Cross-Talk between IGF/IGFR and Psoriasin (S100A7) Enhancing Growth and Metastasis of Breast Cancer

THESIS

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Abstract

Breast cancer is the most common type of malignant tumors and the second most common cause of cancer-related deaths in women worldwide. Breast cancer metastasis is the main cause of death from this disease. Psoriasin (S100A7), a member of the S100 family proteins, is characterized by having calcium-binding domains and is involved in multiple physiopathological processes in the cell such as cell cycle progression and cellular differentiation. Overexpression of S100A7 is a well known factor for inducing progression of the preinvasive to invasive form of breast cancer and its presence is correlated with poor prognosis. Insulin-like Growth Factor (IGF), especially types 1 and 2, has also been shown to play an important role in cellular growth and proliferation of breast and other types of cancer. The purpose of this study was to investigate the cross-talk between S100A7 and IGF-induced signaling mechanism on breast cancer progression and metastasis. We have shown that overexpression of S100A7 in ERα− breast cancer cell lines MDA-MB-231 and SCP6 enhances IGF-induced growth, wound healing, chemotaxis and chemoinvasion. MDA-MB-231 overexpressing S100A7 cell lines showed enhanced metastasis compared to vector control cells. Furthermore, delineation of signaling mechanisms revealed that S100A7 overexpression also enhanced
IGF-induced IGFR phosphorylation and activation/phosphorylation of downstream signaling molecules Akt and ERK. These studies suggest that S100A7 may modulate breast cancer progression and metastasis through enhancing IGF-induced signaling and activation of cell survival molecules Akt and ERK, which have been shown to enhance proliferation. In summary, our studies indicate that crosstalk between S100A7 and IGF/IGFR-induced signaling enhances metastatic potential and proliferation of breast cancers.
Dedication

To my beloved parents who raised me to seek all kinds of knowledge and wisdom, to my sincere wife who supported me without waiting for the reward, and to all Libyans who paid their souls for our beloved country Libya, I dedicate this work.
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I would like to sincerely thank Dr. Ramesh K. Ganju for all his kind support and help, and for being my advisor in my master studies, Dr. Xue-Feng Bai, for his kind acceptance to be in the Master’s Examination Committee, Dr. Mohd Nasser for helping me master many laboratory techniques which were vital to complete my research, and Yadwinder Deol for all his kind advising in my experiments. I also would like to extend special thanks to Dr. Virginia Sanders and Elizabeth Grubenhoff at the IBGP program for their limitless support and kind help in my master courses.
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CHAPTER 1: INTRODUCTION

BREAST CANCER:

Breast cancer is the most common malignant tumor, and the second most common cause of cancer-related deaths in women. Breast cancer metastasis is the main cause of death from this disease. The incidence of breast cancer is more than a million cases per year worldwide\(^1\). There is estimated to be more than one hundred thousand new diagnosed breast cancer cases in the United States annually. One-third of women diagnosed with breast cancer will die because of this disease. Western countries in North America and Northern Europe have a higher incidence compared to intermediate countries in Southern Europe and Latin America. However, the countries with lowest of breast cancer are in Africa and Asia. The widespread use of mammography in the United States made a linear increase in the early detection of the breast cancer cases\(^2\). However, even with the early detection of the tumor, the survival rate has not widely changed from the 1930s to the early 1990s\(^3\). However, in North America, Western Europe, and Australia, deaths from breast cancer have started to decline through the combined actions of early cancer detections and the increased efficacy of the cancer treatment\(^4\) by using new biological agents which mostly work on blocking signaling pathways in many cancers.
There are several established risk factors for breast cancers, and others continue to be investigated\(^5\). An important risk factor for malignant mammary neoplasia is prolonged and/or strong estrogen stimulation to breast tissue especially in genetically susceptible people\(^6\). It has been shown that steroid hormones, progesterone, estrogen and growth factors acting through receptors can drive the development and progression of breast cancers\(^7\). A woman with a first-degree-relative with breast cancer has two to three times higher risk of breast cancer than the general population, and this risk is increased further if a relative had bilateral tumors and/or had breast carcinoma at an early age\(^8\). Other risk factors are early menarche, nulliparity, late age of first parity and late menopause \(^9,6\). Prolonged exposure to exogenous estrogen has also shown a two and half fold increase in the overall risk for breast cancer\(^9\). A new study was done on women who were on hormone replacement therapy, and the study shows that the breast carcinoma risk is highly increased in those women compared to the control women\(^10\).

There are two important features to determine the microscopic types of breast cancer. First, breast cancer is defined by extent of invasion. That is breast cancer limited to the epithelial layer of the breast is called “carcinoma in-situ”, while breast cancer that extends beyond the epithelial layer, through the basement membrane, and invades into the surrounding stroma which is called “invasive carcinoma”. Second, breast cancer is classified according to histological origin and may fall into one of two categories: lobular or ductal carcinomas. This breast cancer nomenclature was taken from the Wellings et al study; ductal carcinoma indicates tumor arising from the breast ductal system, and lobular carcinoma indicates a tumor originating from the breast lobular
However, carcinoma in-situ could be further subtyped into “ductal Carcinoma in-situ” (DCIS), or “lobular Carcinoma in-situ” (LCIS).

Five percent of all breast carcinomas or about two thirds of familial breast cancer have been linked to two genes alterations. These two genes are BRCA1 and BRCA2. BRCA1 is located on chromosome 17q, and BRCA2 is located on chromosome 13q-12. Additionally BRCA1 shows a predisposition to ovarian and fallopian carcinomas.

Breast cancer molecular analysis has shown a large number of genes that are differentially expressed from the progression of benign to malignant cells with the potential for metastasis. Early breast cancer diagnosis has raised the requirement for identifying the molecular alterations which could serve as tissue markers to detect the potential for invasion and metastasis.

**S100 PROTEINS:**

S100 proteins are a family with multi-gene calcium-binding sites. S100 proteins consist of 18 human gene members each encoded by a separate gene. The presence of two Ca\(^{2+}\)-binding motifs of the EF-hand type is a major characteristic of S100 proteins. Intracellurally, the vast majority of S100 proteins are homodimers in which two related monomers are connected by non-covalent bonds and are related in space by a two-fold axis of rotation as shown in (Figure. 1). The N-terminal ends of S100 proteins have Ca\(^{2+}\)-binding EF-hand motifs which are called helix-loop-helix (HLH) domains. The domains have also been called “half-EF-hands” and are the sites where Ca\(^{2+}\) binds with only with a weak affinity. However, the domain at the C-terminal end has a one hundred times higher affinity for Ca\(^{2+}\) binding as compared to the N-terminal.
This relation was shown in each of the S100A4, S100A6, S100A7, S100A8, S100A10, S100A11, S100A12 and S100B proteins. This unique characteristic was detected by using nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, and by using multiple wavelength dispersion (MWD) \(^{22}\). Many S100 proteins are implicated in many cancer processes by altering their level of expression in malignant cells in comparison with the normal cells.

**Figure 1. Structure of the S100 dimer, (A) Ca\(^{2+}\)-free and (B) Ca\(^{2+}\)-loaded S100B2 dimer, which is taken as an example of S100 dimer.** The S100B monomer is in yellow and the other monomer is in blue. In Roman numerals are helices, (I-IV in one monomer, and I’-IV’ in the other monomer). Ca\(^{2+}\) binding causes the S100B monomers shift helix III in relation to the other helices and leads to the reorientation of the hinge region (H), as seen when comparing figures A to B \(^{19}\).
Several studies have been shown that dimers of S100 proteins have the same functional and structural motifs. However, the main difference is in the primary sequence of each helix is the region of the hinge and/or the extension of the C-terminal with structural changes in the interhelical angles in the dimers loaded with Ca\(^{2+}\). This may have an importance in recognizing the target proteins and specifying the individual roles of S100 members. Ca\(^{2+}\) binding causes the hydrophobic residues to become exposed, and it may represent the S100 site which used to link the target proteins. However, the hydrophilic residues for individual S100 proteins may play an important role in stabilizing S100 protein interactions with target protein\(^{19}\).

Calcium is considered the most common universal second messengers that work intracellularly. It acts on many physiological regulatory and stimulatory functions, such as nerve impulse conduction, transmission, and muscle contraction. However, calcium has a vital role in gene expression, enzymes interactions, cellular growth and differentiation, and programmed cell death. Furthermore, calcium plays an integral role in tissue necrosis\(^ {19}\).

Recent studies have suggested that S100 proteins which are Ca\(^{2+}\) sensing proteins are important in Ca\(^{2+}\)-dependent regulatory functions. These include protein phosphorylation, activation and regulation. S100 proteins also participate in the inflammatory response, contraction-relaxation cycle, calcium hemostasis, and cytoskeleton component dynamics. S100 proteins directly interact with tubulins, intermediate filaments, actin, myosin and tropomyosin\(^ {23}\). Furthermore, S100 proteins have a proliferating function in the cells and they participate in transforming cells from
benign to malignant. Figure (2) demonstrates the various functions of S100 proteins\textsuperscript{19}.

Figure 2. Intracellular and extracellular functions of S100 proteins which are regulated through Ca\textsuperscript{2+} dependent binding mechanisms.\textsuperscript{19}

Most of the S100 proteins have been shown to interact with p53\textsuperscript{19} and modulate p53 activity. S100A4 and S100B were shown to inhibit p53 phosphorylation, which in turn reduced its transcriptional, and ultimately tumor-suppressor activity. On the other hand, S100A2 has been shown to increase the transcriptional activity of the cells\textsuperscript{24}. S100A4 has been shown to enhance p53-dependent apoptosis. Therefore, functional determination of S100 proteins within the cell is dependent on the balancing effect of these proteins\textsuperscript{18}.
Despite the high degree of sequence homology between S100 proteins, they are very unique in regard to their biochemical pathways and their distribution in different tissues. An important finding is related to the extracellular interaction of S100 with RAGE and stimulation of several biological signaling pathways. Most importantly, RAGE expression is present in pulmonary and breast neoplasia (Hsieh et al. 2003). As shown in (Figure 3) various intracellular effects of S100 and RAGE have been shown intracellularly that S100 proteins also interact with p53. As a result of this interaction, p53 cannot bind to the target sites in gene promoter regions, and is therefore unable to initiate gene transcription activation\textsuperscript{19}.

\textbf{Figure 3. S100 and RAGE interaction}, many of the S100 proteins are found in nuclear and cytoplasmic compartments and they are excreted into the extracellular compartment. Many extracellular activities are RAGE mediated and sequentially involve the MAPK pathway. Finally, the result of pro-survival/ anti-apoptotic genes are regulated by NF-\textkappa B transcription factors. Intracellular functions of S100 could be mediated by transcription factors interactions. As S100A7 interacts with Jab1, this leads to Jab1 redistribution from cytoplasm to the nucleus. Nuclear Jab1 further participates in many diverse nuclear pathways. For example, it interacts with Bcl3 to stimulate NF-kB dependent transcription and also, with c-jun to stimulate AP-1 related genes\textsuperscript{19}.
Many of the p53-related genes are pro-survival genes. In other cases, p53
sequesters calcium in the cytoplasm, which thereby prevents cells from completing
apoptosis. S100A4 has a continuous role in promoting tumor invasion primarily by
interacting with proteins and other genes than p53.

**PSORIASIN (S100A7):**

A new member of the S100 protein family is called Psoriasin (S100A7). S100 A7,
along with 16 other S100 genes, have been shown to form a group of clusters at
chromosome 1q21, which is known as the “epidermal differentiation complex.” The
S100A7 gene is located on chromosomes 1q21 and 2q-22. The S100A7 was identified as
11.4 kDa protein and has a common homology with and close chromosomal proximity
with the other genes in the S100 family. S100A7 has been shown to be induced in the
epidermal squamous epithelial cells from skin with psoriasis. S100A7 was firstly
described to be highly expressed at the mRNA level in the inflamed tissue of psoriasis
(madsen et al. 1991).

S100A7 protein has an N-terminal calcium binding site as it does not have the
space that is provided by the additional amino acids in comparison to the other S100
genes. Based upon S100A7 crystal structure and the sequence of its amino acid,
S100A7 is one of the most unique proteins in the family of S100 Proteins. S100A7
expression was found to be present in both intracellular and extracellular compartment,
and was exclusively produced in the epithelial layer.

Although the physiologic function of S100A7 is not yet well known, the S100
proteins are thought to have an effect on calcium mediated signal transduction and other
physiologic cellular events by direct interactions with target proteins. Furthermore, S100A7 works as a chemotactic agent that attracts polymorphonuclear cells and CD4⁺ lymphocytes to the epidermal surface of the skin, leading to the infiltrate characteristics of psoriasis. Figure (4), demonstrates the biological function of S100A7 and its effect on the immune cells.

Figure 4. A representation to demonstrate the possible effects of altered expression of S100A7 on immune and epithelial cell motility by intracellular actions and extracellular actions in psoriasis pathogenesis and breast cancer invasion.

S100A7 has a strong association with pre-invasive cancers including squamous cell carcinomas of the head and neck, skin, the cervix, mammary gland and the
S100A7 expression is correlated with its chemotactic functions in the inflammation, as firstly hypothesized for psoriasis\textsuperscript{29}. S100A7 is not expressed in the normal epithelial tissues. However, its expression increases during the initial steps of tumor progression. Cellular hyperplasia and atypical hyperplasia are associated with increased S100A7 expression. However, the main expression is in the pre-invasive carcinoma in-situ especially in ductal carcinoma in-situ (DCIS). S100A7 has emerged as one of the highly expressed preinvasive carcinoma genes by using relatively unbiased serial analysis of gene expression (SAGE) assays to detect the global expression levels\textsuperscript{30}. This pattern of S100A7 expression is seen in both in DCIS and invasive carcinoma. Results of an experimental study were performed at our laboratory. Figure (5) illustrates the high expression level of S100A7 in both DCIS and invasive carcinoma.

![Figure 5. Expression of S100A7 in human breast tissue microarray (TMA).](image)

Paraffin-embedded, formalin-fixed specimens were analyzed for S100A7 by immunohistochemistry. (A) Normal breast tissue (B) Strongly (+) Invasive carcinoma, micropapillary (no DCIS) (C) Strongly (+) invasive carcinoma (no DCIS) (D) Focally strongly (+) invasive carcinoma (E) DCIS, invasive carcinoma is only focally (+) (F) High grade DCIS (no invasive carcinoma).

There is little information on the regulatory factors and processes that control S100A7 actions. However, there are some strong factors that are considered as positive factors that lead to breast and skin tumors. These factors include UV exposure, serum

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depletion, and decrease in substrate attachment\textsuperscript{31}. The stimulatory processes may be mediated via estrogen receptors (ERs) and/or receptors of the retinoic acid or activator protein-1 (AP-1)\textsuperscript{28,32,33}. Those precancerous factors are also dominant in invasive ductal carcinoma of the breast. As their levels increase, the anoxic insult increases and the cellular polarization and attachment to the basement membrane decrease\textsuperscript{21}.

Several studies have shown the main role of S100A7 in early tumor progression\textsuperscript{17,31,34}. These studies have shown expression of S100A7 has a strong association with the absence of the expression of the estrogen receptors (ER). Previous studies have shown the strong association with ER-negative with breast carcinomas, in comparison to ER-positive tumors. S100A7 expression in invasive breast carcinomas is mostly a sign of poor prognostic outcome. S100A7 is expressed highly in the DCIS, however, this direct correlation with the prognosis is much difficult to recognize\textsuperscript{34}. Figure (6), illustrates the relationship of S100A7 expression and Estrogen effect on the breast cells\textsuperscript{35}.

S100A7 is connected with many proteins throughout the in-vitro interactions. One of the most common interactions is homodimerization\textsuperscript{36}, and the important interaction with \textit{c-jun} activation domain binding protein 1 (Jab 1)\textsuperscript{37}. This basically occurs in the breast cancer cells and the interaction can be diminished by a mutation in the amino acid at the binding site and decreases the physiological effects of that interaction\textsuperscript{38}. 
Figure 6. Expression of S100A7 has a strong association with the absence of the expression of the estrogen receptors (ER). Effect of estrogen on S100A7 in a time-dependent manner after doxycyclin induction of ERβ. (A) MCF7-β (clone c1 shown; similar results are obtained using clone c3) cells depleted of estrogen were plated in 5% CS, left overnight and then treated for 48 h with 0.75 μg/ml doxycycline (Dox +) or left untreated (Dox -), followed by treatment with 5 nM E2 or vehicle alone for the times (hours) indicated. 100 μg of protein per sample was assayed for psoriasin/S100A7 expression by western blotting. (B) Cells from several different MCF7-β clones (c1, c2, c3, c4) and parental MCF7 rTA alone expressing control (rTA) were depleted of estrogen, then treated with (+) or without (-) 0.75 μg/ml doxycycline for 48 h (top panel, western blot for ER β) followed by 5 nM E2 for 72 h. 100 μg of cell lysate was assayed for S100A7 or ER β; M= molecular mass marker; actin serves as protein loading control.

This interaction leads apparently to redistribute Jab1, leading to its accumulation in the nuclear compartment and stimulation of Jab1. The other observation was found that S100A7 transfected ER-negative mammary cells may show an increase in the growth and the ability to invade in the in-vitro assays. Figure (7) shows the increase in the Jab1 expression in the nuclear compartment with increased S100A7 and EGFR expression levels in breast carcinomas, as was illustrated by using the immunohistochemistry in breast cancer tissue.
Figure 7. Shows expression of Jab1 in the nuclear compartment parallel to EGFR and S100A7 increment in expression in the breast cancer tissue. Levels of expression of Jab1, EGFR and S100A7 were detected by using the immunohistochemistry method in the breast cancer tissue. Tumor number 188 shows increase in nuclear Jab1 expression with high EGFR and S100A7 expression. Tumor number 226 shows cytoplasmic and nuclear intermediate level of Jab1 expression correlated to intermediate EGFR and no S100A7 expression. Tumor number 230 shows decreased nuclear but increased cytoplasmic Jab1 expression associated with the absence of EGFR or S100A7 expression.

INSULIN-LIKE GROWTH FACTOR RECEPTORS (IGFR):

Insulin-like growth factor-I (IGF-1) is a polypeptide which has both a circulating hormone and tissue growth factor characteristics. Most of IGF-1 is produced hepatically and regulated by anterior pituitary gland growth hormone. Besides the nutritional factors, insulin has an important role in regulating the IGF-1 factor. However, some studies have shown that IGF-1 could be released from the tissue directly as it has paracrine and autocrine functions besides the endocrine physiological activities. IGF-1 has an important physiological role in postnatal growth and neonatal growth. On the other side, IGF-1 has important roles in growth, metastasis and increase the incidence of some invasive carcinomas such as breast carcinomas. Physiologically, insulin is a well known
anapolic hormone which works on liver, muscle and fat tissues. It is primarily influenced by the glucose level and glucose metabolism. However, many recent studies show its important effect on proliferation, anti-apoptosis and mitogenicity.

Insulin-like growth factors (IGFs) act mainly through a Protein Tyrosine Kinase (PTK) known as type 1 insulin-like growth factor receptor (IGF-1R). The system of insulin-like growth factors consists of soluble ligands such as IGF-1, IGFR1-R and insulin; multiple receptors, and binding proteins. There are some receptors belong to the tyrosine kinase signaling system as IGF-1R receptor which is related closely to insulin receptor (IR).

On the physiological aspects, activation of IGF-1R is important in the fetal growth. IGF-1 complex system is basically consists of ligands as IGF-1 and IGF-2 with their related receptors, IGF binding proteins and the signaling pathways starting from IGF-1R and Insulin receptor (IR). This signaling pathway is very important in physiological cellular growth and tissue differentiation. However, this also plays an important role in metaplasia and neoplasia. The IGF-1R axis of signaling basically consists of the two receptors (IGF-1R and IGF-2R), IGF-1 and IGF-2 ligands, and minimally six binding proteins with ligand modulating proteases. As illustrated in Figure (8), circulating IGF-1 is mainly released by liver with growth hormone stimulation.
**Figure 8. IGF signaling system,** IGF-1 and IGF-2 are able to bind and excite the activity of IGF-1R. The bioavailability of IGF-1 is remodeled by IGFBPs family. However the bioavailability of IGF-2 is remodeled by both IGFBPs and binding to IGF-2R. As IGF-1 or IGF-2 binds to IGF-1R, the receptor goes into receptor cross-linking and autophosphorylation, creating many docking sites for IRS-1, IRS-2 and Shc. This activate class I phosphatidylinositol 3' kinase which activate PDK1 and mTOR-containing complex mTORC2 lead to activate AKT has many cellular activities as disinhibiting of mTORC1 and elevate protein synthesis level and eventually cell growth. Increase in glucose to glycogen biotransformation through GSK-3β, and in survival and proliferation rate through foxo transcription factors46.

IGF-1 and IGF-2 are able to stimulate IGF-1R clustering and causes autophosphorylation. This leads to stimulation of the downstream pathways. On the other side, the IGF-2R is not able to stimulate any further downstream signaling pathways. However, it has a regulatory function at the extracellular IGF-2 level via receptor-mediated endocytosis which is precedes degradation of IGF-2 in the lysosomes48. On activation, phosphorylated IGF-1R stimulates the adaptor protein signals such as IRS-1, IRS-2 and Shc. Recent studies show that IRS-1 is required for mitogenicity and IRS-2 for
motility response in the cell\textsuperscript{49}. On activation by IGF-1R, the phosphorylated IRS-1 attach to the subunit of PI3K leading to increment of the membrane bound PIP3. This helps to recruit AKT to the membrane as it will be activated by phosphorylation by PDK1 and mTORC2 complex\textsuperscript{50}. Activated AKT leads to anti-apoptotic effects by inhibiting the phosphorylation of the pro-apoptotic factors such as BAD and other members in Foxo family and increasing the expression of the anti-apoptotic factors like BCL-2, BCL-XL and NF-\textit{k}B\textsuperscript{51}. AKT also has another important biological function which is glucose regulatory function. This function is mediated by using GSK-3\textbeta activity which is very vital in protein biosynthesis and cellular growth which is mediated through TORC1 activity\textsuperscript{52}. On the opposite side, the activated Shc may trigger RAS/MAP kinases system and transmits the mitogenic signals by using activated nuclear ELK1\textsuperscript{53}.

Breast tissue is composed of epithelial and stromal elements. During puberty, duct development occurs as terminal end bud (TEB). These TBs are located at the immature ductal leading ends and from there extends to the breast fat component\textsuperscript{54}. Estrogen functions to initiate the pubertal process and has an important effect on breast tissue development. Recently, it has been shown that estrogen has a synergistic effect with IGF-1 on the breast tissue during puberty with the effect of Growth Hormone (GH) as it triggers IGF-1 release which acts with estrogen to form TEB (Kleinberg \textit{et al}). IGF-1R levels are increased in breast carcinomas in comparison to benign or normal epithelia\textsuperscript{55,56}.

Recent studies have shown that the overexpression of IGF-1R could be in relation with decreasing IGF-1R transcription as a result of decrease p53 expression\textsuperscript{55,57}. IGF-1R overexpression is related to tyrosine kinase activation and has a poor prognostic effect on
breast carcinoma survival rate\textsuperscript{58}. However, breast carcinoma cell lines which are hormonally dependent and have low aggressive phenotype may correlate with high IGF-1R and IRS-I expression levels\textsuperscript{59,60}.

Metastasis includes invasion, intravasation, angiogenesis, survival and extravasation of the tumor. Recent studies show that IGF-1 signals has an important role in regulating all steps of metastasis. IGF signals modulate the mobility and invasion of many breast carcinomas in-vitro. Many studies show that excitation of IGF to the mobility and invasiveness of MDA-MB-231, MDA-MB-435, MDA-435A/LCC6, T47D and many other breast carcinomas cell lines \textsuperscript{61,62,63,64}. Metastatic cancer cells are able to pass through the basement membrane and also through the extracellular matrix, then disseminate either through the blood vessels and/or lymphatic vessels to distant sites from the original cancer. Protease is degrading class of enzymes that can proteolize the ECM. These classes of enzymes are very vital for metastatic cells to invade the surrounding tissue. One of these proteolytic enzymes is matrixmetalloprotease (MMP) which is comprised basically of Zinc-dependent endopeptidases. An important member of MMP family is MMP-2 which is regulated basically by IGF-1 \textsuperscript{61,65}.

RAS family has many kinds of GTPases. These enzymes have a critical role in the regulatory switches which are parallel with many other physiological and biological functions. There are five classifications for these proteins into five families; Ras, Rho, Rab, Sar 1/Arf, and Ran \textsuperscript{66}. Rho overexpression is generally associated with local and distant metastasis of breast carcinoma cells\textsuperscript{67} and a poor prognostic breast carcinoma subtype is called inflammatory breast cancer\textsuperscript{68}. One of the most important a
farnesyltransferase inhibitors is Tipifarnib (Zarnestra; Johnson & Johnson, New Brunswick, NJ). This agent inhibits MCF-7 breast cancer cell xenografts growth which follows dose-dependent style\textsuperscript{69}. The most important side effects of this agent are low WBC and platelets, and the toxicity to central and peripheral nerves. However, recent studies show a positive response has occurred in patients have ER- positive breast carcinomas\textsuperscript{70}.

PI3K pathway is also critical in various physiological functions, such as growth and proliferation, cellular survival, angiogenesis, and motility\textsuperscript{71}. PI3K mutation was detected in about 40\% of breast carcinomas. This indicates how PI3K is important in developing of the breast carcinoma\textsuperscript{72}. It was clear that mammalian target of rapamycin (mTOR) was involved in the amplification and mutation which result in activating PI3K/AKT pathway in breast cancer. Everolimus, Temsirolimus and Sirolimus are mTOR antagonists that used as an adjuvant treatment for breast carcinomas, especially in the ER- positive breast cancers. Figure (9) shows the inhibitory and stimulatory effects of various pathways as AKT, PI3K and the mTOR inhibitors and their site of actions\textsuperscript{70}. 
Figure 9. IGF/IGFR and its inhibitory and stimulatory effects of various pathways such as AKT, PI3K and the mTOR inhibitors, and their site of actions⁷⁰.
CHAPTER 2: METHODS AND MATERIAL

CELL CULTURE

For analyzing the role of S100A7 in breast cancer, we used ERα- cancer cell lines MDA-MB-231 and SCP6. Cell lines were cultured in complete medium “Dulbecco’s Modified Eagle’s Medium” (DMEM) which is basically; (10% fetal bovine serum which it was inactivated by heat (FBS)\(^1\), and 1 % penicillin-streptomycin). Cells were split in duration of 18-24 h, according to the growth rate of the cell lines.

TRANSWELL MIGRATION ASSAY

Transwell migration Assay is used to analyze the metastatic and invasive potential of cell lines \textit{in vitro}. Cells were serum starved overnight and transferred to transwell plates and then incubated for 6 to 24 hr in presence and absence of IGF. The transwell plate has 12 inserts that contain two chambers per well which are separated by a membrane attached to the removable insert. \(1\times10^5\) cells in serum free medium were placed on the upper chamber and the lower chamber contained 600 \(\mu\)L of serum free medium with or without IGF (100 ng/ml). Cells were permitted to migrate through semi-

\(^1\) FBS was heated to 60 C for 30 min to inactivate proteins that might interfere with cell culture or any assays that FBS was used in.
permeable polycarbonate membranes of the transwell migration plates (BD Bioscience). At the end of the assay, cells that migrated to the other side of the membrane were fixed and stained with Hema3 staining. Cells attached to the membrane were stained and photographed (Zeiss), and eventually manually counted.

**SCRATCH WOUND ASSAY**

Breast cancer has been shown to metastasize to various organs. In addition, IGF has been shown to play an important role in breast cancer metastasis. In order to determine if S100A7 enhances IGF-induced metastasis, we have performed scratch wound assays, which determine the invasive properties of the cells under *in vitro* conditions. In this assay, after making a wound, the wound closure was compared between S100A7 overexpressing and vector control cells in the presence and absence of IGF. Briefly, S100A7 overexpression MDA-MB-231 and SCP6 cells were grown in 6-well plates to 80% confluence and were serum starved overnight and scratched with a sterile 200µl pipette tip to create wound. Cells were washed by using Serum Free Media (SFM) and incubated with or without 100ng/ml IGF up to 36 hrs. Photographs were taken at 0 hr time, 12 hr, 24 hr and 36 hr.

**CELL STIMULATION**

IGF has been shown to enhance proliferation and invasive capabilities of cells through activation of its receptor IGFR and various downstream signaling molecules, such as Akt and ERK. We have analyzed the effect of S100A7 overexpression on IGF-induced signaling mechanisms. Briefly, MDA-MB-231 and SCP6 breast cancer cell lines were grown in a monolayer to 70-80% confluence and incubated in Serum Free Media.
(SFM). Medium was removed, and the cells were washed with SFM. Cells were stimulated by 100 ng/ml IGF for 0, 5, 15, 30 and 60 min and immediately placed on ice, then washed twice with ice cold PBS (1X), and either stored in -80 C or lysed.

PROTEIN ISOLATION

Immediately after stimulation, plates of cells were put on ice for starting the lysis process. Extra media was aspirated and 150 µL ice-cold radio immune precipitate assay (RIPA) lysis buffer which has phosphatase and nuclease inhibitors was added to each plate. RIPA buffer was prepared by using the following instructions: 150 mM sodium chloride, 1.0 % NP-40 or Trion X-100, 0.5% sodium deoxycholat, 0.1 % SDS (Sodium dodecyl sulphate), and 50 mM Tris, pH 8.0.

The lysed cells were scraped off of the plate and then transferred to Eppendorf tubes on ice. These tubes were rotated for 30 min at 4 C, and then centrifuged at 12,000 RPM at 4 C, and supernatants were collected and either stored at -20 C or used for immediate protein estimation. The pellets were discarded.

Protein estimation was done as modified version of the microplate Assay protocol (Bio-Rad): 25 µL reagent S to 1 mL reagent A and vortex at room temperature. Four concentrations of BSA (1.52 mg/mL, 0.76 mg/mL, 0.38 mg/mL, and 0.19 mg/mL) were used for making standard curve. We added 5 µL of standards and samples in duplicate to each well in a 96-well plate. Then, 25 µL of the A and S reagent mixture, followed by 200 µL reagent B was added each well and the plate was placed on a shaker at room temperature for 15 min. The absorbance of each well was read at 645 nm with a spectrophotometer. The protein was estimated based on the standard curve.
WESTERN BLOT

10-60 µL of cell lysates containing 50 µg of protein in SDS Sample Buffer sample were prepared according to the instructions of the manufacturer (Invitrogen). Samples were boiled for 5 min. 1 X running buffer was made from NuPAG ® Antioxidant for every 1 L from buffer. Buffer was added to the running chambers as all of the chambers are filled with the buffer. Samples and Precision Plus Protein Dual Color Standard (BioRad) markers were loaded into a pre-cast 4-12% BisTris polyacrylamide gel and run at no more than 150 V until the running dye get to the bottom level of the gel cassette. We used Bio-Rad Semi-Dry Transfer Cell system for transferring the separated proteins to a nitrocellulose membrane at 16 V for 45 min. The membrane was blocked by using 5 % non-fat dry milk in tris-Buffered Saline tween-20 (TBST) for 30 min. Primary antibodies were incubated overnight at 4° C with shaking and for 2 h for the secondary antibodies at room temperature with shaking.

STATISTICAL ANALYSIS

Two-tailed student t-test was used to compare vector control and S100A7 overexpressing cells. A p-value of less than 0.05 was considered as significant. On graphs, * indicates p<0.05, and ** indicates p<0.01 compared to vehicle.
CHAPTER 3: RESULTS

Overexpression of S100A7 in breast cancer cells

In Dr. Ganju’s laboratory, S100A7 expression was analyzed in human breast cancer tissue microarrays. Paraffin-embedded, formalin-fixed specimens were analyzed for S100A7 by immunohistochemistry using S100A7 specific antibody. High expression was observed in sample obtained from lymph nodes containing metastatic carcinoma (Figure 10A). Similarly, high S100A7 expression was observed in different types of invasive and high-grade ductal carcinoma in situ (Fig. 10B). However, not much expression was observed in normal breast tissue (Fig 10B i).

Figure 10. Expression of S100A7 in human breast tissue microarray (TMA). (A) Expression of S100A7 in human breast metastatic carcinoma in lymph node. (B) S100A7 expression in human breast tissue microarray (TMA). Paraffin-embedded, formalin-fixed specimens were analyzed for S100A7 by immunohistochemistry. (i) Normal (ii, iii) Invasive carcinoma (iv, v) Invasive carcinoma with focal DCIS (vi) DCIS with focal invasive carcinoma.
Since S100A7 was shown to be overexpressed in high-grade ductal carcinoma and invasive breast cancer patient samples, we determine the role of S100A7 in enhancing breast cancer growth and metastasis by overexpressing S100A7 in ERα- MDA-MB-231 and SPC6 breast cancer cell lines. These cells were shown to express a low amount of S100A7. We transfected S100A7 in MDA-MB-231 and SCP6 cells by using IRES2-EGFP-S100A7 plasmid using Lipofectamine reagent according to the manufacturer’s instructions. Ten μg of plasmid DNA was used for the transfection and after 24 hour of transfection, cells were selected by using 500 μg/mL G418 for 2-3 weeks for stable S100A7 expression. Resistant clones were isolated and expanded in culture.

We analyzed the expression of S100A7 in MDA-MB-231 and SCP6 cells by Western blotting and confocal microscopy. As shown in Figure 11A and B, a high amount of S100A7 was expressed in S100A7 transfected MDA-MB-231 (A) and SCP6 (B) cell lines compared to vector control, as detected by Western blot analysis. Similar amounts of protein were loaded in each as equal amounts of GAPDH expression was observed in both vector and transfected cell lines. Similarly, high amounts of GFP fluorescence was observed in S100A7 compared to vector control cells as observed by confocal microscopy (Fig. 11C).
Figure 11. Overexpression of S100A7 in ERα− breast cancer cells. S100A7 overexpressing and vector control cells were analyzed for S100A7 expression by Western blot analysis (A and B). GAPDH was used for equal loading. S100A7 and vector control cells were either analyzed by phase contrast microscopy (C left panel) or confocal microscopy (C right panel).

Effect of S100A7 overexpression on IGF-induced growth

IGF has been shown to play an important role in breast cancer progression and metastasis. Similarly, S100A7 has been shown to play an important role in breast cancer and its expression is correlated with poor prognosis. However, not much is known about the crosstalk between S100A7 and IGF/IGFR signaling in breast cancer. We determined if S100A7 overexpression in cells has any effect on IGF-induced proliferation. Proliferation was measured using MTT assays. We observed enhanced IGF-induced proliferation in S100A7 overexpressing cells compared to vector control (Figure 12). This study suggests that S100A7 modulates IGF-induced proliferation of breast cancer cells.
Figure 12. IGF induced the growth of S100A7 overexpressing MDA-MB-231 cells. IGF-induced proliferation of S100A7 overexpressing MDA-MB-231 and Vec cells was analyzed using MTT assay. Briefly, cells (6000) were subjected to proliferation after seeding 5x10^3 cells in 96-well tissue-culture plates. Next day, cells were serum starved and different concentrations of IGF were added to the wells as indicated. The proliferation of the cells was measured at different days using MTT assay as a percentage increase in the absorbance reading at 570 nm with respect to the control (day 0).

Effect of S100A7 overexpression on IGF-induced migration in breast cancer cell lines MDA-MB-231 and SCP6.

Although S100A7 has been shown to modulate EGF/EGFR-induced migration in breast cancer cells\textsuperscript{71}, not much is known about its role in IGF-induced migration. In this study, we determined whether S100A7 overexpression enhanced IGF-induced migration in breast cancer cell lines MDA-MB-231 and SCP6. Migration assays were done as described in our Methods section. Migration was induced using different concentrations of (0, 50 and 100 ng/mL) to show the overexpression of S100A7 in breast cancer cell lines; MDA-MB-231 as is shown in Figure 13. (A and B) and SCP6 as shown in Figure 14(C and D). (A) MDA-MB-231 breast cancer cells were treated with different concentrations 0, 50, and 100 ng/mL IGF or SFM in vector cell. (B) SCP6 breast cancer
cells were treated with different concentrations 0, 50, and 100 ng/mL IGF or SFM in vector control. Cells were allowed to migrate through semi-permeable polycarbonate membranes of transwell migration plates. The upper chamber contained 120 µL of 1 x 10^6 cells/mL which were suspended in SFM and bottom chambers contained 600 µL SFM with or without 50 or 100 ng/mL IGF. Cells adherent to the membrane were fixed and stained using HEMA stain. Membrane were photographed (Zeiss) and cells were manually counted at the end of the migration. Statistical analysis by two-tailed variance t-test : \( *p < 0.01 \) in comparison to vehicle.

Figure 10. Effect of S100A7 overexpression on IGF-induced migration in breast cancer cell lines. S100A7 or vector control expressing MDA-MB-231 (A) or SCP6 (B) breast cancer cell lines were treated with 0, 50 and 100 ng/mL IGF concentrations and subjected to migration assays for 6 hrs. Cells migrated to lower chambers were counted in five different fields and fold increase in migration was determined compared to untreated cells.
**Effect of S100A7 overexpression on IGF-induced wound healing**

IGF has been reported to stimulate migration and wound healing abilities of breast cancer cells both *in vitro* and *in vivo*\(^\text{17}\). We determined if S100A7 overexpression has any effect on IGF-induced wound healing. The wound healing assay stimulates an environment in which cancer cells can metastasize and invade into the adjacent areas. We first evaluated metastasis and invasion of MDA-MB-231 and SCP6 in absence of IGF. Then, we compared the IGF-mediated metastasis and invasion of S100A7 overexpressed cells to vector control.

Upon visual examination of wound closure in Figure 14 A and B, it is clear that 50 ng/mL IGF enhances wound healing/invasion of highly metastatic MDA-MB-231 and SCP6 breast cancer cell lines overexpressed S100A7.

This experiment shows that S100A7 overexpression show stimulation of IGF-induced wound healing/invasion in highly metastatic breast cancer cell lines MDA-MB-231 and SCP6.
Since S100A7 overexpression was shown to enhance migration and wound healing capabilities of MDA-MB-231 and SCP6 cells, Dr. Ganju’s laboratory determined if S100A7 overexpression had any effect on the metastatic potential of these cells \textit{in vivo}.

As shown in Figure 15, S100A7 overexpression in MDA-MB-231 luciferase expressing cells enhanced metastasis: Mice (n=10 per group) were injected intracardiacally with either luciferase positive S100A7 overexpressing or vector control MDA-MB-231 cells. Mice were analyzed for bioluminescence weekly by IVIS imaging. (A) Representative picture of the bioluminescence at 6th week. (B) Radiance flux (average) of different groups of mice.
**Figure 15. S100A7 overexpression in MDA-MB-231 luciferase expressing cells enhances metastasis:** Mice (n=10 per group) were injected intracardially with either luciferase positive S100A7 overexpressing or vector control MDA-MB-231 cells. Mice were analyzed for bioluminescence weekly by IVIS imaging. (A) Representative picture of the bioluminescence at 6th week. (B) Radiance flux (average) of different groups of mice.

**Effect of S100A7 Overexpression on IGF-induced IGFR phosphorylation.**

IGF has been shown to enhance activation of downstream signaling pathways by enhancing phosphorylation of tyrosine residues present in the cytoplasmic domain of IGFR. In order to determine the effect of S100A7 overexpression on IGF-induced IGFR phosphorylation, we analyzed the IGF-induced IGFR phosphorylation at different time periods by Western blot using pIGFR-specific antibodies in breast cancer cell lines MDA-MB-231 (Figure 16 A) and SCP6 (Figure 16 B).

As shown in Figure 16 (A) and (B), IGF induced IGFR phosphorylation was enhanced in S100A7 overexpressing breast cancer cells lines compared to vector control.
**Figure 16. S100A7 overexpression enhances IGF-induced IGFR phosphorylation.**

MDA-MB-231 (A) and SCP6 (B) cell lines were grown in a monolayer to 70-80% confluence and incubated in SFM. SFM was removed, cells were washed with SFM and stimulated with 100 ng/mL IGF different time periods. Equal amounts of cell lysates (50 µg) were run on SDS PAGE and then transferred and blotted with pIGFR specific antibody. The blots were stripped and re-probed with GAPDH to confirm equal loading.

**S100A7 overexpression enhances AKT pathway in breast cancer cell lines**

IGF has been shown to enhance activation of many signaling pathways including Akt. Akt is activated upon phosphorylation of serine residue at 473 and has been shown to enhance cell survival. In order to determine the effect of S100A7 overexpression on IGF-induced AKT phosphorylation, we analyzed the Akt phosphorylation by western blot using pAKT-specific antibodies which recognize phosphoserine reside at 473.
position in breast cancer cell lines MDA-MB-231 and SCP6.

As shown in Figure 17 (A) and (B), IGF induced Akt phosphorylation was enhanced in S100A7 overexpressing breast cancer cells lines compared to vector control.

![Figure 17. S100A7 overexpression enhances IGF-induced Akt phosphorylation.](image)

**Figure 17. S100A7 overexpression enhances IGF-induced Akt phosphorylation.** MDA-MB-231 (A) and SCP6 (B) cell lines were grown in a monolayer to 70-80% confluence and incubated in SFM. SFM was removed, cells were washed with SFM and stimulated with 100 ng/mL IGF different time periods. Equal amounts of cell lysates (50 µg) were run on SDS PAGE and then transferred and blotted with pAkt specific antibody. The blots were stripped and re-probed with GAPDH to confirm equal loading.

**Effect of S100A7 overexpression on ERK pathway in breast cancer cell lines**

IGF has been shown to enhance proliferation of cells through activation of the ERK pathway. In order to determine the effect of S100A7 overexpression on IGF-induced ERK phosphorylation, we analyzed the ERK phosphorylation by western blot using pERK-specific antibodies in breast cancer cell lines.

As shown in Figure 18 (A) and (B), IGF induced ERK phosphorylation was enhanced in S100A7 overexpressing breast cancer cells lines compared to vector control.
Figure 18. S100A7 is overexpressed by IGF induction stimulated ERK phosphorylation. MDA-MB-231 (A) and SCP6 (B) cell lines were grown in a monolayer to 70-80% confluence and incubated in SFM. SFM was removed, cells were washed with SFM and stimulated with 100 ng/mL IGF different time periods. Equal amounts of cell lysates (50 µg) were run on SDS PAGE and then transferred and blotted with pERK specific antibody. The blots were stripped and re-probed with GAPDH to confirm equal loading.
CHAPTER 4: DISCUSSION

Breast cancer is the most common cancer in women, and it is the second most common cause of cancer-related deaths after lung cancers in women. Western countries as North America and Northern Europe have higher incidence in comparison to the rest of the world. Breast carcinomas can be divided according to the level of invasiveness into invasive or in-situ. Furthermore, these types can be subdivided according to the microscopic picture into lobular and ductal carcinomas. Many genes are well recognized to have a strong relation to breast cancer etiology, such as BRCA1 and BRCA2. Mutation to these genes show increase incidence of breast carcinomas among this population.

There are many risk factors that are related to breast cancer pathogenesis, such as exposure to more than normal levels of estrogen and/or duration in the life of the woman. Other vital risk factors for breast cancer development are family history positivity, especially history with first-degree-relative breast cancer. In addition, obesity has been shown to play an important role in the development of breast cancer. Other factors that may influence development of breast cancer include inflammation. Our studies indicate that molecules associated with obesity, such as IGFR, and inflammation-associated molecules, such as S100A7, may crosstalk with each other to modulate breast
cancer growth and invasion.

S100A7 is one of the most important members in S100 protein family. It is expressed among the intracellular and extracellular compartment in the epithelial cells layer in skin and breast \(^2^1\). S100A7 is an important chemotactic factor for immune cells and plays an important role in the development of inflammatory disorders, such as psoriasis. Recently, overexpression of mS100A7a15, the mouse homolog of S100A7 in the skin, has been shown to develop psoriasis-like disease in mice\(^7^5\). In addition, Dr. Ganju’s laboratory has shown that overexpress of mS100a7a15 in mammary glands develop hyperplasia and enhance tumor growth and metastasis in orthotopic mouse models. These effects were mediated though pro-metastatic and pro-inflammatory signals (unpublished data).

We have shown that overexpression of S100A7 in breast cancer cell lines enhances IGF-induced wound healing and chemotaxis. This indicates that S100A7 may enhance the IGF-induced invasive capabilities of breast cancer cells. S100A7 overexpressing breast cancer cell lines have been shown to have enhanced metastatic capabilities as shown in Figure (15). IGF, which binds to the IGFR receptor, plays an important role in breast cancer development. IGFR has been shown to play an important role in fetal growth\(^4^3\). IGF upon binding to IGFR enhances signaling which has an important effect on cellular growth and tissue differentiation, besides its role in metaplasia and neoplasia \(^4^5\). This signaling system as shown in Figure (8) works mainly through autophosphorylation of the downstream factors, such as AKT, IRS and RAS.

IGF1R has many biological signaling pathways, and it enhances signals through
autophosphorylation. Our studies also indicate that S100A7 enhances IGF-induced IGFR phosphorylation, indicating that S100A7 plays an important role in activation of IGFR receptor. In addition, we also observed that S100A7 enhances activation of IGF-induced downstream signaling molecules, such as Akt and ERK kinase. Both Akt and ERK have been shown to play an important role in cell survival and proliferation of breast cancer cells.

IGF/IGFR signaling has been shown to regulate obesity, which is a risk factor for breast cancer development. S100A7 plays in important role in inflammation. Our studies indicate that crosstalk between S100A7 and IGF/IGFR signaling may link obesity with inflammation that may mediate the development of breast cancer.
References

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