of recurrence among other types of breast cancer. This leads to a low survival rate of this type of breast cancer (Jaitak, 2014).

Basal like and triple negative breast cancers:

Based on gene expression profiles, breast cancers were classified into 5 categories; Basal like, Luminal A, Luminal B, ErbB2+ and Normal breast-like groups (Perou et al., 2000). Among these groups, the basal type is considered the most

aggressive. The basal like breast cancer displays a gene expression pattern similar to basal cells of breast acini. The majority of basal like cancers are triple negative. However 15-45% of them are positive to one or more of the three receptors. On the other side, some of the triple negative cancers do not belong to the basal type given their gene expression profile. (Rakha et al., 2008).

Activating protein-1 (AP-1):

AP-1 is a dimeric transcription factor that is formed by combinations of Jun and Fos proto-oncogenes families members. It can form either a heterodimer of one member of Fos family and one member of Jun family or homo-dimer from the Jun family members (Chinenov and Kerppola, 2001). The Fos family includes 4 proteins (c-Fos, FosB and Fos related antigen 1 and 2 -Fra-1 and Fra-2-), while the Jun family is formed of 3 proteins (c-Jun, JunB and JunD) (Vesely et al., 2009). The AP-1 dimer binds a specific DNA sequence known as TPA Responsive Element (TRE) which is T G A C/G T C A (Angel and Karin, 1991).

Structure: The structure of different members of Fos and Jun family of proteins shows great similarity. They all have a DNA binding domain which is called the basal domain (DBD) and a leucine zipper domain, that both together forms the bZIP motif (Cohen et al., 1989). The leucine zipper domain induces binding both partners to form the AP-1 dimer. All the Jun family and both c-Fos and FosB have, in addition, trans-activation domains in the C-terminal and N-terminal ends. These trans-activation domains mediate the recruitment of the co-activators that in turn enhance gene expression (Wisdon and Verma, 1993). They are absent in both Fra-

1 and Fra-2. (Cohen et al., 1989) suggested that the DNA binding of the AP-1 complex is detected by regions from both protein partners.

Function: In normal cells, AP- 1 participates in regulation of most cellular processes including cell proliferation, migration, apoptosis and differentiation. In addition to its role in normal cells AP-1 member plays important role in different pathological processes including oncogenesis, and cancer progression (Dai et al., 2003; Eferl and Wagner, 2003; Milde-Langosch, 2005). The different combinations of the Jun and Fos family members determines the different effects of the AP-1 complex (Milde-Langosch et al., 2004).

Regulation of AP-1:

AP-1 proteins are the downstream targets of a number of surface receptors involved in regulation of expression and activity in response to different environmental stimuli. These include for example EGF, LPA, TGF receptors. The first pathway that is well documented to induce AP-1 is the RAS/RAF/MEK/ERK pathway (Verde et al., 2007). In addition, the most recently described mitogen activated protein kinase (MAPK) pathway; MEK5/ERK5 is also involved in the activation of c-Fos and subsequently Fra-1. Additional MAPK pathway regulates cjun activity through is the P38/JNK pathway (Verde et al., 2007).



Figure 1: The structure of AP-1 (Hess et al, 2004)

Our focus here will be related to the regulation of AP-1 in response to mitogenic stimuli during cell cycle re-entry (Figure 2). In normal cells, AP-1 proteins show a highly regulated sequential pattern of expression during the cell cycle reentry in response to growth factors. This pattern is very important for regulation of cell cycle progression and cell division. Immediately after growth factor stimulation, c-Fos expression briefly increases. As such, c-Fos is one the immediate early genes which are stimulated immediately. This is followed by a drop of c-Fos and an increase of Fra-1 expression, which continues until late G1 phase (Cook et al., 1999; Kovary and Bravo, 1992). The increase of Fra-1 is partially induced by c-Fos via a TREs on the Fra-1 promoter (Cohen et al., 1989). In addition to activation of c-Fos, ERK1/2 phosphorylates Fra-1 at serines S252 and S265, which leads to activation, stabilization, and nuclear transport of Fra-1 (Basbous et al., 2007) . In normal cells, the half-life of Fra-1 under serum stimulation is 5.5 hours; starting about 2 hours after serum induction; while it is only 1 hour in serum starved cells (Gruda et al., 1994). This increase in half life is due to phosphorylation caused by mitogenic induced ERK1/2 activation (Basbous et al., 2008). Both c-Fos and Fra-1 are constitutively unstable proteins because they are degraded by ubiquitin independent proteasomal degradation induced by instability element in the cterminal domain. When c-Fos or Fra-1 is phosphorylated this instability element is inactivated leading to increase the half-life and subsequent accumulation of Fra-1(Basbous et al., 2007; Bossis et al., 2003). This occurs, for example, under any condition that induces the MEK/ERK kinase pathway either physiological, like

serum induction or pathological e.g. after carcinogenic transformation (Mechta et al., 1997).

In western blot, Fra-1 is represented by 3 bands of different electrophoretic mobility ranging from 34 to 42 KD. These bands represent different phosphorylation states. The lowest band defines the newly synthesized Fra-1 and the higher (slower) bands represent the phosphorylated forms of Fra-1 which is more stable and represent the active forms of Fra-1 (Casalino et al., 2007).

Fra-1 activation leads to expression of Cyclin D1 which in turn is initiated by cyclin dependent kinases (CDKs). The CDKs then induces the S-phase of the cell cycle i.e. G1/S transition (Burch et al., 2004; Casalino et al., 2003; Cook et al., 1999). This highly regulated pattern of Fra-1 expression is very important for the overall regulation of cell cycle entry and cell proliferation (Yang et al., 2006)

AP-1 and cancer:

c-Fos was first recognized as the human and mouse homologue of the retroviral protein v-Fos which is able to induce osteosarcoma in mice. The study of the difference between c-Fos and v-Fos lead to the understanding of the function and regulation of the c-Fos protein and its role in cancer transformation (Miller et al., 1984). Moreover, the AP-1 factor was separately identified as a transcriptional factor able to induce transcription of both viral and vertebrate genes through the binding to the sequence TGACTCA following treatment of cells by phorboesters (Lee et al., 1987). Later, the link between c-jun and then c-Fos to AP-1 was recognized ((Bohmann et al., 1987; Curran and Franza, 1988).



Figure 2: Pattern of c-Fos and Fra-1 protein expression in normal rat fibroblasts after lysophosphatidic acid (LPA) activation (Cook et al, 1999).

Moreover, Ras mutant transformed epithelial cells were used as a model to study the role of AP-1 in cancer. Ras involves mutation which mediated transformation occurs through RAF/MEK/ERK pathway (Casalino et al., 2003). When the cells are transformed by RAS the Fra-1 member of the Fos family predominates and mediates the growth and metastatic ability of these cells. These studies of Ras transformed cells highlighted the predominance of the Fra-1 over c-Fos in mediating the function of AP-1 in cancer cell (Andreolas et al., 2008; Casalino et al., 2007)

Fra-1 and metastatic breast cancer:

Expression of Fra-1 is known to increase in many types of cancer. For example, it increases in breast, ovarian, colon and prostate, and thyroid cancers at the switch from non-invasive to metastatic state (Young and Colburn, 2006).

In RAS transformed thyroid cell lines Fra-1 is up-regulated and it shows a peak at the G2/S phase of the cell cycle. This increase of Fra-1 is due to both transcriptional auto-regulation and posttranslational phosphorylation which prevent proteasomal degradation of Fra-1 (Casalino et al., 2003). Fra-1 enhances cell progression in these cells through induction of cyclin A2 expression in G2. Also, Fra-1 enhances the invasiveness and migration of these transformed cells (Casalino et al., 2007).

In clinical tumor samples, Fra-1 is highly expressed in tumor tissue as compared to the adjacent tissues and in the TNBC as compared to the luminal and to the Her2 overexpressing tumors (Kharman-Biz et al., 2013). Studies of clinical samples of breast cancer have shown a correlation between the high level of Fra-1 and the more aggressive phenotype of cancer (Logullo et al., 2011).

In breast cancer cell lines, Fra-1 increases in many types of aggressive (e.g. MDA-MB-231 and BT549) cancer cell lines as compared to less aggressive types (e.g. MCF-7 and MDA-MB-468) both on the mRNA and protein level (Figure 3) (Belguise et al., 2005; Zajchowski et al., 2001; Zhao et al., 2014). Furthermore, when Fra-1 was over-expressed in MCF-7 cells they became more aggressive as shown by increased proliferation, migration ability, invasiveness and growth in matrigel. On the other side, when Fra-1 was silenced in MDA-MB-231 cells their metastatic behavior abated. Loss of Fra-1 leads to decrease cell invasion, migration, proliferation and growth in matrigel. Also it changed the shape of the colonies in matrigel from the stellate appearance to rounded smooth colonies; a sign that indicates decreased metastatic ability of these cells (Belguise et al., 2005). In addition, knockdown of Fra-1 suppresses the metastatic potential of tumors formed by these cells when they are introduced in nude mice (Desmet et al., 2013).

These evidences suggest that Fra-1 plays an important role in the metastatic process. Fra-1 mediates this process by promoting expression of genes that enhance cell invasion and motility through diverse mechanisms. Some of them are collagenases that helps cells to invade through the surrounding tissues like Matrix metallloprtineases 1 and 9 (Belguise et al., 2005; Gordon et al., 2009; Kimura et al., 2011). Others are cytokines that enhance cell growth and motility like IL-6 and transforming growth factor β (TGF β) (Luo et al., 2010; Ndlovu et al., 2009) and (Zerbini et al., 2003). Some others recruit tumor associated macrophages (TAM) to the tumor vicinity which by its turn secrete some paracrine factors that enhance tumor migration. Examples of this are CSF-1 and osteopontin. Another category is

exemplified by cyclooxygenase2 (COX2) which is an enzyme that produces prostaglandines that inhibits cell apoptosis and stimulate angiogenesis. In addition to that Fra-1 induces transcription of other transcriptional factors which by their turn regulate components of the cytoskeleton and cell motility machinery like snail, slug and ZEB1/2 (Chen et al., 2009; Shin et al., 2010).

Regulation of Fra-1 in TNBC cell lines:

More than one pathway upstream of Fra-1 share in the maintenance of high level of Fra-1 in TNBC cell lines. The protein level and/or activity of these factors correlates with the level of Fra-1 in breast cancer cells. For example, PKC0 and pERK levels are higher in highly invasive breast cancer cells compared to the less invasive ones (Figure 3) and mediates the increased Fra-1 level in these cells through increasing Fra-1 stability This occurs through activation of the JNK and ERK1/2 pathways. However, different TNBC cell lines show different roles of either of these two pathways. For example, in MDA-MB-231 cells the main activation occurs through the ERK1/2 pathway while in BT549 cells the main activation occurs through the JNK pathway. Knockdown of PKC0 or inhibition of ERK pathway using UO126 leads to substantial reduction in the level of Fra-1 (Belguise et al., 2005; Belguise et al., 2012; Chen et al., 2009). Another member of the PKC family; namely PKC- α , increases in MDA-MB-231 cells as compared to MCF-7 cells (Urtreger et al., 2012). PKC-α regulates epithelial to mesenchymal transition in breast cancer cells through the regulation of Fra-1(Tam et al., 2013).



Figure 3: PKCθ and pERK level correlates with Fra-1 across different types of breast cancer cell lines (Belguise et al 2010) The PI3K / AKT pathway is another potential mediator of the increase of Fra-1 in highly metastatic tumor cells. For example in clinical samples pAKT was found to increase in the TNBC tumor samples (Umemura et al., 2007). Recent work by (Zhao et al., 2014) showed that AKT also play a role in the increase of Fra-1 in different TNBC cell lines. They found that inhibition of AKT lowered the Fra-1 level in these cells. However the role of AKT pathway in this process is still controversial and contradictory results has been shown.

Mixed linkage kinase 3 (MLK3) is a mitogen-activated protein kinase that is highly expressed in breast cancer cell lines and enhance breast cancer migration and metastasis. Over-expression of MLK3 in MCF10A cells increased cell migration and invasiveness in an AP-1 dependent manner and increased Fra-1 expression level (Chen et al., 2010).

Other Ap-1 members in metastatic breast cancer:

Other than Fra-1, C- Jun was the only AP-1 member that was reported to be increased in the metastatic breast cancer cell lines (Du et al., 2010) and to play some role in induction of tumor invasion (Zhao et al., 2014).



Figure 4: Summary of the pathways that was found to share in regulation of AP-1 in metastatic TNBC cell lines.

Autocrine role in regulation of cancer cell aggressiveness and metastasis:

The tumor micro-environment plays an important role in the regulation of aggressiveness of these tumors. This environment includes factors secreted by the tumor cells itself and perform different functions in the tumor microenvironment and they are globally called the cell secretome (Chen et al., 2008). Some of these secreted substances act as autocrine factors that through surface receptors control different functions of the tumor cells including cell migration invasion, proliferation and apoptosis (Jacobs et al., 2008). Also these autocrine factors represents one of the mechanisms by which the cells can sustain what is called "self-sufficiency in growth signal" which considered one of the hallmarks of cancer.

Some of these autocrine factors secreted by polyoma virus transformed rat fibroblast has transforming properties that allow these cells to be able to grow in serum free medium i.e. complete absence of any external growth factors or mitogens. Also, conditioned medium from these cells allowed normal rat fibroblast to grow in a serum independent manner (Kaplan and Ozanne, 1982; Kaplan et al., 1981)

Moreover, conditioned medium from MDA-MB-231 cells was found to increase MCF10A cells proliferation and to increase the synthesis of both IL-6 and cyclin D1 protein levels in these cells. Also this conditioned medium increased the proliferation of MCF10A cells (Lieblein et al., 2008). Several autocrine factors was found to mediate the aggressiveness of the TNBC cells. Examples:

Colony stimulating factor-1 (CSF-1): CSF-1 was found to mediate an autocrine loop that contribute to the aggressiveness of MDA-MB-231 cells (Patsialou et al., 2009).

Nodal: Both Nodal and its receptors are overexpressed in less differentiated breast cancer cell lines compared to the more differentiated ones. This indicate that nodal play an autocrine role in these cells. This loop shares in promoting the aggressiveness of the MDA-MB-231 cells and enhances the metastatic phenotype of the breast cancer cells (Quail et al., 2012).

Osteopontin: it is a chemokine that is secreted by different types of cancer cells. Osteopontin binds works through binding to integrin or CD44 cell surface molecules to enhance tumor cell aggressiveness. Also, it mediates autocrine loop that is able to induce metastasis in different malignant cell lines including the MDA-MB-231 cells.

IL-6: it plays an important role as an autocrine and paracrine factor that enhances the aggressiveness and the metastatic ability of breast cancer. IL-6 performs its role through the JAK-STAT pathway (Grivennikov and Karin, 2008).

Lysophosphatidic acid (LPA): it is a membrane phospholipid which secreted by many types of cells and act as a growth factor and is mitogenic to many cells. It acts through G protein associated receptors on the cell surface. And it induces Fa-1 synthesis.

LPA also was found to play important role as an autocrine factor to enhance the aggressiveness of the breast cancer cell lines. A study by (Du et al., 2010; Eder et al., 2000) showed that the secretion of the LPA is double in the MDA-MB-231 as

compared to MCF-7 cells. Also LPA enhances MDA-MB-231 cell migration through stimulating both PI3K and ERK signaling (Du et al., 2010). Fra-1 was reported to play a direct role in enhancing membrane phospholipid production this role is aside from its role as a transcriptional factor. Phospholipid production is a process that is necessary for cell growth and cell division (Motrich et al., 2013).

These factors and other soluble molecules secreted in the cancer cell secretome are potential candidates to mediate an autocrine loop that keeps the high level of Fra-1 one in MDA-MB-231 cells.

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CHAPTER TWO

A UNIVERSAL ROLE OF FRA-1 IN AGGRESSIVE CANCER CELL LINES AND AN IMPACT ON AN AUTOCRINE LOOP

ABSTRACT

Fos related antigen 1 (Fra-1) is a component of the dimeric AP-1 transcription factor that plays an important role in cell cycle regulation and cancer initiation and progression. Fra-1 is highly increased in invasive types of breast cancer when compared to the noninvasive types. In normal fibroblasts, Fra-1 shows a strictly regulated pattern of expression during G0 to G1 transition in response to growth factors. My thesis research is focused on the significance of the excessively high level of Fra-1 in serum starved MDA-MB-231 cells and how this phenomenon is maintained in absence of any external stimuli.

To answer this question we characterized the pattern of expression of Fra-1 and other AP-1 family members as a function of cell cycle in invasive breast cancer cell lines comparing them to non-invasive and normal cells. A major result is the excessive level of Fra-1 produced by MDA-231 under serum starvation and its maintenance all-through the cell cycle. We also identified a role for Fra-1 in keeping MDA-MB-231 cells growing under serum starved state, albeit very slowly.

Another major finding of our work demonstrated an ability of MDA-MB-231 cells to secrete in their medium some factors(s) that, in turn, can induce Fra-1, as tested in MCF10A maintained in serum free medium. We found that both the MEK/ERK and MLK mediated pathways are involved in this process. These secreted factors were found to have a significant role in controlling migration of neighboring cells, an effect that was mediated through Fra-1. The ability of these factors to self-control MDA-MB-231 cells is yet to be tested.

In addition, we extended these results to invasive cancers from other tissue origins, e.g. prostate and colon cancers, suggesting a universal role of Fra-1 in invasive cancers.
INTRODUCTION

Ap-1 is a dimeric transcription factor composed of one member each from the small Fos and Jun families (Chinenov and Kerppola, 2001; Vesely et al., 2009). AP-1 potential role in cancer was revealed in the course of their discovery in two separate conditions. Fos and Jun genes and their proteins were discovered as mediators of tumor promotion in early studies of the effect of phorbol ester treatment. A common sequence showed up upstream of phorbol induced genes as a conserved TGAG/CTCA DNA binding sequence. This element was called TRE for TPA-Responsive-Element and AP-1 was named for Activator Protein 1(Lee et al., 1987). Approximately at the same time, v-Fos and v-Jun RNA sequences were found by chance in the genomes of rare retroviruses with unexpected oncogenic properties (Miller et al., 1984). They were shown to be produced by accidental reverse transcription of c-Fos and c-Jun transcripts that generated oncogenic properties (Chen and Barker, 1985; Eva et al., 1987).

Many early studies of Fos and Jun demonstrated their essential role in various steps of the cell cycle in response to treatment with growth factors (Cook et al., 1999). One aspect is linked to their restricted temporal expression and consequently that of their targets. For example AP-1 plays a major role in the G1 \rightarrow S as well as G2 \rightarrow M transitions via controlling cyclin D1, and, respectively, cyclin A(Casalino et al., 2007).

One factor has contributed some difficulties in studying Ap-1 properties. The vast majority of the experiments have been carried out in very different conditions, with

changing parameters variability such as cells from different species, from different tissues; in different states normal cells vs differentiated cells, or transformed cells (using various carcinogens); cells from tissues, or cell lines; using different growth factors (For details refer to our review above). An additional problem has been the long delay until the discovery of Fra-1 a major member of the Fos family (Cohen and Curran, 1988). Despite some differences (at least temporal) both c-Fos/c-Jun and Fra-1/c-Jun are called AP-1 and it is not always possible to know from the literature which of the two is active in any specific manuscript.

Based upon old and new data, c-Fos is the first mRNA synthesized in the nucleus (immediate early gene) followed by c-Jun, and forming c-Fos-/Ap-1(Fambrough et al., 1999). C-Fos disappears early too (Cook et al., 1999). Fra-1 expression is delayed to "delayed early gene"; presumably helping cells past the restriction point by inducing cyclinD1. This results in shift from c-Fos/AP-1 to Fra-1/AP-1 observed in the delayed early G1 phase. This switch is mediated by an interactive swap of binding sites, in which c-Fos induces Fra-1 via an AP-1 binding site in the Fra-1 promoter (Chambard et al., 2007) and in return Fra-1 turns off c-Fos by binding to c-Fos AP-1 binding site(Ito et al., 1990). Also the suppression of ERK activity leads to reduced phosphorylation of c-fos and its degradation (reviewed in Chambard et al., 2007). Overall, the results strongly suggest that Fra-1 has more functions than c-Fos, which fits with the fact that embryonal Fra-1 null, but neither c-Fos nor Jun induces an early embryonal lethal (Eferl et al., 2004).

While these results suggest that all cells need Ap-1 to go through cycling in and division, it is evident that AP-1 mediates the expression or repression of genes

that mediate many functions such as movement, migration as well as role in differentiation, death etc (Dai et al., 2003; Eferl and Wagner, 2003; Milde-Langosch, 2005). These multiple functions agree with the results of ChIP-Seq which vary between 16,000 and 20,000 hits (Biddie et al., 2011).

Recently, Fra-1 protein levels were found to increase significantly in highly aggressive breast cancer cell lines (e.g MDA-MB-231) as compared to the non – invasive types such as MDA-MB-468 and MCF7 (Belguise et al., 2005). This increase was found to play role in induction of metastatic abilities of these cells through induction of expression of genes that enhance cell migration and invasion such as MMP2 and MMP9 (Belguise et al., 2005) and/or repression of genes that suppresses these processes like TSCL1 (Bahassi el et al., 2004).

Three of the kinase pathways were found to share in inducing the high level of Fra-1 in these highly aggressive breast cancer cell lines. The first one is the canonical pathway- MEK/ERK pathway- that normally induce the AP-1 activity under serum induction. The induction of this pathway in the metastatic breast cancer cell lines occurs through the increased activity of PKC Θ . PKC Θ in addition works through the JNK pathway (Belguise et al., 2012). The third pathway is the PI3k through AKT (Zhao et al., 2014).

In this work we are exploring in more depth the role of Fra-1 in metastatic cells and the forces that keep these cells to a sustained to high level. We showed that high level of Fra-1 is maintained over all the stages of the cell cycle and, finally

that it has a role in keeping the cells progressing through the cycle even in absence of any growth factors.

RESULTS

Altered Cell cycle dependence of Fra-1 expression in metastatic breast cancer cell lines compared to normal cells and non-invasive tumor cells: Recent studies of MDA-MB-231 and other invasive metastatic breast tumor cells demonstrated large increases in the accumulation of Fra-1 protein levels in contrast with non-invasive tumor cells (e.g. MDA-MB-468 and MCF7) (Belguise et al., 2005). To further understand potential alterations in the control of AP-1 factor in response to progression into metastasis, we carried out extensive cell cycle analyses. As expected, the Fra-1 expression pattern of MCF10A normal cells resembles that observed for many normal cell types and species (Cook et al., 1999; Kovary and Bravo, 1992). Upon reentry of normal cells into the cycle, Fra-1 levels increase from very low (+) to its maximal around 3 hours (++) ("immediate early G1"), reach a maximum around 12 hours ("delayed early G1") (+++), and decrease again after 18 hours (" progression in S phase") (++) (Figure 5 A).

The Fra-1 level in non-invasive MDA-MB-468 pattern leans toward that of normal cells. In contrast, MDA-231cells showed two major differences; a high (++++) Fra-1 expression level and independency of the cell cycle (Figure 5 A)

The metastatic-specific Fra-1 expression pattern is not restricted to breast tumors: The metastatic specific Fra-1 behavior as observed in breast tumor cells was compared in pairs of non-invasive and invasive/metastatic cells from different tissues, namely, prostate one metastatic pair (PC3 ++) and one non-invasive (Du145), and four colon: one metastatic pair (SW620 +++), one intermediate



Figure 5: The expression pattern of Fra-1 during cell cycle reentry was shifted from normal in metastatic cancer cell lines : (A) western analysis of Fra-1 in non-tumorigenic and tumorigenic breast epithelial cell lines: The MDA-MB-231, MCF10A, MDA-MB-468 and the MCF7 cells were all subjected to serum starvation then stimulated with serum for the time periods indicated in the figure. (B) Different colon and prostatic cancer cell lines were compared for Fra-1 protein level using western blot. The cells were harvested during exponential growth (X), under serum starvation (0) and after 8 hours of reinduction with 10% FBS (8)

invasive (SW480 ++) and 2 noninvasive (HT29 and Caco2, both -). We found that, the Fra-1 level was high in the serum starved state in the highly invasive types of colon (SW480 and SW620) and prostate (PC3) cancer, when compared to the non-invasive types for colon (Caco2 and HT29) and for prostate (Du145) cancers. However, under serum stimulation there was no significant difference of the Fra 1 level between these cells (Figure 5 B). Thus the metastasis-specific Fra-1 expression extends to from breast cancer to metastatic tumors from other organs.

Metastatic-dependent "G0" escape of MDA-231 cells:

As expected and shown by FACS analysis (Figure 6), growth factor deprivation of normal MCF10A cells leads to total gathering in a single 1x presumably G0 peak. Most non- invasive 468 cells, arrested at 1x as well, except for a small 2x peak of cells presumably in G2/M. In the case of metastatic cells, the 2x peak was more prominent, even if cells that reached G2/M border were lost. To prevent cell division followed by reentry into the 1x peak group of the 2nd cycle, cells were arrested at the G2/M border by treatment with nocadozole and further incubated for 18 hours or 30 hours. Most evident at 30hrs, a large bubble containing MDA-231 genomes exclusively from a single cycle were found in S as well as in G2/M population indicating that MDA231cells can overcome deprivation starvation and divide albeit slower than serum fed cells..

As shown in (Figure 6 B), in serum starved cells, about (96.75% \pm 0.64) of the MCF10A cells were arrested in the G0/G1 stage of the cycle versus only (75.53% \pm 1.24) of the MDA-MB-231 cells (P value = 0.00006). About 30.76% \pm 0.53 of MDA-MB-231 cells were blocked at the G2/M, demonstrating a considerably reduced G0 arrest (only 47.01 \pm 0.21). Whereas as expected for "normal" cells,

MCF10A cells showed a complete arrest in the G0/G1 stage which is indicated by the persistence of almost all the cells in the G0/G1 stage in both 18 and 30 hours (95.7 % \pm 0.74 and 96% \pm 0.5) of Nocodazole blockage. The MDA-MB-468 cells also showed a continued progression through the cell cycle but with a lower rate than MDA-MB-231 cells . When AP-1 action was inhibited using dominant negative form (A-Fos) the progression of the MDA-MB-231 cells through the cycle was reduced (Figure 7)

We further studied the ability of the MDA-MB-231 cells to survive and *divide* in serum free medium and the role of Fra-1 in mediating this property using a CKK-8 cell counting kit. MDA231 cells *continued* to grow up 96 hours in serum free medium. This ability was lost when MDA-MB-231 cells were stably transfected with Fra-1 shRNA (Figure 8 A). The capacity of MDA231 to grow in serum free in tissue culture was followed up to 3 weeks (Data not shown). The cells survived and grew in serum free medium for several generations (labelled MDA-MB-231_sf) were further grown and compared to the parental cells (MDA-MB-231) for Fra-1 level. The MDA-MB-231_sf showed a *slightly higher level of Fra-1 (Data not shown)*.











Figure 7: A-Fos reduces the ability of MDA-MB-231 cells to progress through the cell cycle in absence of serum. (A) The expression of Fra-1 in MDA-MB-231 cells using a doxicyclin inducible promotor: (B) The same Nocodazole block experiment repeated for MDA-MB-231/Flag-afos. Cells were induced for A-Fos production using Doxycyclin.



MDA-MB-231/flagA-Fos

MDA-MB-231/flagA-Fos

Figure 8: Both Fra-1/shRNA and A-Fos were able to reduce MDA-MB-231 cells growth in presence and absence of serum: (A) MDA-MB-231 cells were stably transfected with scrambled sequence (MDA-MB-231 V) or with Fra-1 shRNA (MDA-MB-231 Fra-1 shRNA). Both cells were plated in 96 well plate at a density of 5x104 then allowed to grow with and without serum for up to 96 hours. The cell number was estimated using CCK8 cell counting kit. (B) MDA-MB-231/flagA-Fos cells were plated in 6 well plates at a density of 3x105 overnight. After that group of them were incubated with 10%FBS or in 0.05% FBS (serum free medium). Each group was devided into 2 groups which was either induced with Doxycyclin to produce A-Fos or not. After 72 hours the cells were trypsinized and counted then replated at a density of 3x105 again with or without doxycycline.

Co-culturing MDA-MB-231 cells with MCF10A or MDA-MB-468 cells and exposing either cells to MDA-MB-231 condition medium (CM) induced Fra1 expression: The continued growth and sustained high Fra-1 levels in invasive cancer in the absence of growth factors raised questions about the mechanism; we considered and test for the presence of secreted autocrine factors. The first step was to find if the MDA-MB-231 cells secrete factors that have the ability to increase cellular Fra-1 levels on their own self. We used two approaches. First, MDA-MB-231 cells were co-cultured with the MCF10A and MDA-MB-468 cells using trans-well system. Second, the effect of CM from MDA-MB-231 cells was tested on MCF10A, MCF-7 and MD-MB-468 cells. These latter two cell lines have been chosen as they all have normally low levels of Fra-1 compared to MDA-MB-231 cells. As shown in (Figure 9) Fra 1 level was highly increased when MCF10A cells that were cocultured with MDA-MB-231 cells (Figure 9 A). Similarly, when the MCF10A cells were incubated with CM from MDA-MB-231, Fra-1 level was significantly increased in MCF10A cells (Figure 9 B). I got similar results for both experiments with MDA-MB-468 cells (Figure 9 C). On the other hand neither CM from MDA-MB-231 cells nor co-culture of MCF7 with MDA-MB-231 cells increased Fra-1 level in MCF7 cells (Data not shown).

The ability of MDA-MB-231 cells to produce factor(s) which induce Fra-1 in MCF10A and MDA-MB-468 cells is Fra-1 dependent: To test the role of high Fra-1 level in MDA-MB-231 cells in the secretion of some factors in the CM that is able to induce Fra-1 level in other cells, we utilized again the Doxycycline inducible A-Fos in MDA-MB-231 cells. CM from cells that is MDA-

MB-231/flag-afos induced with Doxycylcine lost the ability to induce Fra-1 in MDA-MB-468 cells as compared to the CM from the non-induced cells (Figure 9 D)

Co-culturing MDA-MB-231 cells with MCF10A cells and exposing MCF10A to MDA-MB-231 CM increase MCF10A migration ability:

Fra-1 is known to control the expression of many genes that enhance cell migration and metastasis. Therefore, we tested whether the co-culture and/or CM treatment have a functional role in promoting cell migration and metastasis, we found that migration of the MCF10A cells increased when they were co-cultured with MDA-MB-231 cells (Figure 10 A). In addition we tested the role of MDA-MB-231 CM in increasing cell migration in MCF10A cells using transwell migration assay and wound healing assay. Our results shows that CM significantly increased the migration through 8µm transwell pores. The CM experiment was conducted in two different scenarios (Figure 10 B). Conditioned medium was added in the upper chamber and serum free medium was used as a control and cells were allowed ing to migrate towards10% FBS. Alternatively, the serum free medium versus conditioned medium were added to the lower chamber. In both cases the number of migrating cells significantly increased with CM as compared with the control (the average number of cells in HPF in the first method was15.4 versus 8.2, P value=0.0001 (Figure 9 B left) while it was 21.6 versus 6, P-value=0.00001, in the second method. (Figure 9 B right). Also, CM from MDA-MB-231 significantly increased wound closure (-6.04 \pm 3.1 vs 12.44 \pm 0.59, p= 0.002) (Figure 10C)).



Figure 9: Incubation MCF10A and MDA-MB-468 cells with the conditioned medium from MDA-MB-231 cells or their co-culture with MDA-MB-231 cells increases Fra-1 level in the MCF10A cells: (A) MDA-MB-231 cells were cultured with MCF10A through transwell system then cells were harvested and proteins were extracted and analyzed using western blot (B) Conditioned medium from MDA-MB-231 cells was added to MCF10A cells for 6 hours then cells were harvested and Fra-1 level was established by western blot and quantitated by densitometry averaging 4 samples. (C) Conditioned medium from MDA-MB-231 cells was added to MDA-MB-468 cells and the MDA-MB-468 cells were co-cultured with MDA-MB-231 cells and then Fra-1 protein level was measured using western blot. (D) CM from non induced versus doxyxyclin induced MDA-MB-231/FlagA-Fos cells were added to MCF10A cells for the indicated time periods then cells were harvested and studied with western blot.



Figure 10: Co-culture of MCF10A with MDA-MB-231 cells or adding MDA-MB-231 CM to MCF10A increased the migration of MCF10A cells: (A) MCF10A cells were cultured on the 8um pore transwell insert membrane with MDA-MB-231 cells cultured in the lower chamber. The cells were fixed using formaldehyde and stained with crystal violet, then the cells were webbed off the upper surface of the membrane using a cotton swab. (B) The effect of MDA-MB-231 CM on MCF10A migration through 8µm pore transwell: MCF10A cells were cultured in the upper chamber of transwell inserts in MDA-MB-231 CM versus serum free medium (left graph) or MCF10A cells were cultured in the upper chamber in serum free medium with the lower chamber filled with either CM or serum free medium (right graph). Cells were counted per HPF and representative pictures were taken. (C) Wound healing migration assay was used to measure the effect of MDA-MB-231 CM on MCF10A cell migration. The results were quantitated using the adobe Photoshop software detecting the percent closure of the wound after 24 hours. Increase of Fra-1 level was demonstrated with western blot.



Figure 11: The knock down of the FRa-1 using shRNA negated the effect of CM fom MDA-MB-231 cells on MCF10A migration: (A) Control cells

infected with scramble shRNA virus gene and cells infected with Fra-1 shRNA gene (Fra-1 shRNA-3 and Fra-1 shRNA-5) were harvested and Fra-1 protein level was detected using western blot. (B) Wound healing assay was applied to measure the conditioned medium effect on scrabbled and Fra-1 shRNA viral infected MCF10A migration.

CM induced MCF10A cells migration is suppressed by a knock down of Fra-1 in MCF10A cells:

To test if the increase of cell migration when the MDA-MB-231 CM is added to MCF10A cells is mediated through Fra-1, Fra-1 shRNA was expressed into the MCF10A cells using retro viral vector. Two of the four shRNA tried showed successful knockdown of Fra-1 and stable cell lines were produced (Figure 11A) The effect of CM on wound healing cell migration assay was evaluated using the wild type versus the Fra-1 knocked down cell lines (Figure 11B). When Fra1 was knocked down in MCF10A cells using Fra-1 shRNA, the ability of CM from MDA-MB-231 cells to increase the migration in MCF10A cells was significantly reduced.

MEK and MLK3 inhibitors suppressed the MDA-MB-231 CM induction of Fra-1 in MCF10A :

In order to address the signal transduction pathway responsible for the induction of Fra-1 by conditioned medium, two different pathways inhibitors were used in order to investigate the role of MEK pathway; the U0126 and PD-98059 MEK inhibitors were used. Both MEK inhibitors were able to alleviate the effect of conditioned medium on the level of Fra-1 in MCF10A cells (Figure 12)



Figure 12: Both MEK inhibitor and MLK3 inhibitor reduced the effect of conditioned medium on the Fra-1 level in MCF10A cells. MCF10A cells were incubated with MDA-MB-231 CM with and without PD98059 and U0126 (MEK inhibitors) (A) or CEP 5147 (MLK inhibitor) (B) The cells was harvested and level of Fra-1 was detected using western blot.

DISCUSSION

In this work we tried to shed more light on the role of Fra-1 and its regulation in the invasive breast cancer cell lines hoping to discover new targets of treatment for these types of breast cancer. Our main focus was on the role of Fra-1 in maintaining the growth of the invasive TNBC cell lines under serum starved state and a potential role of an autocrine loop. The autocrine loop we are exploring is both maintained by and work through Fra-1.

As shown in Figure 1, level of Fra-1 in MDA-MB-231 cells was constitutively high even after 72 hours of serum starvation and persisted at the same high levels at alltime points after serum treatment. This pattern is completely different from that observed in normal MCF10A cells where Fra-1 expression starts later in mid G1. Interestingly the pattern of Fra-1 in MDA-MB-468 cells was intermediate, with an earlier induction (1hr) compared to MCF10A cells.

We have demonstrated that in invasive breast cancer cells (MDA-MB-231), Fra-1 over-expression plays a central role. Fra-1 is known to be essential for allowing cells to go through the cell cycle as it specifically controls cyclin D1 to allow G1/S transition and cyclin A to allow G2/M transition (Burch et al., 2004; Casalino et al., 2003; Cook et al., 1999). This suggested that the properties of Fra-1 in MDA-MB-231 cells may be shared (Chinenov and Kerppola, 2001)that aggressive cell lines of both colon and prostate origins have a Fra-1 expression pattern similar to that of the MDA-MB-231 cells. To the best of our knowledge, the relation between the aggressiveness of cancer and the Fra-1 level when the cells are at the state of

serum starvation hasn't been investigated before. In addition to that the difference in Fra-1 level between the metastatic and the non-metastatic prostate cancer was not published before. These results reflect a universal function of Fra-1 in metastatic cancers which may introduce Fra-1 as candidate for universal cancer treatment. Despite the common strategy nowadays is toward the individualized therapy, the scientists are still hoping to find a universal treatment for cancer (Corcos, 2013).

Because of the persistence of the high level of Fra-1 in MDA-MB-231 cells, we studied its role in keeping the growth of these cells and their progression through the cell cycle under serum starved states. Figure 6 showed that in both MDA-MB-231 and MDA-MB-468 cells, the cells continued to progress through the cell cycle even under serum starvation. However, in MDA-MB-231 cells the degree of the progression was significantly greater. On the other hand as, expected, normal MCF10A cells were completely arrested in G0/G1 phase when under serum starvation. Interestingly, the MDA-MB-231 cells were able to grow in serum free medium up to at least 3 weeks. Figure 8 C quantitates this growth during the 1st 96 hours and shows that the number of MDA-MB-MDA-MB-231 was able to significantly increase when the cells were grown in serum free medium up to 96 hours albeit their rate of growth was slower than cells growing in serum. Fra-1 knockdown shows that Fra-1 plays an essential role in the ability of the cells to grow in serum free medium This finding relates Fra-1 to one of the most fundamental "Hallmarks of cancer" which is " self-sufficiency in growth signaling" (Hanahan and Weinberg, 2000).

Another major finding we discovered is the intrinsic ability of MDA-MB-231 to secrete a Fra-1 inducing factors resulting in a perpetual loop (). These factors have a significant role in controlling migration and proliferation of neighboring cells. The direct proof that these factors function in an autocrine manner to induce self-control of MDA-MB-231 cells is yet to be tested. To study these factors we first co-cultured MDA-MB-231 cells with the MCF10A cells using a transwell system. The other method we used to confirm the presence of such factors is by growing MCF10A cells and MDA-MB-468 cells in CM from MDA-MB-231 cells. In both cases the Fra-1 increased in MCF10A or the MDA-MB-468 cells (Figure 9). Also the migration and proliferation of the MCF10A cells increased in both cases (Figure 10). In addition, Fra-1 knockdown in MCF10A cells reduced the ability of CM in inducing cell migration in these cells (Figure 11). This indicates that Fra-1 could potentially mediate the action of some autocrine factors that is secreted by the MDA-MB-231 cells. We also investigated the pathway in MCF10A that mediates the increase of Fra-1 when treated with CM. Our results suggest the Raf/MEK/ERK and MLK pathways to play some role in this process (Figure 12). Further work is needed to dissect the pathways through which conditioned medium induce Fra-1 in MCF10A cells.

Some studies before showed the role of autocrine factors in the regulation of aggressiveness of breast cancer cell lines and suggested different pathways. For example (Lieblein et al., 2008) suggested a role for STAT 3 in mediating such a loop. In our work we are able to provide evidence for a central role of Fra-1 as a mediator of such an autocrine loop.

MATERIALS AND METHODS

Cell lines:

MDA-MB-231 cell line was a gift from Dr. Basson lab. MDA-MB-231, MCF7 and MDA-MB-468 were maintained in DMEM medium with 10% FBS. MCF10A were obtained as a gift from Conrad lab. They were maintained in DMEM/F12 medium supplemented with 5% horse serum (HS) (Atlanta Biologicals), 20 ng/ml Epidermal Growth Factor (EGF) (Sigma, St. Louis, MO), 100 ng/ml Cholera Toxin (CT) (Sigma), 10 µg/ml Insulin (INS) (Sigma St. Louis, MO), 500 ng/ml hydrocortisone (HC) (Sigma), and 2.5 mM L-glutamine (Mediatech St. Louis, MO).

DU145 and PC3 were a gift from Hollenhorst lab. The former were grown on eagle's minimum essential medium (EMEM) supplemented with 10% FBS. While the later were grown on F12K medium (Life technologies, 21127-022) with 10% FBS.

Colon cancer cell lines were generously provided from Basson lab. Caco2 cells were grown in DMEM medium with 20% FBS. While SW620, SW480 and HT29 were grown on RPMI medium with 10% FBS. The T84 was grown on DMEM/F12 with added 2.5 mM L-Glutamine and 5% FBS.

All cells were grown at 37 °C in humidified 5% CO₂ incubator.

Antibodies:

Fra-1 Antibody (Santa Cruz (N-17), sc-183) and (R-20), sc-605). c-Fos (Santa Cruz (4) sc-52). Phospho-FRA1 (Ser265) (D22B1) Rabbit mAb #5841 . p-c-Jun Antibody (Santa Cruz (KM-1): sc-822). C-Jun antibody (Santa Cruz (D) sc-44). Jun D (Santa Cruz (329), sc-74, rabbit IgG).

Secondary antibody: Goat antirabbit IgG HRP Stress gene SAB300

ECL Sheep Anti mouse IgG HRP Amresham Bioscience UK limited NA931V

926-32213 IRDye® 800CW Donkey anti-Rabbit IgG

926-68072 IRDye® 680RD Donkey anti-Mouse IgG

Cell cycle re-entry:

MCF10A, MDA-MB-231, MDA-MB-468 and MCF-7 were serum starved for 48 hours except MCF10A which was starved only for 24 hours because they are too fragile to be starved for 48 hours. After that one group of the cells was harvested. For the rest of the cells the medium was replaced with new medium with 10% FBS. Then the cells were grown for 1,3,6,12,18 and 24 hours. The cells were harvested the specified time points.

Western blot was conducted using anti c-Fos, Fra-1, pc-Jun, c-Jun and Jun D antibodies.

Flow cytometry was conducted using cells stained with propidium iodide to confirm the different phases of the cell cycle (Data not shown).

Western blot:

Protein assay was done using Biorad DC protein assay kit (Biorad 500-0116) and 25µg of total proteins from each sample were loaded on 12% SDS page gel and run on 60V till they reach the separation gel then continued on 120 V. Then the cells were transferred to PVDF membrane (BioRAD, 162-0177).

The indicated primary antibody was added in a concentration of 1:1000 for 1 hour at room temperature then the membrane was washed three times for 10 minutes each. The secondary antibody was added for 1 hour then washed three times for 10 minutes each.

Nocadazole block:

The cells were plated in 10cm dishes, in four groups; exponential, serum starved, nocadazole (Sigma-Aldrich, St. Louis, MO).for 18 hours and 30 hours. First the exponential cells were harvested then the other groups were serum starved for 48 hours. After which the serum free group was harvested. Later on the nocadazole was added in a concentration of 250 ng/ml to the other two groups. After that nocadazole was added to the other groups for the indicated time periods and then the cells were harvested then fixed with 50 % ethanol then stained with propedium iodide. Finally studied using Flowcytometery.

Co-culture:

Trans well was used to co-culture MDA-MDA-MB-231 cells the MCF10A cells as follow:

In the first day the MDA-MB-231 cells was plated in a density of 6*10^5 cells/well in a 6 well plate.

When the cells reached 70 % confluence the cells were washed twice with serum free medium and then 0.05% FBS medium was added to the cells.

At the same time the medium for the MCF10A was changed to 2% horse serum medium without any other additions.

Next day the membrane was equilibrated by incubation in medium for at least 1 hour. Then 2*10^5 of MCF10A cells were cultured on each trans well membrane in a medium supplemented with 0.05% serum then Incubated overnight.

After 16 hours the trans wells containing the MCF10A cells were transferred to the wells that contain the MDA-MB-231 cells and incubated for 24 hours. Finally the MCF10A cells were trypsinized and then collected in PBS containing 2% serum, then centrifuged and the lysis buffer was added.

Preparation of condition medium:

MDA-MB-231 cells were plated in the 6 wells plates in a density of 7.5*10^5 cells / well, next day the cells were washed twice with serum free medium and then 1.5 ml medium with 0% FBS was added. Then the cells were incubated for 24 hours and the condition medium was harvested and kept at -70 degrees Celsius.

Wound healing migration assay:

MCF10A cells were seeded in 6 well plates in a density of 3*10^5 cells / well , next day (when the cells are 95% confluent)the cells were washed twice with serum free medium then medium with 0% serum medium was added for 24 hours.

Then after 24 hours, a straight wound was made using P200 pipette tips, then washed once with PBS. After that in only one group the conditioned medium was added while in the other group serum free medium was added. In both groups the mitomycin C (Sigma-Aldrich, St. Louis, MO) was added and then pictures were taken and the cells were incubated later for 24 hours after that another group of pictures were taken.

Trans well migration assay:

First the medium was changed for the MCF10A to 2% horse serum medium without any other additions. Next day the membrane was equilibrated by incubation in the medium for at least 1 hour. Then 2*10^5 cells were plated on each trans well. The

cells incubated till they adhere to the membrane and then the medium in the lower chamber was changed to complete MCF10A growth medium.

The medium the lower chamber was changed to condition medium in one group of cells and to serum free medium for the other group then incubated for 1 hours.

Then fixation and staining were done as follow;

First the medium was removed and the membranes were washed twice using ice cold PBS, the cells at the upper surface of the membrane were removed using a cotton swab, then for fixation of the cells 3.7% formaldehyde was used for 20 minutes . The cells were washed three times then using PBS. Staining of the cells was done by crystal violet (0.5 crystal violet in 10 % ethanol) for 10 minutes. Finally it was washed with tape water till the water run clean. The cells were examined later using high magnification and the number of cells was counted per high power field (HPF).

Preparation of the Fra-1 shRNA infected cells:

A- Preparation of retrovirus;

First day, the 293 GPG cells were plated in a density of 1*10^6 in wells of 6 well plate. Next day 1ug of DNA/well was added to 0.5 ml of with 2ul of Opti-mem medium, 2ul of lipofectamine 2000 were added to 0.5 ml of Opti-mem medium and then incubated for 5 minutes at room temperature. The DNA then was combined to the lipofectamine 2000 and then incubated at room temperature for 30 minutes. The medium for 293 GBG cells was then aspirated very gently and the cells were washed with opti-mem twice then the DNA lipofectamine mix was added and incubated for 6 hours after which 1ml of 293GBG transfection medium was added

without antibiotic for overnight incubation. Next day, change the medium to fresh transfection medium on days 4, 5, 6, 7 post transfection, the virus was collected on each day after harvesting at fresh 1.5 ml of transfection medium for next day collection. the virus contain medium was filtered through 0.45 um syringe filter , then the virus was aliquot and stored at -80 degree Celsius .

B- Infection with retrovirus;

The cells were then seeded the night before the infection at density of 5*10^4/ well in 6 well plate. On the day of infection 500ul of the virus contain medium was added to 500 ul of growth medium with 8 ug/ml of polybrene, and then the mixture was added to the cells and incubated for 4 to 5 hours after which 1 ml of growth medium was added for overnight incubation and then in the morning replaced with fresh medium.

Drug selection was done using puromycine at concentration of 1ug/ml.

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CHAPTER THREE

DECIPHERING THE ROLE OF FRA-1 AND OTHER AP-1 FAMILY MEMBERS IN MDA-MB-231 CELLS

INTRODUCTION:

Activating protein -1 (AP-1) is a dimeric transcription factor that is formed of one member of Fos family of proteins and one member of Jun family. The Fos family includes four proteins (c-Fos, Fos related antigen 1 and 2 (Fra-1 and Fra-2) and FosB) while the Jun family contains 3 proteins (c-Jun, JunD and JunB) (Vesely et al., 2009). In normal cells AP-1 shares in regulation of many physiological processes including cell proliferation, migration, apoptosis and differentiation. AP-1 was found since its early discovery to be involved in cancer; however, different members were found to play different roles (Dai et al., 2003; Milde-Langosch et al., 2004). One of the recently discovered features is the very high level of Fra-1 in invasive versus the non-invasive breast cancer cell lines. This high level of Fra-1 was found to be involved in increasing the aggressiveness of these cancer cell lines (Belguise et al., 2005). One report suggested a role of the canonical MEK/ERK pathway in keeping this high level of Fra-1 induced by ectopically high level of PKC- Θ (Belguise et al., 2012). Our previous work showed that a very prominent feature of these cells is the maintained high level of Fra-1 in all stages of the cell cycle and suggested a role of an autocrine loop in keeping Fra-1 level high in these cells (Abd El Fattah et al, Manuscript). In this work we are exploring associated roles of the other members of other AP-1 family. Also we are trying to shed more light on the regulation and distribution of Fra-1 in these metastatic breast cancer cell lines and its role in enhancing the aggressiveness of these cells.

RESULTS:

Regulation of Fos and Jun proteins during the phases of the cell cycle in highly metastatic breast cancer cell lines:

The AP-1 family of proteins have a highly regulated pattern of expression through the cell cycle in normal cells (Cook et al, 1999). This pattern is mandatory for maintaining normal regulation of the cell cycle. Our previous work (Abd El Fattah et al, Manuscript) showed that the Fra-1 protein level in MDA-MB-231 cells is kept high during all phases of the cell cycle including when the cells are under serum starvation. So, it was expected that the pattern of expression of other members of the AP-1 proteins in these cells along the cell cycle will be shifted from normal.

We conducted a full study of the level of Fra-1 and other AP-1 family members including c-Fos, c-Jun, and Jun D in different breast cancer cell lines along different stages of the cell cycle using serum starvation followed by serum stimulation for the time periods indicated (*Figure 13*).

Contrary to the finding of Fra-1, c-Fos showed a similar pattern of expression during cell cycle re-entry in all cell types which was very similar to what was found before in the normal fibroblasts (REF). It showed an increase within one hour of serum stimulation followed by immediate decrease within the next two hours which is early in the G1 phase.



Figure 13: Western analysis of Fos and Jun family members in nontumorigenic and tumorigenic breast epithelial cell lines: : The MCF10A (A), MDA-MB-231 (B), MDA-MB-468 (C) and the MCF7 (D) cells were all subjected to serum starvation then stimulated with serum for the time periods indicated in the figure. Fra-1, c-Fos, c-Jun and JunD protein levels were detected using western blot. Because members of the Jun family are regulated similarly to Fra-1, c-Jun and Jun D expression was also analyzed. Similar to Fra-1, c-Jun showed an equally high level at all stages of the cell cycle including during the serum starved state in MDA-MB-231 cells . However, Jun D was always high in MCF10A even under a serum starved state; while, in MDA-MB-231 cells, it was so low under serum starvation and increased after the induction with FBS.

The increased Fra-1 in MDA-MB-231 cells is both due to increase expression and increased stability of Fra-1:

We asked whether the increase of Fra-1 in MDA-MB-231 is due to increased expression or stability. To this end, we studied the stability of Fra-1 in MDA-MB-231 cells under serum starvation using a cycloheximide chase assay and western blot. The cells were serum pre-starved for 24 hours followed by incubation with cycloheximide as shown in **Figure 14**. The western blot shows that the lower 2 bands of Fra-1 (MW≈34 KD) disappeared early within the first hour. (**Figure 14**: arrow heads). These bands represent the newly synthesized non-phosphorylated Fra-1. However, the upper band, which represents the phosphorylated Fra-1, persisted for up to 24 hours after the cycloheximide addition or for a total period of 48 hours. This means that the stability of Fra-1 in MDA-MB-231 is highly increased. The overall picture from this experiment indicates that the Fra-1 protein is increased in MDA-MB-231 cells due to both an increased expression and stability.


Figure 14: The high level of Fra-1 in MDA-MB-231 cells is due to both increased expression and stability: (A) Western blot from cells that were serum starved for 24 hours and then incubated with cycloheximide for the indicated times. (B) Western blot of Cell extracts from MDA-MB-231 cells was were treated or not treated with SAP (C). Western blot of cell extracts from MDA-MB-231 propped with anti-Fra-1 and anti-pFra-1 antibodies



Figure 15: Fra-1 was found to be both cytoplasmic and nuclear in serum starved MDA-231 cells: (A) Western blot analysis of nuclear and cytoplasmic fractions of MDA-MB-231. (B) Western blot analysis of the chromatin bound Fra-1 versus free Fra-1.

Study of the chromatin bound and nuclear Fa-1 levels showed that Fra-1 is both cytoplasmic and nuclear in serum starved MDA-MB-231 cells: To study the distribution of Fra-1 in MDA-MB-231 cells under serum starvation

we did first nuclear Fractionation which showed that Fra-1 is distributed both in

the cytoplasmic and the nuclear fraction in serum starved MDA-MB-231 cells (**Figure 15** A). Similar results were found when I studied the chromatin bound versus free chromatin in serum starved MDA-MB-231 cells (**Figure 15** B).

A dominant negative form of AP-1 (A-Fos) was able to reduce the aggressive properties of the MDA-MB-231 cells:

We utilized a doxycycline inducible system to express A-Fos in MDA-MB-231 cells. A-Fos was able to suppress the migration of MDA-MB-231 cells both in presence and absence of serum (Figure 15 A). Also induction of A-Fos inhibited the ability of these cells to grow on agar (figure 4 B).





DISCUSSION

In this work we studied the change in the pattern of expression AP-1 family members other than Fra-1 along the cell cycle in the highly metastatic breast epithelial cells. As we did before, we compared it to the non-metastatic ones (MCF-7 and MDA-MB-468 cells) and to the non-tumorigenic MCF10A cells. We found that c-Jun is the one that has a pattern similar, yet to lesser extent, to Fra-1 while Jun D pattern was opposite to Fra-1 and c-Jun.

Also we further investigated the stability of Fra-1 in the MDA-MB-231 cells and its distribution. We found that the stability of the Fra-1 in MDA-MB-231 cells was incredibly increased as compared to the normal cells. Also we detected that Fra-1 in these cells is both nuclear and cytoplasmic. To discern the functional role of Fra-1 in these highly aggressive cell lines we utilized A-Fos which is the dominant negative form of AP-1. The introduction of the AP-1 into the MDA-MB-231 cells leads to the cells' loss of their ability to grow in agar and diminished cell migration.

Results from our previous study showed that MDA-MB-231 cells maintained a high level of Fra-1 over all the stages of the cell cycle (Abd El Fattah et al, manuscript). These results raised two questions. What is the pattern of Fra-1 partners: the interchangeable c-Fos and the essential Jun partners? To answer this question we compared the expression of c-Fos, c-Jun and Jun D in the same cells (*Figure 13*). The pattern of c-Fos expression was indifferent among all the studied cell types as it increased early in the first hour and returned nearly to the pre-stimulated state within 3 hours. On the other hand, c-Jun in MDA-MB-231 cells showed a pattern

similar to Fra-1. However, Jun D showed variations between different cell lines that was opposite to what occurred with Fra-1 and c-Jun. This result suggests a protective role for Jun D in breast cancer. In previous studies, results regarding the role of Jun D in cancer remained controversial (Shaulian, 2010). The overall findings of this experiment suggest that Fra-1 and c-Jun are the main players among the AP-1 members in TNBC cell lines, as previously suggested (Baan et al., 2010; Zhao et al., 2014). To the best of our knowledge this is the first study describing the changes of the cell cycle dependent expression profile of AP-1 family members in cancer cells lines. An earlier study (Mechta et al., 1997) was carried out with a very artificial cancer model; a RAS transformed fibroblast. They found that RAS transformation leads to increased Fra-1 and c-Jun expression in the serum starved state which is refractory to serum stimulation, albeit at much lower levels than what was shown in our model i.e. MDA-MB-231 cells.

Our next question was if the increase of Fra-1 in MDA-MB-231 cells is due to increased expression or decreased rate of degradation. To answer this question, we utilized a cycloheximide stability assay which showed that the high level of Fra-1 in MDA-MB-231 cells is due to both increased expression and increased stability (**Figure 14**). The phosphorylated band of Fra-1 persisted for up to 48 hours after cycloheximide addition, which is 8 times the half-life of Fra-1 in normal fibroblast after serum stimulation. However, with the serum free medium in normal cells Fra-1 half-life was reduced to one hour only (Gruda et al, 1994). More work is needed to confirm these results.

In order to understand more the role of this high level of Fra-1 in advancing the progression of these cells into metastasis, we studied the distribution of Fra-1 inside the MDA-MB-231 cells. Fra-1 shows different distribution in different cell lines. In normal cells the distribution of Fra-1 is mostly nuclear. However, in cancer cells the distribution is both nuclear and cytoplasmic (Song et al., 2006). This was confirmed in our results. In addition, western blot study of the nuclear versus cytoplasmic fraction of Fra-1 showed a slight electrophoretic shift in the nuclear protein versus the cytoplasmic one which may reflect a difference in the degree of phosphorylation.

MATERIALS AND METHODS

Cell lines:

MDA-MB-231 cell line was a gift from Dr. Basson lab. MDA-MB-231, MCF7 and MDA-MB-468 were maintained in DMEM medium with 10% FBS. MCF10A were obtained as a gift from Conrad lab. They were maintained in DMEM/F12 medium supplemented with 5% horse serum (HS) (Atlanta Biologicals), 20 ng/ml Epidermal Growth Factor (EGF) (Sigma, St. Louis, MO), 100 ng/ml Cholera Toxin (CT) (Sigma), 10 µg/ml Insulin (INS) (Sigma St. Louis, MO), 500 ng/ml hydrocortisone (HC) (Sigma), and 2.5 mM L-glutamine (Mediatech St. Louis, MO).

All cells were grown at 37 °C in humidified 5% CO₂ incubator.

Antibodies:

Fra-1 Antibody (Santa Cruz (N-17), sc-183) and (R-20), sc-605). c-Fos (Santa Cruz (4) sc-52). Phospho-FRA1 (Ser265) (D22B1) Rabbit mAb #5841 . p-c-Jun Antibody (Santa Cruz (KM-1): sc-822). C-Jun antibody (Santa Cruz (D) sc-44). Jun D (Santa Cruz (329), sc-74, rabbit IgG).

Secondry antibody: Goat antirabbitIgG HRP Stress gene SAB300

ECL Sheep Anti mouse IgG HRP Amresham Bioscience UK limited NA931V

926-32213 IRDye® 800CW Donkey anti-Rabbit IgG

926-68072 IRDye® 680RD Donkey anti-Mouse IgG

Cell cycle re-entry:

MCF10A, MDA-MB-231, MDA-MB-468 and MCF-7 were serum starved for 48 hours except MCF10A which was starved only for 24 hours because they are too

fragile to be starved for 48 hours. After that one group of the cells was harvested. For the rest of the cells the medium was replaced with new medium with 10% FBS. Then the cells were grown for 1,3,6,12,18 and 24 hours. The cells were harvested the specified time points.

Western blot was conducted using anti c-Fos, Fra-1, pc-Jun, c-Jun and Jun D antibodies.

Flow cytometry was conducted using cells stained with propidium iodide to confirm the different phases of the cell cycle (Data not shown).

Western blot:

Protein assay was done using Biorad DC protein assay kit (Biorad 500-0116) and 25µg of total proteins from each sample were loaded on 12% SDS page gel and run on 60V till they reach the separation gel then continued on 120 V. Then the cells were transefered to PVDF membrane (BioRAD, 162-0177).

The indicated primary antibody was added in a concentration of 1:1000 for 1 hour then the membrane was washed three times for 10 minutes each. The secondary antibody was added for 1 hour then washed three times for 10 minutes each.

Cycloheximide chase assay:

The MDA-MB-231 cells were plated in 10 mm cell culture plates till they are 90% confluent then the cells were washed twice with serum free medium then the cells were incubated for 24 hours. One group of plates was harvested as control (0 point). The rest of the cells were divided into two groups. The 1st group DMSO was added and to the second group cycloxeimide (Sigma-Aldrich, St. Louis, MO) was added.

The plates from both groups were harvested after 1, 6, 12, 24 hours and Fra-1 level was compared using western blot.

Nuclear Fractionation:

Two buffers were prepared. Buffer A consisted of 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.05% NP40 , pH 7.9 while buffer B was formed of 5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH 7.9.

1 ml of buffer A was prepared with added cocktail of ant proteases and ant phosphatases A 500 µl of buffer a was added per 100mm plate of cells on ice and scraped thoroughly, left on ice for 10 min. Then the samples were centrifuged at 4°C at 3000 rpm for 10 min. 4. Remove The supernatant was removed and kept (this will contain everything except large plasma membrane pieces, DNA, nucleoli) and 10 µl were saved for Bradford assay. On ice the pellets were re-suspended in 374 µl of buffer B with 300mM NaCl (high salt helps lyse membranes and forces DNA into solution). The samples were homogenized by passing in 30 G syringe needles several times. Kept on ice for 30 min. then centrifuged at 24,000 g for 20 min at 4°C. The supernatant was liguated and saved at -70.

Chromatin bound proteins:

About 4*10^ 6 cells per sample were rinsed with ice cold PBS then washed with ice-cold CSK buffer then the cells were scraped from the plate. The extract was centrifuged at 500x g then the CSK-Triton buffer was added to the pellet at 10^7 cells/ml then incubated for 10 min on ice. After that the nuclei were pelleted (designated fraction P1) by centrifugation at 1,500Xg for 5 min at 4°C. The

supernatants (fraction S1) (cytoplasmic and un- bound nuclear proteins) were removed and further clarified by centrifugation at 16,000X g for 10 min at 4°C. The pelleted nuclei then were washed with 1 ml of CSK-Triton buffer, pelleted by centrifugation then suspended in CSK-Triton buffer at 10^7 nuclei/ml.

The washed nuclei were then used for Western blotting analysis directly or treated with nuclease (10^7 nuclei/ml in CSK-Triton buffer containing 160 U of DNase I/ml and 50 mM MgCl2 on ice for 10 min) to release chromatin-bound proteins after. Nuclear remnants were then pelleted by centrifugation as before, and the proteins released into the superna- tant (fraction S2) by the nuclease treatment were separated from the proteins remaining in the pellet (fraction P2).

CSK buffer (10 mM HEPES [pH 7.4], 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2).

CSK-Triton buffer: (CSK buffer containing 0.5%TritonX-100, and Proteinase and phosphatase inhibitors cocktails

Production of doxycycline inducible Flag/afos stable MDA-**MB-231 cell line**: To generate inducible Lentiviral vector pINDUCER20-Flag-AFos, the Flag-AFos fragment was excised as a 321bp Sal1-EcoR1 from pCMV-500 Flag- AFos (a gift of Dr.Schwartz, Richard, MSU) and cloned into Sal1-EcoR1 cleaved pENTR1A (a gift of Dr. Brian Schafhausen, Tufts Medical School) to generate pENTR1A-Flag-AFos. Then Flag-AFos was recombined into pINDUCER20 (a gift of Dr. Brian Schafhausen, Tufts Medical School) from pENTR1A-Flag-AFos by Gateway LR clonase II enzyme mix (Life Sciences). Lentiviral supernatants were generated by transient transfection of psPAX2, pMD2.G (gifts of Dr. Kathey Gallo, MSU.)

pINDUCER20-Flag-AFos to 293T cells according to Invitrogen Lipofectamin 2000 transfection protocol and harvested 48h after transfection. Stable cell lines MDA-MB-231 expressing indicated Flag-AFos were generated by lentiviral transduction in the presence of 8ug/ml polybrene followed by selection with G418 (Meerbrey et al., 2011).

Wound healing migration assay:

MDA-MB-231 cells were seeded in 2 of the 6 well plates in a density of 5*10⁵ cells / well. Next day the cells of one of the plates were washed twice with serum free medium then medium with 0.05% FBS medium was added. Doxycylin was added to 3 wells in each of the plates then all the cells were incubated for 48 hours. After 48 hours, straight wounds were made using P200 pipette tips, then washed once with PBS and pictures were taken. The cells were allowed to further grow for 24 hours then pictures were taken again for the same points as the first ones. The surface area was calculated using the image-J linked T-scratch bundle.

Growth on soft agar:

Inducible MDA-231/Flag-AFos was cultured in DMEM containing 10% FBS medium with and without the addition of doxycycline (final 50µg/mL) for 48 hours. Then single-cell suspensions of 2.00 x 105 cells were plated per 60-mm culture dish in 2 mL of DMEM, 10% FBS and 20% agar with and without doxycycline (final 50µg/mL)

on a layer of 6mL of the DMEM containing 5% FBS and 30% agar with and without doxycycline (final 50µg/mL). Three weeks after plating photographs of the colonies were taken. During the three weeks 200µL of DMEM containing 10% FBS with and without doxycycline (final 50µg/mL) was added twice weekly for feedings.

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REFERENCES

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regulated transcription provides insights into the invasiveness of triplenegative breast cancer. *Cancer Res.* 74:3983-3994. **CHAPTER FOUR**

SUMMARY AND FUTURE WORK

SUMMARY AND FUTURE WORK

Expression of the Fra-1 transcription factor is increased in metastatic relative to non-metastatic breast cancer cell lines and plays an important role in the metastatic ability of these cells. Scientists are still far behind in finding the appropriate target for therapy in these highly metastatic types of cancer. Understanding the significance and the regulation of Fra-1 in these cells is important for finding new targets.

In this work we compared the protein expression levels of Fra-1 and other members of the AP-1 family during cell cycle re-entry in non-tumorigenic MCF10A cells and different breast cancer cell lines. A major finding was that, in MDA-231 cells, Fra-1 and c-Jun show excessive level of expression under serum starvation that persists all through the cell cycle. This is compared to the MCF10A and MDA-MB-468 cells that showed a reduced level of Fra-1 under serum starvation followed by increase of the protein level after serum induction that persisted till the late G1 (about 18 hours after induction). However, JunD showed an opposite picture that it was expressed all the time in MCF10A cells even under serum starvation while it was inhibited by serum starvation in cancer cells. Our next step is to examine the level of the AP-1 family members in the exponentially growing cells. In addition we are going to study the regulation of AP-1 members at the transcriptional level in same cell lines during cell cycle re-entry. Also because c-fos showed a pattern that looked nearly similar in all cell lines, we decided to ask whether c-fos still play a role in regulation of Fra-1 in MDA-231 cells by using c-fos-shRNA. Another point to resolve is the

significance of the reversed picture of the JunD and if it plays any role as a tumor suppressor.

Besides studying the expression level of AP-1 members as a function of cell cycle, we also studied the stability and the spatial distribution of Fra-1 in MDA-231 cells. Our results showed that Fra-1 is highly stable in MDA-231 cells compared to previous reports about the half-life of Fra-1 in normal rat fibroblasts. Furthermore, the persistence of the Fra-1 in these cells is attributed to increment of both expression and stability. In addition to that the distribution of Fra-1 was found to be both nuclear and cytoplasmic which is contrary to normal cells that showed Fra-1 is only nuclear (Ref). Our plan is to study the stability and distribution of Fra-1 in both MCF10A cells and MDA-MB-468 cells to compare to the MDA-MB-231 cells.

In the next step we showed that the MDA-231 cells does not arrest in the G0/G1 stage of the cycle under serum starvation using Nocodazole block . To test if this progress is mediated by AP-1 we utilized the dominant negative form of AP-1 that is called A-Fos . The expression of A-Fos in MDA-231 lead to partial suppression of the power of the MDA-231 cells to progress through the cell cycle under serum starvation. In addition knocking down Fra-1 using Fra-1 shRNA inhibited the ability of these cells to grow in serum free medium. We plan to study the relation between the amount of Fra-1 and the distribution of the cells along the cell cycle phases using double staining with PI and Fra-1 antibody. The aim is to get a more accurate evaluation of the effect of the absence of Fra-1 on the progression through the cell cycle.

We additionally used A-Fos to study the role of Fra-1 in inducing the oncogenic and metastatic properties of MDA-231 cells. First we studied the long term growth of these cells in absence AP-1 activity. When the cells are passed for several times in presence of A-Fos they undergo a decrease in number compared to the control cells. After the third passage all the cells carrying the A-Fos die in crisis. Cells that resist death are further grown but when they are induced by Doxycycline they fail to express A-Fos. Our plan is to use apoptosis and senescence specific markers to address the cause of growth delay in these cells after expression of A-Fos.

Further work is needed to test if the delayed growth of these cells by A-Fos is a mere arrest of the cells in the G0 stage or associated with cell death which could be due to direct induction of apoptosis or associated with cell senescence followed by crisis. To address this issue we are going to use some apoptosis and cell senescence assays.

Second, we studied the effect of A-Fos induction on migration of MDA-231 cells using wound healing assay. This experiment showed reduction of the migration ability of the MDA-231 cells after A-Fos induction. Again MDA-231 cells lost the ability to grow on soft agar when A-Fos was induced.

Further work is needed to unravel the mechanisms through which AP-1 enhances these malignant properties. *Utilizing A-Fos enables us to study different genes that control these processes* and how their expression is affected by the absence of AP-1 function. Also we can use Fra-1shRNA or c-junshRNA for more specific exploration of the role of the different members of the AP-1 family. This work will give us more solid

conclusion about the central role of AP-1 in these highly malignant cancer cells and its value as a target for therapy. From this we would move to trying oligonucleotide (ODN) therapy against AP-1 as a new cancer therapy for the highly aggressive types of breast cancer .

The current study also revealed an autocrine loop as a suggested mechanism of Fra-1 persistence in MDA-231 cells under serum deprivation status. Such mechanism was suggested by the elevation of the Fra-1 level in MCF10A and MDA-MB-468 cells after either co-couture with MDA-231 cells or incubation with the condition medium of the MDA-231 cells. Additionally both conditioned medium from MDA-231 and coculture with MDA-231 increased migration of MCF10A and MDA-MB-468 cells. However, when we knockdown Fra-1 in MCF10A using Fra-1shRNA this effect on migration has diminished indicating that Fra-1 shares in mediating the effect of CM on MCF10A cells. Also, induction of A-Fos expression in MDA-231/flag-afos cells attenuates the ability of the MDA-231 CM to induce Fra-1 in MCF10A and MDA-MB-468 cells. This points out the role of Fra-1 in regulating the factors that is secreted by MDA-231 cells to mediate the autocrine loop.

Finally we were able to show that the high level of Fra-1 during serum starvation in metastatic cell lines is not restricted to breast cancer cell lines. We found similar properties in both aggressive colon and prostate cancer cell lines. Work is in progress to test the other properties of Fra-1 in these other types of cancer. Additionally, we are planning to examine more types of cancer like melanoma and lung and liver cancer cell lines. These findings points to a universal role of Fra-1 in inducing cancer metastasis and progression of cancer. This would add more value to a new therapy

based on the suppressing the action of AP-1 in highly aggressive cancers like for example the use of AP_1 specific ODNs.