REGULATION OF THE OPIOID GROWTH FACTOR – OPIOID GROWTH FACTOR RECEPTOR AXIS IN THE PROGRESSION OF TRIPLE NEGATIVE BREAST CANCER AND ITS POTENTIAL AS A THERAPEUTIC AGENT

A Thesis in
Anatomy
by
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ABSTRACT

Triple negative breast cancer (TNBC) is an extremely aggressive form of breast cancer characterized by tissues lacking estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor (HER2). TNBC accounts for approximately 15% of all breast cancers diagnosed worldwide, and frequently affects younger women, women of color, and those from lower socioeconomic countries. TNBC has an increased likelihood of recurrence and a twofold greater mortality risk compared to other types of breast cancer, as well as a tendency to metastasize outside the breast. The financial burden for patients with TNBC is considerable, as costs are increased by 50% compared to other breast cancers.

The median survival rate for patients with metastatic TNBC is only 13 months. Lack of hormone receptors and frequent de novo or acquired chemotherapy resistance renders almost all therapies ineffective. Thus, there is a critical need to develop TNBC treatments that target the underlying biology of tumor growth. A novel biological pathway has been studied in a wide variety of human cancers that may provide knowledge on progression of TNBC and serve as an effective treatment.

The opioid growth factor – opioid growth factor receptor axis is a determinant of cell proliferation in both normal cells and neoplasia. OGF inhibits cell proliferation by upregulating the cyclin dependent inhibitory kinases p16 and p21, arresting cells in the transition from G0/G1 phase to S phase of the cell cycle and halting DNA synthesis.

The proposed experiments investigated the presence of the OGF-OGFr axis in MDA-MB-231 TNBC cells in vitro and in vivo and assessed whether expression of peptide or receptor changes with cancer progression, and whether OGF serves as an effective treatment for TNBC.
For cell culture studies, log phase cells were compared with confluent and post-confluent cultures to mimic early and advanced stages of cancer growth.

Semi-quantitative immunohistochemistry and western blot analysis comparing log phase and confluent cells, as well as small and large tumors, revealed that both OGF and its receptor are significantly reduced in advanced stages of squamous cell carcinoma of the head and neck, as well as ovarian, cancers. These observations were extended and confirmed in human breast cancer using MDA-MB-231 and MCF-7 cancer cell lines grown in vitro and in vivo. A mechanism of action involving OGF inhibition of growth was demonstrated utilizing BrdU incorporation that indicated a decrease in DNA synthesis in TNBC cells treated with OGF. Confluent cells were less responsive to OGF treatment than log phase cells.

The data suggest that OGFr expression is reduced in TNBC with tumor progression, and that tumor progression may lead to deficits in the OGF-OGFr axis. Nonetheless, OGF was shown to significantly decrease cell growth relative to controls, indicating its potential as an effective biological treatment for TNBC.
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LIST OF ABBREVIATIONS

AI
aromatase inhibitor

ANOVA
analysis of variance

BCS
breast-conserving surgery

BRCA1
breast cancer 1, early onset (gene)

BRCA2
breast cancer 2, early onset (gene)

BrdU
bromodeoxyuridine

BSA
bovine serum albumin

°C
degrees Celsius

cm
centimeter

d
day

DMEM
Dulbecco’s modified Eagle’s medium

DNA
deoxyribonucleic acid

ER
estrogen receptor

et al.
and others

g
gram

G
gauge

µg
microgram

G₀
G zero/resting phase of cell cycle

G₁
gap 1 phase of cell cycle

G₂
gap 2 phase of cell cycle
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>$[^3]H$</td>
<td>tritium</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor</td>
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<td>IACUC</td>
<td>International Animal Care and Use Committee</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<td>l</td>
<td>length</td>
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<tr>
<td>L-15</td>
<td>Leibovitz’s medium</td>
</tr>
<tr>
<td>LDN</td>
<td>low-dose naltrexone</td>
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<tr>
<td>M</td>
<td>molar</td>
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<td>M phase</td>
<td>mitotic phase of cell cycle</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>µm</td>
<td>micrometer</td>
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<tr>
<td>NGS</td>
<td>normal goat serum</td>
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<tr>
<td>NTX</td>
<td>naltrexone</td>
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<tr>
<td>OCT</td>
<td>optimal cutting temperature embedding compound</td>
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<tr>
<td>OGF</td>
<td>opioid growth factor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>OGFr</td>
<td>opioid growth factor receptor</td>
</tr>
<tr>
<td>p16</td>
<td>cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>p21</td>
<td>cyclin-dependent kinase inhibitor 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<tr>
<td>PPE</td>
<td>preproenkephalin A gene</td>
</tr>
<tr>
<td>PR</td>
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<td>%</td>
<td>percent</td>
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<td>π</td>
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</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>S</td>
<td>synthesis phase of cell cycle</td>
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<tr>
<td>SCCHN</td>
<td>squamous cell carcinoma of the head and neck</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPB</td>
<td>Sorensen’s phosphate buffer</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>TNBC</td>
<td>triple-negative breast cancer</td>
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Chapter 1

INTRODUCTION

1.1 Cancer Incidence and Disease

Cancer is a major public health problem in the United States and throughout the world. It is currently the second leading cause of death in the United States, and is expected to surpass heart disease as the leading cause of death within the next few years (Siegel et al., 2015). These rising numbers are due in part to population growth and aging, but also to increasing cultural adoption of many cancer-associated lifestyle choices including smoking, lack of physical activity, and a “Westernized” diet (Jemal et al., 2011).

In 2008, an estimated 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide (Jemal et al., 2011). The number of reported cases continues to rise, and current statistics predict over 1.6 million cancer cases and 589,000 cancer deaths this year in the United States alone (Siegel et al., 2015). The lifetime probability of being diagnosed with an invasive cancer is higher for men (43%) than for women (38%), and it is estimated that one in 3 women and one in 2 men in the United States will develop cancer within his or her lifetime (Siegel et al., 2012, Siegel et al., 2015).

1.1.1 Breast Cancer Incidence and Disease

Breast cancer is the most frequently diagnosed neoplasm, and is the leading cause of cancer-related deaths in females worldwide (Jemal et al., 2011, Van Asten et al., 2014). The median age at time of diagnosis is 61 years, but can affect women as young as the late 20s (Siegel et al., 2012, Hulka and Moorman, 2001). In general, about 20% of breast cancers occur in
women younger than 50 years, while 40% occur in women aged 65 and older (Siegel et al., 2012). As many as 60% of all breast cancers are diagnosed at a localized stage with early detection through mammography (Siegel et al., 2012, Jemal et al., 2011).

In 2008, breast cancer accounted for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths worldwide, about half of which occurred in economically developing countries (Jemal et al., 2011). Over 225,000 new cases of breast cancer and more than 40,000 related deaths are reported each year in the United States, with nearly 3 million women surviving with a history of invasive breast cancer (Siegel et al., 2012). Despite these staggering figures, the prognosis for women diagnosed with localized breast cancer is improving each year, with a five-year relative survival rate of 98.6% (Jemal et al., 2011).

1.1.2 Risk Factors

There are a variety of reproductive and hormonal factors that can increase the risk of developing breast cancer. These factors include age at menarche and menopause, late age at first birth, nulliparity, increased use of oral contraceptives, or postmenopausal hormone replacement therapy (Key et al., 2006, Jemal et al., 2011). Genetic factors also contribute to this increased risk, as it has been estimated that approximately 7% of all breast cancer cases are due to inheritance (Hulka and Moorman, 2001).

Six genes are known to confer a high risk for developing breast cancer, the most important being the breast cancer predisposition genes, BRCA1 and BRCA2 (Easton, 1999, Campeau et al., 2008). The BRCA1 protein is classified as a tumor suppressor, and functions to maintain proper DNA repair and cell division in healthy cells (Campeau et al., 2008, Henderson, 2012). BRCA1 and BRCA2 gene mutations disrupt these processes and result in chromosome
instability and accelerated cell proliferation linked to increased incidence of breast and ovarian cancer (Easton, 1999, Campeau et al., 2008, Hulka and Moorman, 2001). Individuals with a BRCA1 mutation have an 80-85% chance of developing breast cancer within their lifetime, and a family history of breast cancer can increase this risk by two- to threefold (Hulka and Moorman, 2001).

Other risk factors for the development of breast cancer include socioeconomic status and increased consumption of alcohol (Key et al., 2006, Jemal et al., 2011). The best available strategies to reduce risk include maintaining a healthy body weight through diet, exercise, and minimizing alcohol intake, coupled with early detection through mammography (Jemal et al., 2011).

1.1.3 Current Treatments

The National Cancer Institute reports that more than $20 billion are spent annually in the United States for breast cancer treatment. Surgery and anticancer drugs remain the top two treatment options. Surgical treatment involves the use of mastectomy or breast-conserving surgery (BCS), which together are undergone by 93% of women diagnosed with early stage breast cancer and 73% of women diagnosed with late stage breast cancer (Siegel et al., 2012). While both procedures are used, BCS has become the standard operative treatment for early stage breast cancer over the last two decades, and has the same long-term survival rate as mastectomy while achieving a better cosmetic result (Eggemann et al., 2014, Siegel et al., 2012).

Some patients require mastectomy during early stage breast cancer because of large or multiple tumors, while others may choose mastectomy due to a reluctance or inability to undergo radiation therapy following BCS (Siegel et al., 2012). The majority of patients receive adjuvant
treatment, however, with one-half undergoing radiation therapy alone and one-third receiving both radiation and chemotherapy (Siegel et al., 2012). When these techniques are used appropriately for localized cancers, overall long-term survival is maximized.

Pharmacological treatment involves the use of anticancer drugs, which have been classified into the categories of chemotherapy, immunotherapy, hormonal therapy, and targeted therapy (Siegel et al., 2012). Chemotherapeutic drugs can be used alone or in combination. The combination regimen often involves treatment with doxorubicin and cyclophosphamide, followed by a taxane such as paclitaxel (taxol) or docetaxel (Nabholtz et al., 2003). Paclitaxel is a widely used and naturally occurring antimitotic drug that functions by interfering with microtubule assembly, which halts the cell cycle at the G₂/M phase and thus prevents tumor cell proliferation (Yang et al., 2014). By nature of this mechanism, paclitaxel also inhibits the division of normal cells, which makes it an extremely toxic drug with considerable side effects that often prevent patients from completing a full round of treatment (Yang et al., 2014).

Hormonal drug therapies are also an effective treatment option because as many as 80% of all primary breast cancers contain estrogen or progesterone receptor-positive cells (Van Asten et al., 2014). Hormones such as estrogen can stimulate tumor cell growth, so these drugs function by blocking hormone receptors or by reducing endogenous hormone production. Examples of hormonal drug therapies include administration of receptor modulators such as tamoxifen, or treatment with aromatase inhibitors (AIs) (Van Asten et al., 2014).

Aromatase is a member of the cytochrome P450 enzyme family, which converts androstenedione to estrone and testosterone to estradiol (Van Asten et al., 2014). By blocking aromatase activity, estrogen synthesis is effectively inhibited. AIs do not inhibit the production of estrogen by the ovaries, however, and thus are only administered to postmenopausal patients.
Despite the current efficacy of these drugs, there still remains a large subset of breast cancers which are largely unaffected by current treatment options.

1.1.4 Triple Negative Breast Cancer

Approximately 15% of breast cancers diagnosed worldwide are categorized as triple negative breast cancer (TNBC) (Gluz et al., 2009, Chougule et al., 2011). TNBC lacks the expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2), which renders current hormonal treatment therapies ineffective (Mayer et al., 2014). Chemotherapy treatment options are also limited, as TNBC is frequently associated with de novo or acquired chemotherapy resistance (Chougule et al., 2011).

TNBC often occurs in patients with BRCA1/BRCA2 mutations, and more frequently affects younger women, women of color, and those from lower socioeconomic countries (Bramati et al., 2014). It is a particularly aggressive cancer, with an increased likelihood of distant recurrence and twofold greater mortality risk compared to other types of breast cancer, as well as a tendency to metastasize outside the breast (Gluz et al., 2009, Mayer et al., 2014). The financial burden for TNBC is considerable, as healthcare costs for both inpatient and outpatient care are increased by 50% compared to other breast cancers (Bankhead, 2011).

The median survival rate for patients with metastatic TNBC is only 13 months, and most women will die from the disease despite current systemic therapies (Mayer et al., 2014). Thus, there is a critical need to develop treatments for TNBC that target the underlying biology of tumor growth. A novel biological pathway is known to be present in a wide variety of cancers that has been shown to inhibit cancer growth by modulation of biological processes (Zagon et al., 2009, Zagon et al., 2013b). This pathway has been detected in TNBC. The proposed studies may
provide knowledge on progression of TNBC and facilitate development of effective targets and therapies.

1.2 Endogenous Opioids and the OGF-OGFr Axis


The first two enkephalins identified, [Met⁵]-enkephalin and [Leu⁵]-enkephalin, were reported to have a role in pain modulation and neurotransmission (Akil et al., 1984). [Met⁵]-enkephalin was later identified as a negative growth regulator, and was renamed opioid growth factor (OGF) in order to distinguish its role as a growth factor from that of a neuromodulator (McLaughlin and Zagon, 2012, Zagon et al., 2002). OGF has a wide distribution and has been identified in both prokaryotes and eukaryotes, all vertebrate orders, and both neural and non-neural cells and tissues derived from all 3 germ layers (ectoderm, mesoderm, endoderm) (McLaughlin and Zagon, 2012, Zagon et al., 2002, Zagon et al., 2013a).

OGF is a constitutively expressed pentapeptide encoded by the preproenkephalin A (PPE) gene (Wollemann and Benyhe, 2004, Zagon et al., 2002). It is autocrine produced and secreted, and functions to inhibit cellular proliferation and renewal (Cheng et al., 2007, Zagon et
Furthermore, its inhibitory activity is stereospecific, reversible, noncytotoxic, and nonapoptotic inducing (Cheng et al., 2009a, Zagon et al., 2009).

OGF targets DNA synthesis by upregulating the cyclin dependent inhibitory kinases p16 and p21, which arrests cells in the transition from G$_0$/G$_1$ phase to S phase of the cell cycle (Donahue et al., 2009, Cheng et al., 2009b, McLaughlin and Zagon, 2012). The action of OGF is mediated by interaction with its receptor (OGFr) that is localized on the outer nuclear envelope of eukaryotic cells (Zagon et al., 2002, Cheng et al., 2009a). OGF enters the cell by clathrin-mediated active transport, binds to OGFr, and undergoes nucleocytoplasmic transport that is facilitated by karyopherin β1/Ran signaling pathways (Zagon et al., 2005b, Donahue et al., 2012). Although OGFr exhibits pharmacological characteristics of classical opioid receptors, it shares no homology at the nucleotide or amino acid levels (Cheng et al., 2009a, Zagon et al., 2002).

1.3 The OGF-OGFr Axis and Cancer

The OGF-OGFr axis is an important regulator of growth in both normal and abnormal cells (Zagon et al., 2002). It functions to maintain cell proliferation during homeostasis of tissue and organ development, renewal, and repair (McLaughlin and Zagon, 2014). Disruption of the tonic interactions by disease, mutation, or any other imbalance can amplify cancer progression (McLaughlin and Zagon, 2014). Thus, the OGF-OGFr axis serves as an important regulator in the onset and progression of human cancer and has been identified in many human neoplasia (Donahue et al., 2009, McLaughlin and Zagon, 2012).

The presence of the OGF-OGFr axis has been documented in a number of human cancers, including pancreatic, hepatocellular, colon, renal, ovarian, thyroid, and squamous cell
carcinoma of the head and neck (SSCHN) (Cheng et al., 2008, Avella et al., 2009, Rogosnitzky et al., 2013, Fanning et al., 2012, Goldenberg et al., 2008, McLaughlin et al., 2003a). OGF has been shown to decrease tumor incidence, delay tumor onset and progression, and/or extend survival in immunocompromised mice transplanted with human colon and pancreatic adenocarcinomas, SCCHN, and ovarian cancer (Zagon et al., 2009, McLaughlin et al., 2003a, Donahue et al., 2011). Modulation of the OGF-OGFr regulatory pathway in human cancer can occur by administering a low dose of the opioid antagonist naltrexone (LDN), which blocks endogenous opioids from opioid receptors for a short period of time (4-6 h), leading to an upregulation of endogenous OGF production (Donahue et al., 2011).

1.3.1 OGF Inhibition of Breast Cancer

Both OGF and OGFr are present in the human TNBC cell line MDA-MB-231, as well as a number of other breast cancers (McLaughlin and Zagon, 2014). OGF and LDN have been shown to inhibit breast cancer cell proliferation in vitro, while knockdown of OGFr results in accelerated proliferation of MDA-MB-231 cells (McLaughlin and Zagon, 2014).

Standard treatment for breast cancer includes surgery, radiation, and chemotherapy. Paclitaxel, the chemotherapeutic drug of choice, is associated with various side effects that range from tolerable (nausea, chills, vomiting) to serious (hypotension, difficulty breathing) (McLaughlin and Zagon, 2014). OGF may serve as a safe and non-toxic replacement for paclitaxel, though studies with SCCHN have shown cumulative inhibitory effects on cell and tumor growth when the two drugs are used together (Zagon et al., 2005a, McLaughlin and Zagon, 2014).
The OGF-OGFr axis and the effects of OGF and paclitaxel on human breast cancer tumors in mice have not been studied. Thus, further research on this pathway may provide knowledge on progression of TNBC and enable development of new therapies.

1.4 Conclusions

The OGF-OGFr axis has been shown to be present and functioning in a wide variety of human cancers (Zagon et al., 2013a). It can be manipulated to inhibit cancer cell proliferation, and has added effectiveness when used in combination with standard of care therapy (Zagon et al., 2005a, McLaughlin and Zagon, 2014). However, the effects of OGF on human TNBC are not yet fully understood. Preclinical studies on the OGF-OGFr axis have not been conducted and information on the expression levels of OGF or OGFr in relation to the progression of TNBC in either a mouse model or in tissue culture are unknown.

OGFr expression levels have been studied in ovarian cancer. The number of functional OGF receptors is significantly less than that recorded in normal tissues (McLaughlin and Zagon, 2014). This suggests that downregulation of the OGF-OGFr axis occurs during the transformative phases from normal to neoplastic tissues and further to late-stage cancer. Analysis of human ovarian tissue revealed nearly a 50% decrease in OGFr in late stage (III/IV) tumors compared to benign cysts (Fanning et al., 2012), suggesting that deficits in the OGF-OGFr axis are amplified with cancer progression.

TNBC is an aggressive cancer with an increased likelihood of recurrence, increased tendency to metastasize outside the breast, and higher mortality risk compared to other types of breast cancer (Mayer et al., 2014). It frequently affects younger women and is associated with a considerable financial burden (Bramati et al., 2014, Bankhead, 2011). TNBC lacks hormone
receptors and often results in de novo or acquired chemotherapy resistance, rendering current drug therapies ineffective (Mayer et al., 2014, Chougule et al., 2011).

Thus, there is a critical need to develop TNBC treatments that target the underlying biology of tumor growth. Fortunately, the OGF-OGFr axis may provide knowledge on progression of TNBC and facilitate development of effective targets and therapies (Zagon et al., 2009, Zagon et al., 2013b). The following studies examine the regulation of the OGF-OGFr axis during progression of TNBC, and its potential use as a therapeutic agent.
Chapter 2

OBJECTIVES

Current hormonal and chemotherapy treatment options are ineffective for most patients with TNBC, resulting in a median survival rate of 13 months (Mayer et al., 2014). There is a critical need to develop TNBC treatments that target the underlying biology of tumor growth. A novel biological pathway involving endogenous opioids and opioid receptors has been identified in a wide variety of cancers. This pathway, the OGF-OGFr axis, may provide knowledge on progression of TNBC and facilitate development of effective targets and therapies.

The hypothesis of this research is that the OGF-OGFr axis modulates the progression of TNBC in mice, and that expression levels of OGFr and/or OGF may be downregulated with the progression of neoplasia. Utilizing human breast cancer cell lines MDA-MB-231 and MCF-7, preclinical tissue culture and in vivo studies were conducted. The hypothesis was tested by completion of the following aims:

Specific Aim 1: To investigate peptide and receptor expression of OGF and OGFr in log and confluent MDA-MB-231 TNBC cells and small and large tumors in nude mice.

Specific Aim 2: To examine the effects of OGF and paclitaxel on log and confluent MDA-MB-231 TNBC cell growth and small and large tumor progression in nude mice.
Chapter 3

MATERIALS AND METHODOLOGY

3.1 Cell Culture and Growth

Cell culture experiments utilized the human breast cancer cell lines MDA-MB-231 and MCF-7 (American Type Culture Collection, Manassas, VA). MDA-MB-231 cells were derived from a metastatic pleural effusion in a 51-year-old Caucasian female, whereas the MCF-7 cell line was acquired from a metastatic pleural effusion in a 69-year-old Caucasian female; both were adenocarcinomas. The TNBC cell line MDA-MB-231 was grown in Leibovitz’s L-15 medium supplemented with 2 mM L-glutamine, and the ER⁺/PR⁺ MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium. All media were supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate, and antibiotics (5,000 units/ml penicillin, 5 mg/ml streptomycin), and cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Cells were plated in triplicate wells and counted 24 h later (time 0) to determine seeding efficiency. For treatment studies, 10⁻⁶ M OGF or 10⁻⁹ M paclitaxel (taxol; Sigma-Aldrich Chemicals, Indianapolis, IN) was added at time 0; media and compounds were changed daily. A stock solution of OGF was prepared at a 10⁻³ M dilution in sterile water. Taxol was initially dissolved in DMSO and further diluted to 10⁻⁶ M in sterile water. An equivalent volume of sterile water was added to control wells. Cells were harvested at designated times and counted by hemacytometer using trypan blue exclusion methodology. At least two aliquots per well from two wells/treatment/time point were sampled.
3.2 Animals

Four to five week-old female athymic nu/nu mice were purchased from Charles River Laboratories (Wilmington, MA). A total of 15 mice were acclimated for 48 h after arrival. Mice were housed in pathogen-free isolators in a controlled-temperature room (22-25°C) with a 12-12 h light/dark cycle (light from 07:00-19:00). Sterile rodent diet and water were available ad libitum. All procedures were conducted in accordance with protocol #45490 approved by the Penn State Hershey Institutional Animal Care and Use Committee (IACUC).

3.2.1 Tumor Cell Implantation and Growth

Unanesthetized mice received two subcutaneous injections of MDA-MB-231 cells (8 x 10^6 cells/0.1 ml per injection). The day of tumor cell inoculation was considered day 0. Mice were weighed once per week and observed daily for the appearance of tumors. Individual tumors were considered measurable once the longest dimension reached 5 mm diameter, and volumes were recorded three times per week using a digital Vernier caliper (accuracy ± 0.05mm). Volume was calculated using the formula l x w^2 x π/6, with length (l) as the longest dimension and width (w) as the dimension perpendicular to length.

3.2.2 Tissue Collection and Euthanasia

Tumors were harvested as small or large when tumor volume reached approximately 350 mm^3 or 1000 mm^3, respectively, or when tumors became ulcerated (McLaughlin and Zagon, 2006). Tumor bearing mice were weighed and euthanized with a 0.1 ml intraperitoneal injection of Euthasol ® (Virbac AH, Inc., Fort Worth, TX). Upon sacrifice, tumors and spleens were removed and weighed, and the animals were examined for metastases. Tumors were
photographed at the time of euthanasia using a Kodak EasyShare Z712 IS camera. All mice were sacrificed within 35 d following tumor cell inoculation.

### 3.3 Tissue Preparation

Tumor tissue was frozen in isopentane chilled on dry ice for immunohistochemistry or placed in RIPA buffer and stored at -20°C for western blotting. Tissues were processed within two weeks of collection.

### 3.3.1 Semi-quantitative Immunohistochemistry

Immunohistochemistry was utilized to evaluate the presence and relative levels of OGF and OGFr in cells and tumor tissue following published procedures (Zagon and McLaughlin, 1993, Zagon et al., 2002). Log phase or confluent MDA-MB-231 and MCF-7 breast cancer cells were seeded onto 22-mm diameter cover glasses placed in six-well plates. After 72 h in culture, cells were fixed and permeabilized in 95% ethanol and acetone at -20°C, rinsed in SPB, and blocked with SPB containing 1% normal goat serum and 0.1% Triton X-100 at room temperature for 15 minutes. Cells were incubated with anti-OGF-IgG (CO172) and anti-OGFr-IgG (BO344) polyclonal antibodies diluted 1:200 in SPB containing 1% NGS and 0.1% Triton-X 100 for 2 h at room temperature. The cells were then washed and incubated with goat anti-rabbit IgG (1:1000) for 45 min in the dark and viewed with fluorescence microscopy. Cells incubated with secondary antibody only were considered controls. The mean intensity of staining was determined for at least eight images per cover glass from three cover glasses per group. Cover glasses from each group were randomly selected for analysis.
Tumors were excised and embedded in OCT, sectioned at 10 µm, and processed according to the same protocol with a primary antibody incubation time of 18 h at room temperature. A random sample of three images per section from six sections per tumor were assessed to determine mean intensity of staining. Slides were visualized using an Olympus IX-81 epifluorescent microscope. Slides were randomly selected for analysis.

3.3.2 Protein Isolation and Western Blotting

OGFr expression levels were measured in cells and tumor tissue by western blotting following published procedures (Donahue et al., 2009). Cells were sheared three to four times with a 25G needle, while tumor tissue was homogenized in RIPA buffer containing PMSF protease inhibitor. Equal amounts of protein (40 µg) were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with goat polyclonal IgG antibody to OGFr (1:500) and donkey anti-goat IgG antibody (1:8000). Blots were normalized to GAPDH.

3.4 DNA Synthesis

The effects of OGF on DNA synthesis were evaluated using BrdU incorporation (Zagon et al., 2013b). Log phase or confluent MDA-MB-231 and MCF-7 cells were seeded onto 22-mm diameter cover glasses in six-well plates and treated with 0.2 µl OGF or sterile saline for 72 h; media and drugs were changed daily. Three h prior to fixing cells, 30 mM BrdU (Sigma Chemicals, Indianapolis, IN) was added to cultures. Cells were rinsed, fixed in 1:1 methanol acetone for 20 minutes at 4°C, processed according to previous literature, and stained with anti-BrdU mouse monoclonal antibody (1:200). At least 1000 cells/treatment using at least two cover
glasses/treatment were counted. Labeling indexes were calculated as the number of positively
stained cells divided by total number of cells.

3.5 Statistical Analyses

All statistical analyses were completed using GraphPad Prism 5® (GraphPad® Software
Inc., San Diego, CA). Data were analyzed using Student’s t-test or one or two-way analysis of
variance (ANOVA) with subsequent Newman-Keuls post-hoc tests. P values less than 0.05 were
considered statistically significant.
Chapter 4

RESULTS

4.1 OGF-OGFr Detection in Human Breast Cancer Cells

MDA-MB-231 TNBC cells were grown to either log phase or confluency and immunostained with antibodies to OGF (CO172) or OGFr (BO344) (Fig 4.1A). Antibodies were raised in the laboratory (Zagon and McLaughlin, 1993, Zagon et al., 2002). Assessment of semi-quantitative immunocytochemistry revealed a significant difference in peptide and receptor expression between log phase and confluent cells (Fig 4.1B). Confluent cells exhibited a 20% reduction in OGFr expression and a 7.5% reduction in OGF expression compared to log phase cells. A preliminary growth curve was generated in which cells were counted at 0, 24, 48, 72, and 96 h time points. OGF was shown to significantly inhibit the growth of TNBC cells at the 72 and 96 h time points.

For comparison to cells without hormonal receptors, ER+/PR+ MCF-7 breast cancer cells were also grown to log phase or confluency and immunostained with antibodies to OGF (CO172) or OGFr (BO344) (Fig 4.2A). Assessment of semi-quantitative immunocytochemistry revealed a significant difference in peptide and receptor expression between log phase and confluent cells (Fig 4.2B). Confluent cells exhibited a 28.5% reduction in OGFr expression and a 35% reduction in OGF expression compared to log phase cells. Analysis of the growth curve revealed a significant reduction in cell growth by OGF at the 72 and 96 h time points. As shown by Figure 4.1A and 4.2A, distribution of OGF and OGFr within the log phase or confluent cells was comparable for each type of cell.
Western blot analysis was conducted to evaluate receptor binding expression between log phase and confluent cells (Fig 4.3). OGFr expression was shown to be significantly reduced in both cell lines between log phase and confluent cells. Confluent TNBC cells exhibited a 46% reduction in OGFr expression compared to log phase cells, while confluent MCF-7 cells exhibited a reduction of 89% in comparison to log phase cells.
Figure 4.1 The presence and function of the OGF-OGFr axis in human triple negative breast cancer cells. (A) The presence of OGF and OGFr in log phase and confluent MDA-MB-231 cells as detected by immunocytochemistry. Cultures were stained with antibodies to OGF (CO172, 1:200) or OGFr (BO344, 1:200); cells stained with secondary antibody only served as controls. (B) Data represent the mean intensity of TRITC staining with BO344 and CO172 (mean ± SEM) and were analyzed individually using Student’s two-tailed t-tests. Significantly different from respective log phase cells at **P<0.01 and ***P<0.001. (C) OGF inhibition of cell growth. MDA-MB-231 cells were seeded on 22 mm cover slips and treated with 10⁻⁶ M OGF or sterile water. Cells were counted at 24, 48, 72, and 96 h. Data represent means ± SEM for triplicate samples. Significantly different from control time points at **P<0.01 or ***P<0.001.
Figure 4.2 The presence and function of the OGF-OGFr axis in human breast cancer cells. (A) The presence of OGF and OGFr in log phase and confluent MCF-7 cells as determined by immunocytochemistry. Cultures were stained with antibodies to OGF (CO172, 1:200) or OGFr (BO344, 1:200); cells stained with secondary antibody only served as controls. (B) Data represent the mean intensity of TRITC with BO344 and CO172 (mean ± SEM) and were analyzed individually using Student’s two-tailed t-tests. Log phase cells significantly different from confluent cells at ***P<0.001. (C) OGF inhibition of cell growth. MCF-7 cells were seeded on 22 mm cover slips and treated with 10^{-6} M OGF or sterile water. Cells were counted at 24, 48, and 72h. Data represent means ± SEM for triplicate samples and were analyzed individually using Student’s two-tailed t-tests. Significantly different from control time points at ***P<0.001.
**Figure 4.3** Western blot validation of OGFr protein expression in human breast cancer cells. Histogram represents means ± SEM values from 3 blots and were analyzed individually using Student’s two-tailed t-tests. OGFr expression significantly differed between respective log phase and confluent cells at *P<0.05 or **P<0.01.
4.2 OGF-OGFr Detection in Tumor Tissue

Female athymic nude mice were subcutaneously injected (8 x 10^6 cells/0.1 ml) with MDA-MB-231 TNBC cells into the right scapular and left flank regions. The mice weighed approximately 20 g at the beginning of the study, and no differences in weight were noted in mice with small or large tumors at the time of termination. Tumors were harvested as small or large when tumor volume reached approximately 350 mm^3 or 1000 mm^3, respectively. These volumes correspond to linear dimensions of less than 10.5 mm x 9.5 mm for small tumors and greater than 13.5 mm x 12.5 mm for large tumors. Tumor volume differed significantly between small and large tumors (Fig 4.4A).

Tumors were processed and stained with antibodies to OGF (CO172) or OGFr (BO344) (Fig 4.4C). OGFr was detected in the cytoplasm, and sometimes in the nucleus, of both small and large tumors; no immunoreactivity was observed in sections processed with secondary antibody only. Analysis of semi-quantitative immunohistochemistry revealed a significant difference in peptide and receptor expression between small and large tumors (Fig 4.4B). Large tumors exhibited a 37.5% reduction in OGFr expression and an 18.7% reduction in OGF expression relative to small tumors. Further analysis by western blot showed a 42% reduction in OGFr expression in large tumors as compared to small tumors.
Figure 4.4 The presence of the OGF-OGFr axis in MDA-MB-231 TNBC tumors grown in nude mice. (A) Small (<350 mm$^3$) or large (>1000 mm$^3$) tumors were collected and final volume was recorded. Small tumor volume significantly differed from large tumor volume at **$P<0.01$. (B,C) Tumors were sectioned and stained with antibodies to OGFr (BO344, 1:200) or OGF (CO172, 1:200); tumors stained with secondary antibody only served as controls (inset). Data represent the mean intensity of TRITC with BO344 and CO172 (mean ± SEM) and were analyzed individually using Student's two-tailed t-tests. Expression of OGFr and OGF in small tumors significantly differed from respective large tumors at *$P<0.05$. (D) Western blot validation of OGFr protein expression in small and large tumors. Histogram represents means ± SEM values from 3 blots. OGFr expression significantly differed between small and large tumors at ***$P<0.001$. 
### 4.3 The Inhibitory Effects of OGF on Cell Growth

The inhibitory effects of OGF on DNA synthesis between log phase and confluent cells were evaluated using BrdU incorporation (Fig 4.5). Log phase or confluent MDA-MB-231 cells (Fig 4.5A) or MCF-7 cells (Fig 4.5B) were seeded and treated with $10^{-6}$ M OGF or sterile water for 72 h. Analysis revealed a significant difference in BrdU incorporation between log phase and confluent cells for both control and OGF groups ($P<0.01$ or $P<0.001$). Relative to controls, OGF-treated log phase MDA-MB-231 cells exhibited a 43.8% decrease in BrdU incorporation, while confluent MDA-MB-231 cells exhibited a 29.8% decrease. OGF-treated log phase MCF-7 cells exhibited a 29.8% decline in BrdU incorporation compared to controls, whereas confluent MCF-7 cells showed a 76.7% decline.

Experiments were conducted to examine functional effects of OGF, taxol, and OGF+taxol on cell growth. Log phase or confluent MDA-MB-231 cells and log phase or confluent MCF-7 cells were seeded and treated with OGF, taxol, OGF+taxol, or sterile water for 72 h. Cells were counted at 0, 24, 48, and 72 h time points. OGF, taxol, and OGF+taxol were all shown to have significant inhibitory effects at 24, 48, and 72 h for log phase TNBC cells relative to controls (Fig 4.6A). Growth was reduced 18-31% by OGF, 25-32% by taxol, and 27-35% by OGF+taxol compared to untreated cells. Combination treatment was significantly effective relative to OGF treatment alone at 48 h, and to either OGF or taxol treatment alone at 72 h in log phase TNBC cells ($P<0.05$). In confluent TNBC culture, all three treatments significantly reduced growth at 48 and 72 h (Fig 4.6B). Growth was reduced 12-16% by OGF, 5-20% by taxol, and 14-27% by OGF+taxol. Combination treatment was significantly effective relative to treatment with either OGF or taxol alone at 72 h ($P<0.01$ or $P<0.05$).
OGF and OGF+taxol treatments significantly inhibited the growth of log phase MCF-7 cells starting at 24 h, while all three treatments were significantly effective at the 48 and 72 h time points (Fig 4.6C). All three treatments significantly inhibited the growth of confluent MCF-7 cells relative to controls at 24, 48, and 72 h (Fig 4.6D). Log phase cell growth was reduced 16-35% by OGF, 17-24% by taxol, and 4-24% by OGF+taxol relative to controls (Fig 4.6C). Combination treatment was not significantly effective compared to either OGF or taxol treatment alone in log phase MCF-7 cells. Compared to controls, confluent cell growth was reduced 11-21% by OGF, 10-35% by taxol, and 8-42% by OGF+taxol (Fig 4.6D). In confluent MCF-7 cells, combination treatment was significantly effective compared to either OGF or taxol treatment alone at 24 and 48 h (P<0.001) (Fig 4.6D).
Figure 4.5 The inhibitory action of OGF on DNA synthesis. (A) MDA-MB-231 cells or (B) MCF-7 cells were seeded on 22 mm diameter cover slips and treated for 72 h with OGF (10⁻⁶ M) or sterile water (control). After 72 h, cells were treated for 3 h with 30 µM BrdU. Data represent the percent of BrdU-positive cells (means ± SEM) and were analyzed using ANOVA and Newman-Keuls post-hoc tests. Significantly different from control treated cells at **P<0.01 or ***P <0.001, and between log phase and confluent cells at +P <0.05, ++P <0.01, or +++P <0.001.
**Figure 4.6.** OGF and taxol inhibition of human breast cancer cell growth. (A) Log phase or (B) confluent MDA-MB-231 cells and (C) log phase or (D) confluent MCF-7 cells were seeded on 22 mm diameter cover glasses and treated with 10^{-6} M OGF, 10^{-9} M taxol, both, or sterile water. Cells were counted at 24, 48, and 72 h. Data represent means ± SEM for triplicate samples and were analyzed using ANOVA and Newman-Keuls post-hoc tests. Treatments significantly different from control time points at *P<0.05, **P<0.01, or ***P<0.001. Combination treatment significantly different from individual treatments at +P<0.05, ++P<0.01, or +++P<0.001.
The experiment was then repeated using only OGF as a treatment (Fig 4.7). Relative to controls, OGF significantly inhibited the growth of log phase TNBC cells at 24, 48, and 72 h of growth (Fig 4.7A), and of confluent TNBC cells at 48 and 72 h of growth. OGF reduced the growth of log phase TNBC cells by 16-29% (Fig 4.7A) and of confluent TNBC cells by 13-15% (Fig 4.7B). OGF significantly inhibited the growth of log phase MCF-7 cells at 24, 48, and 72 h of growth. Confluent MCF-7 cells exhibited a significant decline in growth relative to controls at 72 h. OGF reduced the growth of log phase MCF-7 cells by 19-23% (Fig 4.7C) and of confluent MCF-7 cells by 16% (Fig 4.7D). Percent decrease in cell number was then calculated for OGF-treated cells relative to controls at each time point. OGF decreased TNBC cell number by 17-30% for log phase cells and by 12-17% for confluent cells (Fig 4.7E). OGF decreased MCF-7 cell number by 17-32% for log phase cells and by 9-12% for confluent cells (Fig 4.7F).

These experiments were not repeated in vivo due to the high cost of the athymic nude mouse shown to exhibit the best tumor growth for these cell lines.
Figure 4.7 OGF inhibition of human breast cancer cell growth. (A) Log phase or (B) confluent MDA-MB-231 cells and (C) log phase or (D) confluent MCF-7 cells were seeded onto 22 mm diameter cover glasses and treated with 10^-6 M OGF or sterile water. Cells were counted at 24, 48, and 72h. Data represent means ± SEM for triplicate samples and were analyzed individually using Student’s two-tailed t-tests. Significantly different from control time points at *P<0.05, **P<0.01, or ***P<0.001. Percent decrease in number of OGF-treated cells from controls was calculated for (E) MDA-MB-231 and (F) MCF-7 cells. Histograms represent means ± SEM and were analyzed using ANOVA. Percent decrease in OGF-treated log phase cells differed from OGF-treated confluent cells at **P<0.01 or ***P<0.001.
CHAPTER 5

Discussion

Previous research has documented the presence of the OGF-OGFr axis in a wide variety of human cancers, including pancreatic, colon, renal, ovarian, neuroblastoma, hepatocellular adenoma, SCCHN, and breast (Zagon et al., 2013a). Disruption of the pathway can occur by disease, genetic mutation, continuous exposure to opioid antagonists, neutralization of endogenous opioids, or other imbalances that can accelerate neoplastic cell growth (McLaughlin and Zagon, 2014). Conversely, upregulation of the OGF-OGFr axis has been shown to repress cell division in both normal and abnormal cells (Zagon et al., 2009, Zagon et al., 2013b). Given that TNBC is an aggressive cancer that does not respond to current treatments (Mayer et al., 2014, Chougule et al., 2011), this research investigated the presence of the OGF-OGFr axis in TNBC and its potential as an effective therapeutic agent. The data demonstrate that OGF is a prospective biological therapy for treatment of TNBC.

OGF inhibition of human breast cancer cell growth

Both the OGF peptide and its receptor have been identified in the cytoplasm and nucleus of the TNBC cell line MDA-MB-231 (Zagon et al., 2013b, McLaughlin and Zagon, 2014). OGF was reported to inhibit proliferation in these cells in a dose-dependent, receptor-mediated and reversible manner, decreasing cell growth by almost 20% compared to controls (Zagon et al., 2013b). The data presented here confirm these earlier findings, as OGF was shown to significantly inhibit cell proliferation relative to controls in both log phase and confluent MDA-MB-231 TNBC cells and ER+/PR+ MCF-7 breast cancer cells (Fig 4.1C, Fig 4.2C, Fig 4.6, Fig 4.7).
OGF has been shown to inhibit cell proliferation both \textit{in vitro} and \textit{in vivo} in a variety of other human cancers, including ovarian, pancreatic, and squamous cell carcinoma of the head and neck (Donahue et al., 2011, Zagon et al., 2005a, Zagon et al., 2008, McLaughlin and Zagon, 2006). Furthermore, preclinical studies with OGF and chemotherapeutic drugs demonstrated cumulative inhibitory effects on cells in culture and tumors in nude mice in SCCHN and pancreatic adenoma (Zagon et al., 2005a, McLaughlin and Zagon, 2006).

No cumulative effects were seen in previous \textit{in vitro} studies with MDA-MB-231 cells, although addition of OGF to taxol treatment reduced the amount of cell death by 60% (Zagon et al., 2013b, McLaughlin and Zagon, 2014). The data presented here demonstrate cumulative inhibitory effects in MDA-MB-231 cultures and MCF-7 cultures treated with both OGF and taxol (Fig 4.6), possibly because the drug combination targets two separate phases of the cell cycle. Relative to controls, treatment with either OGF, taxol, or OGF+taxol significantly decreased cell proliferation in both cell lines. In TNBC culture, growth was reduced upwards of 35% in log phase cells (Fig 4.6A) and 27% in confluent cells (Fig 4.6B) relative to controls. In MCF-7 culture, growth was reduced upwards of 42% in log phase cells (Fig 4.6C) and 35% in confluent cells (Fig 4.6D) relative to controls. These data support that log phase TNBC cells and MCF-7 cells are more receptive to treatment than confluent cells.

The inhibitory effects of OGF on cell proliferation are mediated by its targeting of DNA synthesis. OGF targets DNA synthesis by upregulating the cyclin-dependent inhibitory kinases p16 and p21, which arrests cells in the transition from G0/G1 phase to S phase of the cell cycle (Donahue et al., 2009, Cheng et al., 2009b, McLaughlin and Zagon, 2012). Decreased cell replication was confirmed using BrdU incorporation showing that OGF-treated cells exhibited significantly lower levels of DNA synthesis relative to controls (P<0.01 or P<0.001) (Fig 4.5).
**OGFr expression is downregulated with cancer progression**

Previous research on ovarian cancer indicated a significant reduction in the number of functional OGF receptors present in cysts and malignant tumors compared to normal epithelial cells (Fanning et al., 2012, Zagon et al., 2013a). Analysis of tissue samples from cysts and late stage (III/IV) tumors revealed a decrease in OGF expression by 29% and 58%, respectively, from normal cells (Zagon et al., 2013a). Expression of OGFr was decreased by 34% and 48% in cysts and tumors, respectively, relative to normal levels (Zagon et al., 2013a). Receptor binding assays on cysts and malignant tumor tissues indicated that tumors had 5.4-fold fewer OGF receptors than cysts, suggesting that deficits in the OGF-OGFr axis are amplified as cancer progresses (Zagon et al., 2013a, McLaughlin and Zagon, 2014).

Data generated in the present study demonstrate similar results, suggesting that OGFr expression in TNBC correlates to the proliferative stage of cancer cells, and presumably to the progression of tumor growth. In both MDA-MB-231 and MCF-7 cells, OGF and OGFr expression was diminished in confluent cells relative to log phase cells (Fig 4.1B, Fig 4.2B, Fig 4.3). Similar results were seen in large tumors relative to small tumors (Fig 4.4B, 4.4D), where western blot analysis revealed reductions in OGFr expression approaching 50%. Loss of OGFr results in decreased binding of OGF to its receptor. Hence, based on these results and given that large tumors were approximately two-fold greater in diameter and volume compared to small tumors, it may be inferred that small tumors have more OGF receptors than large tumors.

The present study confirms and extends findings reported on SCCHN, reporting that large tumors, generally two-fold greater in diameter and volume than small tumors, had 3- to 7-fold less OGF receptors than small tumors (McLaughlin and Zagon, 2006). Moreover, there was a decrease in receptor binding of 31-86% in large tumors compared to small SCCHN tumors.
Collectively, the data suggest that the number of OGFr receptors in SCCHN correlates with the size and, presumably, the progression of the tumors.

Previous studies using human pancreatic and colon cancer have shown different results. OGFr gene expression and binding affinity were determined for human pancreatic adenocarcinoma cells and human colon cancer cells using small, medium and large tumors from nude mice (Zagon and McLaughlin, 2006). Data generated from the study demonstrate that OGFr binding affinity, binding capacity, and transcriptional activity are not dependent on the size or progression of human pancreatic or colon cancer (Zagon and McLaughlin, 2006).

**OGF-OGFr axis deficits are amplified with cancer progression**

To confirm that the OGF-OGFr axis may become defective as tumors become larger or more aggressive, studies were conducted using log phase and confluent TNBC and MCF-7 cells treated with OGF. BrdU incorporation revealed that OGF-treated log phase TNBC cells exhibit a 43.8% decrease in DNA synthesis compared to 29.8% for confluent cells, suggesting that confluent cells are less receptive to treatment (Fig 4.5). Similar results were seen when percent decrease in cell number was calculated for OGF-treated cells relative to controls (Fig 4.6, 4.7E, 4.7F). OGF-treated TNBC cell number decreased upwards of 35% for log phase cells, compared to 17% for confluent cells (Fig 4.7E). OGF-treated MCF-7 cell number decreased upwards of 32% for log phase cells, compared to 12% for confluent cells (Fig 4.7F). These data once again suggest that confluent cells are less receptive to OGF treatment. A defect in the OGF-OGFr axis, such as loss of protein or genetic mutation, would confirm these results.
Conclusions and Future Directions

Although TNBC accounts for only 15% of all cases of invasive breast cancer, it is an aggressive cancer that does not respond to current treatment options (McLaughlin and Zagon, 2014, Zagon et al., 2013b, Chougule et al., 2011, Mayer et al., 2014). TNBC frequently affects younger women, and has an increased likelihood of distant recurrence, twofold greater mortality risk, and a high tendency to metastasize outside the breast (Mayer et al., 2014, Bramatai et al., 2014). The five-year relative survival rate for women with successfully treated ER+/PR+ and/or HER2+ breast cancer is 98.6% (Jemal et al., 2011, Zagon et al., 2013b). The median survival rate for patients with metastatic TNBC is only 13 months, and most women will die from the disease despite current systemic therapies (Mayer et al., 2014).

There is a critical need to develop TNBC treatments that target the underlying biology of tumor growth. The data presented here confirm that OGF can be used to inhibit the proliferation of human TNBC and ER+/PR+ breast cancer in vitro, demonstrating its potential as a successful biological therapy for treatment of TNBC. Future studies should seek to replicate these treatments in animal models, warranting the use of OGF in clinical trials.
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