RICE UNIVERSITY

MUC16/CA125 Regulation by the Proinflammatory Cytokines TNF α and IFNγ and the PPARγ Agonist Rosiglitazone in Breast and Ovarian Cancers

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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Abstract

MUC16 is a high molecular weight transmembrane mucin (TM) carrying the CA125 epitope, a well-known molecular marker for human cancers. TMs are restricted to the apical surface of normal epithelia. TMs not only are over-expressed, but also lose polarized distribution in cancers. Similar to other TMs, MUC16 is overexpressed in a variety of epithelial cancers. In this work the regulation of MUC16 by the proinflammatory cytokines, TNFα and IFNγ, in addition to the PPARγ agonist rosiglitazone, a drug used in the treatment of type 2 diabetes, was explored. MUC16 mRNA and protein expression was mildly stimulated by low concentrations of TNFα or IFNγ when used alone; however, combined treatment with both cytokines resulted in a moderate (3-fold or less) to large (>10-fold) stimulation of MUC16 mRNA and protein expression in a variety of cancer cell types indicating that this may be a general response. Human cancer tissue microarray analysis indicated that MUC16 expression directly correlates with TNFα and IFNγ staining intensities in certain cancers. NFκB is an important mediator of cytokine stimulation of MUC16 since siRNA-mediated knockdown of NFκB/p65 greatly reduced cytokine responsiveness. The 250 bp proximal promoter region of MUC16 contains an NFκB binding site that accounts for a large portion of the TNFα response. Rosiglitazone exerts a dual role in the regulation of MUC16 since at low pharmacologically relevant concentrations it further stimulates MUC16 in combination with cytokines, but at high concentrations inhibits cytokine stimulated MUC16. This regulation is PPARγ dependent. In this work methods were developed to manipulate MUC16 expression that can provide new approaches to treating cancers whose growth or metastasis is characterized by elevated levels of TMs, including MUC16.
Dedication

I dedicate my dissertation work to my family and friends whose love and support sustained me throughout. A special feeling of gratitude for my parents who have always loved me unconditionally and whose good examples have taught me to work hard for the things I want to achieve. This work is also dedicated to my two brothers and my sister who have been a constant source of support, patience and encouragement during the challenges of graduate school. This work is also dedicated to other family members including my sister in law, and most importantly my nieces and nephew that inspire me to be a better person and help me keep going.

I am also dedicating this thesis to four beloved people that mean so much to me and unfortunately left this world during the years of my thesis. Their memories remain very strong in my heart and keep inspiring me.
Acknowledgements

First, I would like to express my sincere gratitude to my advisor and mentor Dr. Dan Carson for the great opportunity and honor of being part of his team. For his continuously guiding and supporting me over the years on my Ph.D. studies. Dan: with your endless patient, motivation, and immense knowledge you have made my experience at Rice unforgettable. You have set an example of excellence as a researcher and mentor and have made a positive impact in my life. You always ignited me with energy, positive attitude and enthusiasm at each meeting. Your guidance has led me to many opportunities and I can never pay you back for all the help you have provided me, I hope you find some kind of satisfaction in this modest paper. Thank you so much!!!!

I would like to thank my unofficial second advisor, Dr. Cindy Farach-Carson. Cindy: I have been so fortunate to have you as a second advisor. It has always been an honor and delight to hear your brilliant ideas and input to my research during our meetings and reviewing of my manuscript. Your have helped me see my research from a different and interesting perspective. You are a tireless and energetic person; your presence and attitude towards life is a tremendous inspiration. Thank you!

I would also like to thank Dr. Larry Rohde from UHCL, my former advisor and forever mentor, whose wise advise and guidance brought me to Rice to the Carson lab.
I would also like to thank my committee members, Drs. Bonnie Bartel, Daniel Wagner and Peter Lwigale who have been an important part in shaping my thesis work through their feedback on my research during yearly progress reviews, as well as Dr. Jeff Jacot who participated in my defense. Critical collaborators I would like to thank include, Dr. Robert Bast and his team including Margie Sutton and Dr. Zhen Lu. From MD Anderson, and Drs. Steve Conlan and Lewis Francis from Swansea University.

I would like to thank Drs. Nikki Delk, Pamela Constantinou, Neeraja Dharmaraj and Eliza Fong for trusting me in collaborating with them in co-authoring their manuscripts.

I would also like to thank the current and past members of the Carson-lab: Drs Pamela Constantinou, Nikki Delk, Neeraja Dharmaraj, Curt Warren, Danielle Wu, Daniel Harrington, Eliza Fong, Brian Grindel, Brian Danysh, Jerahme Martinez, Dan Grigore and Brian Engle; as well as Patricia Chapela, Derek Shenefelt, Ariel Diaz, Julie Liu, Fabio Brasil, Mariane Martinez, Kelsea Hubka, Lindsey Sablatura, and the undergrad Catherine Chantre, for the wonderful times we had together. I greatly appreciate the times we had discussions about our research, data interpretation, the help I received in the reviewing of my manuscripts, and most importantly for a friendly smile, a morning greeting and encouraging words when experiments did not work as expected.
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<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>ACS</td>
<td>American Cancer Society</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>AMOP</td>
<td>Adhesion-associated domain</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen-presenting cells</td>
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<tr>
<td>BRCA</td>
<td>Breast Cancer Gene</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer antigen 125</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Regularly interspaced, short palindromic repeat associated endonuclease</td>
</tr>
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<td>crRNAs</td>
<td>CRISPR RNAs</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DCIS</td>
<td>Ductal carcinoma <em>in situ</em></td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DR</td>
<td>Direct repeats</td>
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<tr>
<td>DSB</td>
<td>Double strand breaks</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<tr>
<td>ErbB2</td>
<td>Erythroblastic leukemia viral oncogene 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>gRNA</td>
<td>Guide RNA</td>
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<td>GPI</td>
<td>Glycophosphatidylinosital</td>
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<td>G2/M</td>
<td>Gap 2/ Mitosis</td>
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<td>GW9662</td>
<td>2-Chloro-5-nitro-5-N-phenylbenzamide</td>
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<tr>
<td>HDR</td>
<td>Homology directed repair</td>
</tr>
<tr>
<td>HGNC</td>
<td>Human Genome Organization Gene Nomenclature Committee</td>
</tr>
<tr>
<td>HSPG2</td>
<td>Heparan sulfate proteoglycan 2</td>
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<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin 12</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin 18</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor kappa-B kinase</td>
</tr>
<tr>
<td>IkBα</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cell</td>
</tr>
<tr>
<td>Indel</td>
<td>Insertion/deletion</td>
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<td>JAK</td>
<td>Janus kinase</td>
</tr>
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<td>kb</td>
<td>Kilobase</td>
</tr>
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<td>kD</td>
<td>Kilodalton</td>
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<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
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<tr>
<td>LMO2</td>
<td>LIM domain only 2</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MMP-7</td>
<td>Matrix metalloproteinase-7</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
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<td>MUC1</td>
<td>Mucin 1</td>
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<td>MUC4</td>
<td>Mucin 4</td>
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<tr>
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<td>Mucin 16</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NIDO</td>
<td>Nidogen</td>
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<tr>
<td>NHEJ</td>
<td>Nonhomologous end-joining</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain enhancer of activated T cells</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OC125</td>
<td>Ovarian cancer 125</td>
</tr>
<tr>
<td>PAMs</td>
<td>Proto-spacer adjacent motifs</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween-20</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitive real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein kinase</td>
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<tr>
<td>ROCA</td>
<td>Ovarian Cancer Algorithm</td>
</tr>
<tr>
<td>ROSI</td>
<td>Rosiglitazone</td>
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<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
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<tr>
<td>SCR</td>
<td>Short consensus repeats</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEA</td>
<td>Sea urchin sperm protein-enterokinase-agrin</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering ribonucleic acid</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRADD</td>
<td>Tumor necrosis factor receptor type 1-associated DEATH domain</td>
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<td>TNF receptor-associated factor 2</td>
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<td>tracrRNA</td>
<td>Transactivating CRISPR RNA</td>
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<tr>
<td>TVUS</td>
<td>Transvaginal ultrasound</td>
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<tr>
<td>TZDs</td>
<td>thiazolidinediones</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VWD</td>
<td>Willebrand factor type D</td>
</tr>
<tr>
<td>ZmpC</td>
<td>The ZmpC zinc metalloproteinase of Streptococcus pneumoniae</td>
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</table>
Chapter 1 : General Introduction

A mucus barrier protects epithelial tissue from harsh conditions. Secreted and transmembrane (TM) mucins including MUC16 comprise the mucus barrier. MUC16’s main role is to protect and lubricate epithelial surfaces. However, MUC16 expression is linked to inflammation and cancer. Due to its high overexpression in ovarian cancer, MUC16 is widely used as an ovarian tumor marker. Nevertheless, the regulation of MUC16 has been understudied. Regulation of MUC16 by proinflammatory cytokines as well as a transcription factor previously shown to modulate the expression of MUC1, namely PPARγ, was the focus of this research.

1.1. Epithelium Overview

A simple epithelium consists of a layer of single layer of cells connected to each other laterally and featuring apical, lateral and basal polarity. They connect to form epithelial sheets that line the cavities and surfaces throughout the animal body, protecting them from the external environment. Epithelial cells are polarized since either side of the cell varies in structure and function. Apical refers to the top of the cell and basal to the base of the cell (Rowley, 2016). Epithelium has several functions including providing a barrier, as a sensory organ, secretory functions, ion transport and adsorption, movement and immunity. Epithelia play a direct role in inflammation and are able to direct
adaptive immunity (Rowley, 2016). There are several different types of epithelial tissues (Fig. 1.1). They are specialized to their function and location in the body. Most epithelia are single cell layers and as such require robust defense mechanisms to maintain the integrity of the epithelial barrier.
Figure 1.1. Epithelial tissue distribution in the human body.

Epithelial tissue is distributed throughout the human body. In general, there are 7 to 8 different types of epithelial tissues. Their first name of the tissue indicates the number of cell layers; simple refers to one layer of cells and stratified refers to more than one layer of cells. Last name of the name refers to shape of the cell; squamous cells are wider than tall; cuboidal cells are as wide as tall; and columnar cells are taller than they are wide. Figure adapted from: http://pulpbits.com/7-tissue-pictures-in-the-human-body/epithelial-tissue.
1.2. Cancer Overview

Cancer is the second leading cause of death in the US, and it has been estimated that about 1.6 million new cancer cases will be diagnosed and about 600,000 people in the United States will die of cancer in 2016. This amounts to 1,630 people per day (American-Cancer-Society, 2016).

Cancer is the term given to a collection of diseases that are related to each other. In general in cancer, there is an uncontrolled growth and spread of abnormal cells that can lead to death. There are both intrinsic and extrinsic factors associated with cancer. Intrinsic factors include genetic and epigenetic states. Extrinsic factors include infectious agents, tobacco use and an unhealthy diet (American-Cancer-Society, 2016).

Cells normally grow and divide to replace old or damaged cells as needed by the body. Tumorigenesis, the formation of tumors, is a multistage process that leads to the transformation of normal cells into malignant cells (Hanahan & Weinberg, 2000). Cells become progressively abnormal, damaged and old cells do not die and new cells form even when not needed. Such aberrant growth can form tumors (NCI, 2015). According to the National Cancer Institute, there are more than 100 different types of cancer. Cancers are typically named depending on the tissue of origin, e.g., breast cancer, brain cancer, lung cancer, etc.
Cancers are categorized depending on the type of cells from which they originated and include carcinomas, sarcoma, leukemia, lymphoma, multiple myeloma, melanoma, brain and spinal cord tumors. Carcinomas are cancers of epithelial cells, cells that form the lining of internal and external parts of organs within the body, and are the most common types of cancer constituting 80 to 90% of all cancers (NCI, 2015; News-Medical, 2016). Depending on the epithelial cell type, carcinomas have specific names. For example, adenocarcinomas are cancers formed in the epithelium of fluid- or mucus-producing cells. The majority of breast, colon, prostate and ovarian cancers are adenocarcinomas (NCI, 2015).

1.3. Breast cancer

1.3.1. Normal Breast
The normal female breast (Fig. 1.2A) is composed of primarily lobules (milk-producing glands), ducts (small tubes that carry milk from the lobules to the nipple), and stroma (fatty and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels)(Going & Moffat, 2004)
Figure 1.2. Diagram of the normal and cancerous breast.

(A) Shows the general structure of the breast and an expanded view of the milk ducts and lobules. (B) Shows that in the case of metastatic cancer, malignant cells can grow in the lobules and spread to other parts of the body.
1.3.2. Breast Cancer

The term breast cancer is used when malignant cells develop in the terminal ductal-lobular unit of milk-producing glands of the breast tissue or the ducts, the channels that allow milk to flow from the lobules to the nipple. Type of cancer can be divided into noninvasive or an invasive carcinoma (Nelson et al., 2016). A non-invasive breast cancer is normally confined to the milk ducts (ductal carcinoma in situ [DCIS]) or lobules (lobular carcinoma in situ [LCIS]). These cancers do not invade normal tissue inside or outside the breast. There is no invasion of the surrounding stroma and there is no metastasis. Nevertheless, a LCIS represents increased risk of invasive lobular or ductal breast cancer (Breast-Cancer-organization, 2015; Nelson et al., 2016). The majority of breast cancers are invasive. Invasive breast cancer is defined as a cancer that grows into normal, healthy tissue (Fig. 1.2B). It invades the basement membrane into adjacent stroma and, consequently, has metastatic potential (Breast-Cancer-organization, 2015; Nelson et al., 2016). The sites in which it will most commonly metastasize are adjacent lymph nodes, liver, lung and bone. Invasive or infiltrating ductal carcinoma account for 70 to 80 % of invasive breast cancers while invasive lobular carcinoma accounts for only 10 %.
1.3.3. Breast cancer incidence and mortality

Breast cancer is the most frequently diagnosed cancer in women and it is the second most common cause of cancer death in women. It is estimated that 246,660 women and 2,600 men will be diagnosed with an invasive breast cancer in 2016. It is estimated that 40,450 women and 440 men will die from breast cancer in 2016 (Siegel et al., 2016).

1.3.4. Breast cancer risk factors

Breast cancer risks include genetic mutations, family and personal history of breast cancer, race and ethnicity, dense breast tissue, certain benign breast conditions and breast radiation. Additionally there are lifestyle risk factors including use of birth control, using alcohol and obesity. Additionally, there are some unclear factors, where research does not show a clear link, such as diet and vitamin intake, chemicals in the environment, tobacco smoke and night work. Controversial or disproven risk factors include use of antiperspirants, use of bras, abortion and breast implants (American Cancer Society 2016). Approximately 15% of women diagnosed with breast cancer have a family member already diagnosed with it and 5-10% breast cancers are linked to gene mutations. The
most common genetic mutations are associated with the _BRCA1_ and _BRCA2_ genes (NCI, 2015).

### 1.3.5. Detection and Treatments

The early stages of breast cancer are asymptomatic causing this disease to go undetected unless regular screenings such as yearly mammograms are used (American-Cancer-Society, 2016; Breast-Cancer-organization, 2015; Nelson et al., 2016). In general breast cancer can be detected by medical history and physical exams, imaging tests, mammograms, breast ultrasound and magnetic resonance imaging (MRI) of the breast. Other tests include, nipple discharge exam, ductal lavage and nipple aspiration, biopsy, fine needle aspiration biopsy, core needle biopsy, surgical (open) biopsy and lymph node biopsy (American-Cancer-Society, 2016; Bevers et al., 2009).

The types of treatments can be divided into local and systemic treatment. Local treatment includes surgery and radiation therapy. Systemic treatment contains chemotherapy, hormone therapy, targeted therapy, and bone-detection therapy (American-Cancer-Society, 2016). Additionally, whole breast radiation might be included in addition to lumpectomy for localized and regional invasive cancer. In some cases only selected metastatic cancers surgery is performed, commonly followed by radiation therapy (Bevers et al., 2009; Nelson et al., 2016).
1.3.6. **Breast cancer staging**

The stages of the cancer are assigned taking into consideration a combination of T, N, and M classifications. T plus a number determines the site and location of the tumor. N is used to indicate that the doctor has evaluated the lymph nodes. M indicates if the cancer has spread or metastasized (Fig. 1.3) ((ASCO), 2015).

Stage 0 is a non-invasive cancer that is localized to the ducts and lobules of the breast tissue. Stage V is the stage the cancer regardless of size that has already metastasized and spread to other areas of the body including distant lymph nodes, chest wall, lungs, brain, liver and bone (Fig. 1.3) ((ASCO), 2015).
### Figure 1.3. Breast cancer staging.

Diagram describes the TNM staging method used in breast cancer. T: determines the size and location of the tumor; N: lymph nodes have been evaluated; and M: cancer has spread or metastasized.
1.4. Ovarian cancer

1.4.1. Normal Ovary

The ovary is an egg (ovo)-producing organ, it is found in pairs, one on each side of the uterus, as part of the vertebrate female reproductive system (Fig. 1.4A). Ovaries are the main source of the estrogen and progesterone hormones. Once the eggs are produced, they travel through the fallopian tubes to the uterus where they can get fertilized and develop into a fetus (American-Cancer-Society, 2016). The approximate size of ovaries from adult women is 3 to 5 x 1.5 to 3 x 0.6-1.5 cm but the size varies depending on follicular derivatives. There are three defined zones; an outer cortex, a central medulla and the hilus (Uhlén et al., 2015).
Figure 1.4. Progression of ovarian cancer.

Image shows a normal ovary (A), and as cancer starts to develop, it can stay in the ovary (B) or it can move away from it (C) and metastasize to surrounding areas and distant sites.
1.4.2. Ovarian cancer

Ovarian cancer is the term used when malignant cells start growing on the ovaries (Fig. 1.4B and C). There are three different cell types in the ovaries, epithelial, germ cells and stroma. Epithelial cells derive from the mullerian duct and cover the ovary, germ cells derive from the endoderm and migrate to the gonadal ridge where they proliferate and differentiate into oocytes, and stroma cells are where estrogen and progesterone are produced (Romero & Bast, 2012). Each cell type can evolve into a different tumor type (Fig. 1.5). The majority of ovarian tumors (90%) are epithelial tumors that originate from cells coating the outer surface of the ovary (Fig. 1.5). Germ cell tumors originate from the egg-producing cells and stromal tumors originate from tissue in charge of the structure of the ovary and hormone production (American-Cancer-Society, 2016; Romero & Bast, 2012).

1.4.3. Ovarian cancer incidence

Ovarian cancer is the most lethal gynecological malignancy in the US and it accounts for 5% of cancer deaths among women. It is estimated that about 22,280 new cases of ovarian cancer will be diagnosed with about 14,240 deaths in the US in 2016 (American-Cancer-Society, 2016). The lifetime risk of a woman to develop ovarian cancer is about 1 in 75 and 1 in 100 of dying from the disease (American-Cancer-Society, 2016).
Figure 1. 5. Types of ovarian cancer.

The diagram illustrates the three different types of cells found in the ovary and their relative location. Each cell type gives rise to a different type of cancer.
1.4.4. Ovarian cancer risk factors

Several risk factors are associated with ovarian cancer and include age, obesity, reproductive history, birth control use, gynecologic surgery, use of fertility drugs, hormone therapy, family history of ovarian cancer, breast cancer, or colorectal cancer, family cancer syndromes, personal history of breast cancer, talcum powder, diet, analgesics, smoking and alcohol use (American-Cancer-Society, 2016; Jones & Drapkin, 2013; Romero & Bast, 2012). Nevertheless, the major risk factors are advancing age, number of ovulatory cycles, and family history of breast, ovarian, uterine or colon cancer associated with mutations of BRCA1, BRACA2 and TP53 genes (American-Cancer-Society, 2016; Romero & Bast, 2012).

1.4.5. Detection and Treatments

Ovarian cancer is frequently referred to as the “silent killer” because even though 80% of patients show early symptoms (Bast et al., 2009), symptoms frequently get dismissed because they are shared with other common conditions such as gastrointestinal, genitourinary and gynecological (American-Cancer-Society, 2016; Bast et al., 2009). Currently, only 20% of ovarian cancers are detected early. When ovarian cancer is detected at an early stage, when the disease is still confined to the ovary, there is a 95% of survival rate (American-Cancer-Society, 2016). Ways to detect ovarian cancer early include, regular women’s
health exam, knowledge of early symptoms and screening test for ovarian cancer. Even though a lot of research has been done in the finding of ovarian markers to be used in the early detection of the disease, currently the most used tests are transvaginal ultrasound (TVUS) and the CA125 blood test (American-Cancer-Society, 2016).

CA125 is high molecular weight heavily glycosylated transmembrane mucin, also known as MUC16, that is expressed in about 80% of ovarian cancers but in less than 1% of normal patients (Bast et al., 1983; Yin & Lloyd, 2001). CA125/MUC16 is shed from ovarian cancers and circulates in serum, and therefore has been used as a first biomarker to monitor ovarian cancer during chemotherapy (Bast et al., 1983). High levels of CA125 after chemotherapy, with 90% precision, indicate residual disease (Romero & Bast, 2012). CA125 levels are also used in combination with age, ultrasound and other biomarkers to detect patients with pelvic masses, which will alert physicians on further treatment or referral to a more specialized oncologist (Moore et al., 2010). Even though CA125 is a good marker, it is still not sufficient to specifically detect early ovarian cancer and there are current clinical trials evaluating CA125 levels to prompt for an ultrasound and perhaps surgery (Lu et al., 2013; Menon et al., 2009).

The most common treatment for ovarian cancer includes surgical resection, surgery combined with postoperative chemotherapy and radiation therapy. In more than one half of the cases ovarian epithelial cancer is bilateral and unfortunately, by the time of detection about 70% of ovarian cancers have spread
beyond the ovaries and is rarely curable ((ASCO), 2015; Uhlén et al., 2015; Uhlen et al., 2010).

Current therapy is generally based on platinum containing agents including carboplatin or cisplatin, and taxanes such as paclitaxel or docetaxel. The standard regime has been expanded by adding the monoclonal antibody directed to the Vascular Endothelial Growth Factor (VEGF), bevacizuma (Bukowska et al., 2016).

1.4.6. Ovarian cancer staging

Stages of ovarian cancer are similar to breast cancer. Additionally, in order to be more specific, letters plus a number is used i.e. T1a: tumor contained within one ovary, NX: The regional lymph nodes cannot be evaluated, M0 (M plus zero): There is no cancer beyond the peritoneal area ((ASCO), 2015). For example, stage I: The cancer localized to the ovaries (T1, N0, M0) (Fig. 1.6) and Stage IV: The cancer that has spread to distal organs (any T, any N, M1) (Fig. 1.6) ((ASCO), 2015).
Figure 1. 6. Ovarian cancer staging.

Figure indicates the different stages of ovarian cancer, the extent of the disease in the surrounding or distal organs, and the survival rate.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Extent of disease</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Limited to ovaries</td>
<td>75-90%</td>
</tr>
<tr>
<td>Ia</td>
<td>One ovary</td>
<td></td>
</tr>
<tr>
<td>Ib</td>
<td>Both ovaries</td>
<td></td>
</tr>
<tr>
<td>Ic</td>
<td>Ruptured capsule, surface tumour, or positive peritoneal washings/ascites</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Limited to pelvis</td>
<td>45-60%</td>
</tr>
<tr>
<td>IIa</td>
<td>Uterus, tubes</td>
<td></td>
</tr>
<tr>
<td>IIb</td>
<td>Other pelvic structures</td>
<td></td>
</tr>
<tr>
<td>IIc</td>
<td>The above plus positive peritoneal washings/ascites</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Limited to abdomen</td>
<td>30-40%</td>
</tr>
<tr>
<td>IIIa</td>
<td>Microscopic metastases</td>
<td></td>
</tr>
<tr>
<td>IIIb</td>
<td>Macroscopic metastases &lt;2cm</td>
<td></td>
</tr>
<tr>
<td>IIIc</td>
<td>Macroscopic metastases &gt;2cm, regional lymph nodes</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Distant metastases outside abdominal cavity</td>
<td>&lt;20%</td>
</tr>
</tbody>
</table>

Figure adapted from: https://ixquick.com/cgibin/serveimageur/Fgeekymedics.com/ovarian-cancer-staging.jpg
1.5. Mucin genes and proteins

1.5.1. Mucins

The mucin or MUC genes encode the mucin glycoproteins. Mucins are high molecular weight, heavily glycosylated proteins produced by epithelial tissues of most metazoans. MUCs protect and lubricate the mucosae of airways, digestive and reproductive systems, and many other organs and tissues in the human body. The human MUC family is composed of twenty-one members, which have been classified as a family based on the common feature of multiple heavily O-glycosylated tandem repeat motifs. The MUC family has been sub-divided into two groups, the secreted and the membrane bound or transmembrane (TM) mucins (HGNC, 2014). The secreted mucins are released from the cells to form a mucus barrier providing protection to epithelial cells that line the respiratory and gastrointestinal tracts, as well as ductal surfaces of the body organs such as kidney, heart, pancreas, liver and breast. The TM mucins on the other hand, span the cell’s outer membrane contributing to the protective mucus gel through the O-glycosylated tandem repeat that extends over 100 nm from the cell surface and beyond the glycocalyx which is about 10 nm (Kufe, 2009).

Characterization of mucins has been a challenge due to their high molecular weight, high degree of glycosylation and size heterogeneity. Nonetheless, NMR studies of MUC1 polypeptide revealed the some α helical structure with little β
sheet character and mostly random coils (Fontenot et al., 1993). Due to their large size mucins can be directly imaged, and this approach has been used to study mucins. For instance, an atomic force microscopic study of ocular mucin proved the multimeric nature of mucins by observing in situ depolymerization upon treatment with dithiothreitol (Brayshaw et al., 2004). Mucins are translated in the rough endoplasmic reticulum whereas the bulk of the glycosylation reactions occur in the Golgi apparatus (Ten Hagen et al., 2003)

1.5.2. Transmembrane mucins

TM mucins share several common features. First, they comprise a large extracellular domain mostly composed of tandem repeat motifs that are good acceptors of O-linked oligosaccharides; these oligosaccharides are arranged around the protein core forming a bottle-brush structure (Bansil & Turner, 2006), a single short transmembrane domain and a relatively small cytoplasmic tail. Additionally, mucins can be N-glycosylated (Duraisamy et al., 2006). The cytoplasmic, carboxy terminal domain, a region containing moderate O-glycosylation and a few N-glycosylation sites and a high proportion of cysteine. The cysteine region is involved in dimerization via disulfide bond formation and polymerization of dimers (Bansil & Turner, 2006).

Of the TM mucins, MUC1, MUC4 and MUC16 are the best characterized. They have a large extracellular domain rich in tandem repeat motifs, a single transmembrane-spanning region, and a small cytoplasmic tail. These three mucins are all initially synthesized as a single protein core, which is post-
translationally cleaved to generate heterodimers. MUC1 is the smallest of the three, with a core protein size of about 200 kDa. This molecular weight more than doubles with full glycosylation of the mucin. The MUC4 core protein has a molecular weight of about 900 kDa. MUC16 is the largest of the three and the largest TM mucin with a core protein molecular weight of approximately 2000 kDa (Hattrup & Gendler, 2008). 

The normal pattern of TM mucin expression is well defined, but can be disrupted under certain conditions including in response to environmental challenges and epigenetic modifications which can alter gene expression and secretion rates (Andrianifahanana et al., 2006). Mucin-producing tissues are assigned to three major categories according to their localization and functional characteristics. These are the respiratory, digestive and reproductive systems. Mucins share overlapping expression among different organs, but their biochemical characteristics can differ depending on the type of organ, development stage and physiological state. Mucins showed distinct differences in mucin glycoproteins and glycopeptide species by the use of pronase digestion from various cancer cells. The assay indicated differences in the distribution of mucin carbohydrate chains but also the different structures of sialylated mucin carbohydrate chains among the various human cancer cell studies (Chandrasekaran et al., 2012; Lan et al., 1987).
1.5.3. *Evolution of MUC1, MUC4 and MUC16*

MUC1, MUC4 and MUC16 appear to have evolved from distinct ancestors (Duraisamy *et al.*, 2006). The only sequence similarity MUC1 has with the other mucins is the presence of a sea urchin sperm protein-enterokinase-agrin (SEA) domain. Studies indicate that the MUC1 SEA domain originated from the heparan sulfate proteoglycan of basement membrane (HSPG2; perlecan). MUC4 has no SEA domains, but contains a NIDO domain evolved from nidogen, an adhesion-associated domain (AMOP) and a Von Willebrand factor type D (VWD) domain. MUC16 contains 56 SEA domains that are found in the chicken gene which appear to have arisen through gene duplication events. The MUC16 SEA domains appeared to have evolved from agrin before the divergence of birds and mammals (Duraisamy *et al.*, 2006).

1.6. *CA125/MUC16 gene and protein*

1.6.1. *Discovery and Structure*

Carbohydrate-antigen-125 was first identified in an antibody screen against the ovarian cancer cell line OVCA433 (Bast *et al.*, 1981). Mice were immunized with the cancer cells, their spleen was harvested and hybridomas were prepared. Clones were screened based on their ability to bind ovarian cancer cells, but not to Epstein-Barr virus immobilized B cells from the same patient. One promising clone was designated ovarian cancer 125 (OC125) and the antigen recognized by the antibody, cancer antigen 125 (CA125) (Bast *et al.*, 1981). Shortly after its
discovery, CA125 was recognized as a mucin-like protein with repeating peptide subunits and abundant glycosylation (Davis et al., 1986). It was found to be present on the short arm of the human chromosome 19, at 19p13.2. Nevertheless, it was not until fifteen years later that the actual protein sequence became available when the MUC16 gene was cloned (O'Brien et al., 2001; Yin & Lloyd, 2001). MUC16 is 3-5 million Da in size and, similar to other mucins, with the vast majority of the protein being its very large ectodomain consisting of 12,068 amino acid with many sites for both O-linked and N-linked glycosylation, a tandem repeat region of 60 subunits containing an identical sequence of 156 amino acids, and a short C-terminal domain composed of 284 amino acids containing three phosphorylation sites (Fig. 1.7) (Hattrup & Gendler, 2008; Hollingsworth & Swanson, 2004; O'Brien et al., 2001; O'Brien et al., 2002). The putative phosphorylation sites provide the potential for MUC16 to participate in signal transduction events (Bast & Spriggs, 2011).
Figure 1. 7. Structural Organization of MUC16.

A) The figure shows the amino terminal heavily glycosylated domain (blue), the tandem repeat region (orange), SEA domains (orange), the transmembrane (TM; purple) domain and the cytoplasmic tail (CT) (dark blue). Indicated are the antibody binding sites in the tandem repeat region (λ) and the area where the MUC16 gene was knocked out shown by an arrow pointing at the region targeted by the gRNA B) A C-terminal truncated version of MUC16 CT (MiniMUC16) fused to the BM40 signal sequence and a poly His tag for detection that is used in transfection studies described in chapter 4. The blue lines in the expanded part and N-terminal region represent a heavily O-glycosylated region.
1.6.2. MUC16’s Role in Cancer

CA125/MUC16 has been studied primarily as an ovarian tumor marker and to
distinguish between benign and malignant disease as well as to monitor
response to therapy (Moore et al., 2008; Moore et al., 2010; Moore et al., 2009;
Moore et al., 2011). Generally, serum of women with ovarian cancer display
CA125 levels above 30-35 U/ml (Skates et al., 1995). Longitudinal monitoring of
CA125 levels is more useful in predicting the probability of ovarian cancer by
using The Risk of Ovarian Cancer Algorithm (ROCA) (Skates et al., 2001; Skates
et al., 1995). Additionally, a collaborative trial of Ovarian Cancer Screening in the
United Kingdom that combined ROCA and ultrasound showed that 47.1% of
women tested positive after combined assessment with CA125 test and
transvaginal ultrasound had stage I or II disease, proving the effectiveness of
CA125 as an ovarian cancer marker in combination with ultrasound (Menon et
al., 2009; Pinsky et al., 2013).

1.6.3. Biological role of MUC16

The MUC16 ectodomain extends approximately 1500 nm above the cell surface
in a brush like pattern providing an initial point of contact between the MUC16-
expressing cell and the external environment. As in the case of ovarian cancer,
MUC16 is likely to represent the initial point of contact between metastasizing
cells and the normal cells lining the peritoneum, namely the mesothelium (Rump
et al., 2004). This interaction of MUC16 with mesothelin, a 40-kDa
glycophosphatidylinositol (GPI) protein commonly expressed on mesothelial cells
that line body cavities; when expressed under malignant conditions enables
peritoneal metastasis (Rump et al., 2004) is only one of several mechanisms that could mediate adhesion of ovarian cancer cells to the peritoneum (Bast et al., 2009). Additionally, since MUC16 is a glycoprotein, other potential binding partners are lectins of the extracellular matrix such as the β-galactoside-specific family of galectins. MUC16 binds to galectin-1, a galectin upregulated in various cancers, which gives MUC16 another way to bind to host tissue (Seelenmeyer et al., 2003). Additionally, in patients with ovarian cancer, MUC16 is capable of interacting with natural killer (NK) cells via interaction with Siglec-9 receptor, a major subtype of lectins, inducing immunosuppression (Belisle et al., 2010).

Presumably as a result of a cell surface proteolytic event occurring approximately 50 amino acids N-terminal of the transmembrane domain, the ectodomain of MUC16 is released from the cell (O’Brien et al., 2001). Since the remaining cell-associated portion of MUC16 is relatively small, the molecular weight of the shed MUC16 is similar to that of the intact molecule. MUC16 release from the surface is likely to be caused by sheddases as is the case for other mucins such as MUC1 (Thathiah et al., 2003). In ocular surface epithelium, neutrophil elastase, MMP-7, MMP-9 and bacterial metalloprotease (ZmpC) all catalyze of the MUC16 ectodomain (Blalock et al., 2008; Felder et al., 2014; Govindarajan & Gipson, 2010; Govindarajan et al., 2012). Additionally, intracellular signaling may impact MUC16 cleavage and shedding. Shedding of MUC16 is stimulated by PKA activation or upon inhibition of PKCβ, EGFR or tyrosine phosphorylation (Bast & Spriggs, 2011). Furthermore, phosphorylation of the MUC16 cytoplasmic tail is associated with increased shedding of the ectodomain (Fendrick et al., 1997).
These studies have provided some hints of the factors that regulate MUC16 expression.

MUC16 is normally found at the apical surface of epithelial cells. In case of malignancies such as cancers when cells lose their polarity, MUC16 is expressed over the entire cell surface and presumably protects the cancer cells from the immune system. The large and abundant barrier provided by MUC16 would prevent contact by NK cells and monocytes required to kill tumor cells (Gubbels et al., 2010). MUC16 additionally may exert an immunoprotective effect by binding to the NK cells inhibitory receptor, Siglec-9. Siglec-9 activation attenuates T and NK cell function and the binding of cell surface MUC16 and Siglec-9 facilitates inhibition of anti-tumor immune responses (Belisle et al., 2010).

MUC16 knockout in breast cancers resulted in cell cycle arrest in G2/M or G1 phase and increased apoptosis. These investigators also found that MUC16 interacts with the ezrin/radixin/moesin domain-containing protein, Janus kinase (JAK2) (Lakshmanan et al., 2012).

Recent studies have focused on the C-terminal portion of MUC16 and its role in cell signaling. A significant body of literature exists on the function importance of the cytoplasmic tail in cell signaling in other mucins such as MUC1 and MUC4 (Hollingsworth & Swanson, 2004). One report suggests that the cytoplasmic tail of MUC16 interacts with Src-family kinases and induces E-cadherin-mediated cell invasion and migration (Boivin et al., 2009). Another report claims that MUC16 plays an important role in tumor growth, tumorigenesis and metastasis.
(Thériault et al., 2011). In this study, MUC16 function was perturbed by stably expressing an anti-MUC16 antibody with an endoplasmic reticulum targeting signal. The result was to prevent cell surface localization of MUC16 in NIH:OVCAR3 ovarian cancer cells. These authors used a second approach employing epitope-tagged, N-terminal region-deleted MUC16 constructs with and without cytoplasmic tail deletions and stably expressed in SKOV3 cells. The antibody-based approach to perturb MUC16 localization did not affect cell growth rate, but cells reached a stationary phase in a short period of time, show low cell density, growth in soft agar decreased and the growth of xenografts were inhibited. Cytoplasmic tail overexpression stimulated anchorage-dependent growth, enhanced migration and invasion as well as xenograft growth and metastases (Thériault et al., 2011). A more recent study showed that the 114 amino acids of the C-terminal portion of MUC16 was sufficient to stimulate growth, promote invasion and increase rate of tumor growth. This response was associated with activation of the AKT and ERK pathways (Rao et al., 2015). Another study reports that the MUC16 C-terminal region promotes nuclear translocation of JAK2 resulting in phosphorylation of the histone-3-upregulating “stemness” specific genes, LMO2 and NANOG (Das et al., 2015). Additionally, another report indicates that the MUC16 C-terminal domain inhibits GSK-3β mediated phosphorylation and degradation of β-catenin, leading to increased β-catenin levels. As a result, the formation of multicellular aggregates was promoted by inhibition of β-catenin degradation (Giannakouros et al., 2015).
1.7. Proinflammatory mediators and MUC16

1.7.1. Cytokines overview

Cytokines are generally small secreted proteins that are important in the interactions and communications between cells. Cytokines regulate the nature, intensity and duration of the immune response by actions on a variety of lymphocytes and other cells. Cytokines is a general name for this class of proteins. They can be subclassified according to their cell of origin or biological activities as lymphokines (cytokines made by lymphocytes), monokines (cytokines made by monocytes), chemokines (cytokines with chemotactic activities), interleukins (cytokines made by one leukocyte and acting on other leukocytes), interferons and tumor necrosis factors. Cytokines bind their corresponding receptors expressed on the surface of a target cell triggering downstream effects, often via tyrosine kinases, to modify cell behavior such as gene expression. Cytokine responses include modulation of expression of membrane proteins, proliferation and secretion of effector molecules (Testar, 2016). Cytokines can act on the cells that secrete them (autocrine actions), or on nearby cells (paracrine actions), or even on distant cells (endocrine action) (Zhang & An, 2007). Cytokines can have redundant activities, can act synergistically or antagonistically, and also can be produced in a cascade in which one cytokine can stimulate a target cell to produce more cytokines. Cytokines are produced by a variety of cells of the immune system including, macrophages, B-lymphocytes, T-lymphocytes, mast cells endothelial cells,
fibroblasts and various stromal cells (Zhang & An, 2007). Activated macrophages are the main producers of proinflammatory cytokines that are involved in the up-regulation of inflammatory responses in tissues.

There are many cytokines including interleukins 1-23 interferons α, β and γ, tumor necrosis factor α and β, transforming growth factor β1, and the monocyte- and granulocyte colony stimulating factors, M-CSF, G-CSF and GM-CSF. Based on their biological actions, proinflammatory cytokines include TNFα, IL-1, IL-6 and chemokines. IL-1, IL-4, IL-12 and IFNγ are considered T-cell activating cytokines (Paul & Seder, 1994). In this research the proinflammatory cytokines TNFα and IFNγ were found to be modulators of MUC16 expression.

### 1.7.2. The TNF family

The tumor necrosis factor (TNF) family is a group of proteins that bind to their corresponding receptors to trigger important biological processes including host defense, inflammation, apoptosis, autoimmunity and development (Zhang, 2004). The TNF superfamily contains 19 ligands and 29 receptors in humans. The interactions between ligands and receptors are normally highly specific and with high affinity, but some ligands have multiple receptors and similarly some receptors bind multiple ligands (Zhang, 2004).

An important member of the TNF family is Tumor Necrosis Factor α (TNFα), also
known as cachexin or cachectin. TNFα is involved in systemic inflammation and is one of the cytokines involved in driving the acute phase reaction. TNFα is produced mainly by activated macrophages, but it can be produced by many other cell types including CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils and neurons (Locksley et al., 2001).

1.7.3. TNFα Signal Transduction

TNFα has two receptors, TNFR1 and TNFR2. TNFR1 is expressed in almost all tissues and can be activated by any form of TNFα. On the other hand, TNFR2 is only found in cells of the immune system and can only be activated by the membrane-bound form of the TNFα homotrimer. Most of the information available is derived from TNFR1 (Theiss et al., 2005). After the binding of TNFα, the receptor forms trimers causing a conformational change of the receptor. This is followed by dissociation of the SODD protein, which allows the binding of the adaptor protein, TRADD, to the intracellular death domain, which in turn will allow further protein binding events. There are three pathways that can be initiated: 1) activation of NFκB; 2) activation of MAPK pathways and; 3) induction of death signaling.

As a result of response to NFκB activation, TRADD recruits TRAF2 and RIP. TRAF2 recruits the protein kinase complex, IKK, enabling activation of RIP. The inhibitory protein, IκBα, normally binds NFκB and inhibits its translocation to the
nucleus. Upon IkBα phosphorylation by IKK, IkBα is targeted for proteasomal degradation liberating NFκB. NFκB, a heterodimeric transcription factor, translocates to the nucleus and mediates transcription of a variety of proteins associated in cell survival and proliferation, inflammatory responses and anti-apoptotic factors (Chen & Goeddel, 2002).

### 1.7.4. The IFN family

The interferon family was discovered as substances that interfere with viral replication (Schroder et al., 2004). Currently interferons are classified into type I and type II depending on receptor specificity and sequence homology. Type I IFNs encompass multiple IFNα subtypes (Sen, 2001) and can be species dependent. All interferons are structurally related and bind a common heterodimeric receptor (IFNAR). Type II IFNs contains only one interferon, IFNγ, which is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a different chromosomal locus. IFNγ is produced by a variety of cells including CD4⁺ T helper cell type 1 (Th1) lymphocytes, CD8⁺ cytotoxic lymphocytes, NK cells, B-cells, NKT and professional antigen-presenting cells (APCs) (Carnaud et al., 1999; Frucht et al., 2001). IFNγ production is controlled by cytokines secreted by APCs, mainly IL-12 and IL-18. These cytokines connect the response to infection with IFNγ production in the innate immune response (Gołąb et al., 2000). IFNγ function also is important in tumor surveillance and protection against tumor development (Dunn et al., 2002).
1.7.5. IFNγ Signal Transduction

The main pathway associated with IFNγ is the Janus family kinase (Jak1 or 3 or Tyk2)-Signal transducer and activator of transcription (STATs 1-6) pathway. This pathway is also used by more than 50 cytokines, growth factors, and hormones in gene regulation (Schroder et al., 2004). The Jak-STAT signaling pathway involves a cascade of events in which receptor engagement by its ligand leads to activation of Jaks or Tyk 2 and subsequent activation and nuclear translocation of STATs. The receptor associated with IFNγ consists of a heterodimer of IFNGR1 and IFNGR2. Before activation these two subunits are associated through their intracellular domains with active forms of Jak1 or Jak2, respectively. When IFNγ binds to IFNGR1, it induces the dimerization of the IFNGR1 chains forming a site recognized by IFNGR2. A complex forms and a cascade of events leads to transcription of STAT target genes through the binding of specific response elements in the promoters of the genes to be regulated (Schroder et al., 2004).

1.8. CA125/MUC16 regulation by cytokines

In spite of the importance of cytokines in cancer and the existing detailed information on the molecular regulation by cytokines of other mucins (Dharmaraj et al., 2010), little is known about regulation of MUC16 gene expression by cytokines (Argüeso et al., 2003; Paulsen et al., 2008). In this regard, previous
studies of cytokine action on MUC16 expression were restricted to cells of the ocular surface epithelium and no detailed mechanism was given. In the present work, I demonstrate that MUC16 expression is stimulated by both TNFα and IFNγ in a variety of cell contexts. I identified NFκB as a key mediator of these responses and a specific NFκB binding site in the MUC16 promoter. I also provide novel information that activation of PPARγ with pharmacologically relevant drugs antagonizes cytokine-stimulated MUC16 expression raising the possibility of using drugs of this class to reduce MUC16 expression in vivo. Finally, I created cell lines either overexpressing a truncated form of MUC16 (MiniMUC16) or in which MUC16 has been knocked out for future studies of MUC16 function.
Chapter 2 : Tumor Necrosis Factor-α and Interferon-γ stimulate MUC16 (CA125) expression in breast, endometrial and ovarian cancers through NFκB

As published in Oncotarget

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2.1. Abstract:

Transmembrane mucins (TMs) are restricted to the apical surface of normal epithelia. In cancer, TMs not only are over-expressed, but also lose polarized distribution. MUC16/CA125 is a high molecular weight TM carrying the CA125 epitope, a well-known molecular marker for human cancers. MUC16 mRNA and protein expression was mildly stimulated by low concentrations of TNFα (2.5ng/ml) or IFNγ (20 IU/ml) when used alone; however, combined treatment with both cytokines resulted in a moderate (3-fold or less) to large (>10-fold)
stimulation of MUC16 mRNA and protein expression in a variety of cancer cell
types indicating that this may be a general response. Human cancer tissue
microarray analysis indicated that MUC16 expression directly correlates with
TNFα and IFNγ staining intensities in certain cancers. We show that NFkB is an
important mediator of cytokine stimulation of MUC16 since siRNA-mediated
knockdown of NFkB/p65 greatly reduced cytokine responsiveness. Finally, we
demonstrate that the 250 bp proximal promoter region of MUC16 contains an
NFkB binding site that accounts for a large portion of the TNFα response.
Developing methods to manipulate MUC16 expression could provide new
approaches to treating cancers whose growth or metastasis is characterized by
elevated levels of TMs, including MUC16.

2.2. Introduction:

Mucins are high molecular weight glycoproteins that are normally found on apical
surfaces of epithelial organs such as trachea, stomach, and reproductive organs
(Gendler & Spicer, 1995), where they serve various functions including protection
against pathogenic infections, hydration and cellular signaling (Hattrup &
Gendler, 2008). To date, 20 mucin genes have been identified and are classified
based on the presence of large, heavily O-glycosylated, tandem repeat motifs
(Duraisamy et al., 2006). Mucins can be membrane bound or secreted
depending on the presence of a membrane spanning region (Hattrup & Gendler,
Of the cell surface bound mucins, MUC1, MUC4 and MUC16 are the best characterized (Bafna et al., 2010).

Mucins are a class of molecules that aid in mucosal defense by providing a large physical cell surface barrier to pathogens and tissue-degrading enzymes (Gendler & Spicer, 1995). Mucins normally function to protect and lubricate the epithelium, but are overexpressed in various cancers (Kufe, 2009), are frequently used as diagnostic markers (Jonckheere, 2010; Rachagani et al., 2009), and are being considered as therapeutic targets (Constantinou et al., 2011). Alterations in transmembrane mucin expression or glycosylation promote the development of cancer and stimulate cell growth, differentiation and invasion (Remmers et al., 2013; Wu et al., 2009). MUC16 (also known as the serum tumor marker, CA 125) is the largest transmembrane mucin being 2-5 MDa including O-linked and N-linked glycosylation. The polypeptide backbone of MUC16 contains 22,000 amino acids (Gubbels et al., 2010; O'Brien et al., 2001), including an N-terminal tandem repeat region composed of 18-60 repeats of 156 amino acids each, and a C-terminal region with a short cytoplasmic (Bafna et al., 2010; O'Brien et al., 2002). Intact MUC16 is the largest membrane glycoprotein known, towering over even other large cell surface mucins like MUC1 and MUC4. As such, it likely represents the initial point of contact with other cells and matrices. MUC16 is believed to play important roles not only in normal contexts such as reproduction, but also in pathological states including cancer and mucosal infections (Bast & Spriggs, 2011; Felder et al., 2014; Gipson et al., 2008). MUC16 has been used as a tumor marker for over thirty years due to its overexpression in ovarian and
other cancers, yet little is known about its regulation. The importance of MUC16 in the diagnosis, progression and therapy of ovarian cancer, and its overexpression in other cancers, demands a need for research on the regulation of this mucin.

Eighty to 90 percent of all cancers originate in epithelial tissue. Membrane bound mucins are overexpressed in many of these cancers as well as in other pathological conditions (Hattrup & Gendler, 2008). The causes of mucin overexpression are not always clear, but include gene duplication (Desseyn et al., 2000), and mucin gene responsiveness to factors in the tumor microenvironment (Hollingsworth & Swanson, 2004). High levels of transmembrane mucins on tumor cells protect these cells from attack by the host immune system as well as from the actions of cytotoxic drugs (Hollingsworth & Swanson, 2004).

The regulation of expression of certain mucins, particularly MUC1, has been well studied and is markedly stimulated by cytokines (Brayman et al., 2007; Dharmaraj et al., 2010; Lagow & Carson, 2002; Mitchell et al., 2002). Inflammatory events trigger cytokine release by immune cells, which invade tumor-associated stroma. Inflammation and cytokine production are one of the proposed “hallmarks of cancer” implicating their significant role in tumorigenesis (Hanahan & Weinberg, 2011). Furthermore, it has been shown that NFkB directly binds to the MUC1 promoter to activate gene transcription (Lagow & Carson, 2002). NFkB generally plays a key role as a mediator of inflammatory
responses, and also has been found to play a crucial role in many steps of cancer initiation and progression (Schmid, 2013). In spite of the existing detailed information on the molecular regulation of MUC1, little is known about regulation of MUC16 gene expression (Argüeso et al., 2003; Paulsen et al., 2008).

At least 20% of all cancers are associated with chronic inflammation, typified by a cytokine-rich environment (Grivennikov & Karin, 2011). This inflammation is most often assessed by histological detection of tumor-associated or infiltrating, cytokine-producing immune cells. Even cancers that do not develop from chronic inflammation often contain high levels of cytokines (Grivennikov & Karin, 2011). Macrophages from tumors secrete inflammatory cytokines including TNFα and IFNγ. TNFα has a tumor-promoting role (Mitchell et al., 2002), and TNFα expression generally increases with tumor stage (Al Obeed et al., 2014). Also, high plasma levels of TNFα correlate with higher tumor stage (Al Obeed et al., 2014). On the other hand, IFNγ has dual roles with both pro-inflammatory and anti-inflammatory properties (Mühl & Pfeilschifter, 2003). Both cytokines have significant physiological importance in regulating immune responses and inflammation. In this study, we link the expression of MUC16 to stimulation by TNFα and IFNγ through NFkB in cell culture and in pathological specimens.
2.3. Materials and Methods:

2.3.1. Cell culture

Well-established cells with known genomic characterization were used. RL95-2 and HEC50, were cultured in DMEM/Hams F12 (Life Technologies; 11330-057). IOSE 261F were cultured in MCDB 106 (SIGMA)/ MEDIUM 194 (MEDIATECH) [1:1, v/v]. SKOv3-ip, were cultured in McCoy’s 5A (Thermo Scientific; SH30270.01). OVCAR-3, were cultured in RPMI 1640 (Gibco®; 11875119). MCF-7 (last profiled on 7/22/13 by STR profiling at Johns Hopkins Genetic Resources Core Facility), were cultured in Minimum Essential Medium (MEM) (Life Technologies; 11095098). All media were supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin (100 U/ml)–streptomycin (100 µg/ml) (Gibco®; 15140-122). MCF-7 and OVCAR-3 cell media also were supplemented with 10 µg/ml of insulin (v/v) (Sigma-Aldrich Inc.; I9278).

2.3.2. Cytokine treatments

Cells were seeded in six well plates in media containing 5% (v/v) charcoal-stripped FBS (Life Technologies; 12676029) and allowed to reach 60-70% confluence. Cells then were gently rinsed with PBS and then serum free medium was added for 24 h. Cells then were treated with tumor necrosis factor alpha (TNFα, ROCHE; 113718430) and or interferon gamma (IFNγ ROCHE; 11040596001) at the concentrations indicated in the text in media containing 10% (v/v) charcoal-stripped FBS. Cells were incubated with cytokines for 48 h
prior to RNA extraction and for six days prior to protein extraction and immunostaining, and for up to 12 days in time course experiments.

**2.3.3. RNA isolation and reverse transcription-PCR**

Total RNA was isolated by using TRIZOL reagent (Invitrogen; 15596-026) and chloroform (Cambridge Isotope Laboratories; DLM-7TB-100). Samples then were treated with DNase according to the manufacturer’s instructions (Ambion; AM1906). Reverse transcription was performed using 1 µg of total RNA in a 10 µl reaction using qScript cDNA Super mix (Quanta; 95048) incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. Real time qPCR was performed using the following primer sequences: MUC16 forward, 5’-GCCTCTACCTAACCCTGATGAA-3’ and reverse, 5’-GGTACCCCATGGCTGTTGTG-3’ (Argüeso et al., 2003) beta actin (ACTB) forward, 5’-GATGAGATTGGCATGGCTTT-3’ and reverse, 5’-CACCTTCACCGGTCCAGTCTTT-3’ (Dharmaraj et al., 2010) and NFκB forward, 5’-ATCTGCGAGTGAACCGAAACT-3’ and reverse, 5’-CCAGCCTGGTCCCGTGAAA-3’ (Sun et al., 2012) and SYBR Green Super mix according to the manufacturer’s instructions (Quanta Bioscience). Samples were cycled as follows: MUC16, (30 sec at 95°C and 30 sec at 59°C for 40 cycles); NFκB p65 (an initial incubation at 50°C for 2 min followed by 40 cycles of 95°C for 10 min, 95°C for 15 s and 60°C for 1 min (Sun et al., 2012)). Relative amounts of mRNA were identified using the comparative threshold cycle method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008) and normalized to that of ACTB.
2.3.4. Immunofluorescence analysis.

Cells (5 x 10^4) were plated in 8-well chamber slides and treated with cytokines for six days. Cells were washed with PBS and fixed with 100% methanol for 10 min at room temperature. Subsequently, cells were washed and allowed to re-hydrate for 10 min at room temperature in PBS. Slides were washed three times with PBS, blocked for 1 h with [3% (w/v) BSA in PBS] and washed three times with PBS. Primary antibody was added and incubated overnight at room temperature at the indicated dilutions: mouse monoclonal anti-MUC16 (OC125); 1:100 in blocking solution. Slides were washed three times with PBS, then incubated with 1:400 dilution of Alexa-fluor 647 goat anti-mouse (Invitrogen; A21235) in blocking solution at room temperature for 1 h in the dark, and washed three times for 5 min at room temperature with PBS. Samples were mounted with Prolong-Antifade with 4\textsuperscript{'}, 6-diamidino-2-phenylindole (DAPI), a fluorescent molecule that binds strongly to A-T regions in DNA in the nucleus, as per manufacturer’s instructions (Life Technologies; P-36931) and viewed by confocal microscopy on a Zeiss LSM710 microscope.

2.3.5. Enzyme-linked immunosorbent (ELISA) assay

Cells were plated (4 x 10^5) in six-well plates and treated with cytokines for six or up to 12 days. Fresh media with cytokines was changed every three days and conditioned media was saved. Protein from cell lysates was isolated using 500 µl of RIPA lysis buffer (Santa Cruz; sc-24948). ELISA assays were performed using CA125 ELISA kit (BQ kits; BQ1013T) following the manufacturer’s instructions.
2.3.6. *siRNA knockdown*

NFκB was knocked down using an oligonucleotide targeting p65 (Santa Cruz; sc-29410/human NFκB p65 siRNA) and a non-silencing, scrambled siRNA control: (5'- AATTCTCCGAACGTGTCACGT-3' (Schroeder *et al.*, 2001). All siRNAs were resuspended in RNAse-free water to a final concentration of 10 µM by adding 330 µl of RNase-free water to 3.3 nmol of lyophilized siRNA for NFκB p65 and by adding 1 mL of RNAse-free water to the lyophilized siRNA to achieve a final concentration of 20 µM for the scrambled control. Oligonucleotides were transfected using Lipofectamine 2000 (Invitrogen; 11668019) according to the manufacturer's instructions. Briefly, cells were plated in antibiotic free media in 12 well plates incubated for 24 h at 37° in a humidified atmosphere of air: CO₂ (95.5, v/v). Oligonucleotides were transfected at a final concentration of 50 nM in Opti-MEM® Reduced Serum Medium, GlutaMAX™ Supplement (Gibco; 51985034). Six h after transfection, media was changed to regular medium plus FBS lacking antibiotics. Twenty four h later cells were treated with or without cytokines for 24 h followed by RNA extraction.

2.3.7. *Immunohistochemistry*

Formalin-fixed, paraffin-embedded tumors were resected from 94 patients and obtained from the archives of Department of Pathology, MD Anderson Cancer Center (Houston, TX). The MDACC Institutional Review Board approved the use of tissue. To provide a positive control, SKOV3-ip cells were grown for 24 h in McCoy’s medium supplemented with 10% (v/v) FBS, 1% (penicillin [100 U/ml]–streptomycin [100 µg/ml], and 1% [w/v] L-glutamine. Cells were harvested in
0.25% (w/v) trypsin (Fisher Scientific, MT-25-052-CI) washed 2 times in PBS, fixed in 10% (w/v) formalin and embedded in paraffin. Oven incubation at 60°C for 20 min was used to deparaffinize slides followed by two 20 min incubations at room temperature in xylene. After slides were rehydrated, antigen retrieval was performed in 6.5 mM sodium citrate buffer (pH 6.0) for 10 min. Blocking was performed with 5% (w/v) bovine serum albumin in PBS. The primary antibody (anti-TNFα mouse monoclonal, 1:200, EDM Millipore MAB1096; anti-IFNγ rabbit polyclonal, 1:250, Novus Biologicals NBP1-19761; or OC125 mouse monoclonal 1:400, Bast Laboratory) were incubated at 4°C overnight. Mouse or rabbit secondary antibody (Jackson ImmunoResearch) was applied for 1 h at room temperature followed by washing 3 times in PBS for 10 min. Diaminobenzidene chromagen (Biocare Medical, BDB 2004L) was added for 1 min per slide followed by 3 additional washes in PBS for 10 min and then hematoxylin staining was performed for 1 min per slide followed by 3 additional washes in PBS for 10 min. Serial sections of paraffin-embedded OVCAR-3 cells and non-immune IgG staining served as positive and negative controls, respectively, and were stained alongside tissue microarray (TMAs) to confirm assay reproducibility. Omission of the primary antibody served as an additional negative control for each immunostaining event. Immunohistochemical staining was evaluated for both overall staining intensity and location of the staining in the tumor or stroma alone versus diffuse staining in both tissues. Total staining intensity was determined as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining).
All slides were evaluated independently by 2 investigators (ZL and MNS) without knowledge of the identity of the patient or clinical outcome.

2.3.8. 5' Rapid Amplification of cDNA Ends (RACE)

RACE PCR was performed to formally determine the MUC16 transcriptional start site. These reactions were performed using the 5'RACE System (Life Technologies; 18374-058) according to the manufacturer’s instructions. Primers used were, GSP1: 5'- CACCACGATTGCACCTGTAG -3' and GSP2 5'- TTAGTGCTCCTGCTCCCTGT -3'. The PCR products then were sequenced using the GSP3 primer 5'- CCAGAGGCAA ATGTTGACCT -3'.

2.3.9. MUC16 Promoter Plasmid Construction

Genomic DNA was purified from MCF-7 cells using a Wizard® SV Genomic DNA Purification System (Promega: A2360). A construct containing 250 bp upstream of the start site of MUC16 transcription (hereafter refer to as 250 bp promoter construct) was amplified by genomic PCR. This PCR product was cloned into pCR 2.1 TOPO (Life Technologies; K456001). This fragment was ligated into the promoter less pGL3 firefly luciferase reporter vector (Promega; E1751). The primers used were Fwd: 5'- AGAGAGAGAGAGAGAGGATCATT -3' Rev 5'- AATGATCCTCTCTCTCTCTCTCTCT -3'. Site directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies Inc; 210518) according to the manufacturer’s instructions.
2.3.10. Transient transfection and reporter assays

MCF-7 or SKOv3-ip cells were plated in 5% (v/v) charcoal-stripped FBS in six-well plates and maintained for 48 h until the cells reached 80 to 90% confluence judged by eye. Transient transfections were performed using Lipofectamine 2000 (Invitrogen; 11668-019) and Opti-MEM (Life Technologies; 51985034) according to the manufacturer’s instruction. Expression and reporter plasmids were added at 500 ng and 1 µg per well, respectively, and 10 ng of pRL-TK plasmid was used per well. After transfection cells were allowed to recover in serum free media for 12 h. Cytokine treatments were added as described above for 24 h. The Dual-Luciferase Assay kit (Promega; E1960) was used to lyse the cells and perform the luciferase assay according to the manufacturer’s instructions. Reporter activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase activity.

2.3.11. Electrophoretic mobility shift assay (EMSA)

Recombinant NFκB p65 protein (Active Motif; 31102)(500 ng) was incubated with 40 ng of oligonucleotide probes for NFκB p65 containing either a consensus (5’-CGACATGATACACTAAGAAATTCTATTCTGCAGACACTGC-3’) or a mutated (5’-CGACATGATACACTAAGAAAAATATTCTGCAGACACTGC-3’) sequence, similar to the NFκB sequence found in the MUC16 promoter by bioinformatics analysis and used in the luciferase assay, in a reaction mixture following the manufacturers instructions containing 2µl of EMSA/Gel-shift binding buffer 5X from EMSA Kit (ThermoFisher; E33075) containing 750 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM Tris, pH 7.4 for 40min. The reaction mixtures
were separated by 12% non-denaturing PAGE. The gel was then stained for 20 min with 1X SYBR® green staining solution; the gel was then washed with dH2O two times for ~2 sec followed by visualization using a Carestream Imaging system.

**2.3.12. Chromatin Immunoprecipitation (ChIP)**

The Chromatrap® kit (Porvair Sciences – Leatherhead, Surrey, England) was used for ChIP assays. MCF-7 cells were grown to 60 to 80% confluency and serum-withdrawn overnight, then treated for 4 h with TNF-α (2.5 ng/mL) and IFNγ (20 IU) or vehicle control prior to collection of chromatin. Fixation, DNA shearing and ChIP were performed following the manufacturer’s instructions. Antibodies included the anti-p65 SC-109X antibody (Santa-Cruz), as well as a control IgG antibody. Primer sequences used to amplify NFκB regulatory region are

1) Fwd: 5'-AGCCTGGTTCCTGGTTTCTAA -3'
Rev: 5'-CCCTTCAAACCTTTTAACGGATT -3'

2) Fwd: 5'-GCCTGGTTCCTGGTTTCTAA-3'
Rev: 5'-TGATCTCAATTCTTCCCTTCAAA-3'

The *NFKBIA* gene promoter served as a positive control for p65 immunoprecipitation, and the GAPDH promoter was used a positive control for RNA polymerase II immunoprecipitation. The *HBB* gene was the negative control for nonspecific pulldown by either the antibody or adsorption by the column beads.
2.3.13. Statistical analysis

All data is shown as the mean ± SD of triplicate determinations of independent biological samples. Statistical analyses were performed using a one-way ANOVA followed by Tukeys post-test using GraphPad InStat software, version 3.05 (GraphPad Software, San Diego, CA). Differences were considered significant when P<0.05, two-tailed test.
2.4. Results:

2.4.1. Basal MUC16 mRNA levels in various cell types differ among normal epithelial cells derived from breast, ovarian and endometrial cancers.

Initially, we determined basal MUC16 mRNA levels in a series of epithelial cells derived from female reproductive tissues: IOSE 261F (Table 2.1 and Fig. 2.1) (a normal ovarian epithelial cell type), SKOv3-ip (Table 2.1 and Fig. 2.1), and OVCAR-3 (Table 2.1 and Fig. 2.1), moderately and poorly differentiated ovarian cancer cells, respectively, which displayed moderate (SKOv3-ip) and very high (OVCAR-3) basal levels of MUC16 mRNA; RL95-2 and HEC50, moderately and poorly differentiated cells, respectively, derived from endometrial adenocarcinomas with moderate basal levels of MUC16 (Table 2.1 and Fig. 2.1); and MCF-7 (breast cancer), which displayed very low basal levels of MUC16 (Table 2.1 and Fig. 2.1).

2.4.2. TNFα and IFNγ stimulate MUC16 mRNA levels in MCF-7 breast cancer cells in a dose-dependent manner.

Pro-inflammatory cytokines stimulate expression of MUC1 and MUC4 in other contexts (Dharmaraj et al., 2010), but little is known about MUC16 responsiveness in this regard. Initially, we determined the dose responsiveness of MUC16 mRNA expression to either TNFα or IFNγ in MCF-7 cells, which contained the lowest basal levels of MUC16 (Fig. 2.1). TNFα was added at
concentrations ranging from 0.25 ng/ml to 25 ng/ml for 48 h. IFNγ was added at concentrations of 2 IU to 200 IU for 48 h. In many experiments with MCF-7 cells, but not with other cells tested, extremely robust stimulation by cytokines was observed (> 50 fold); however, in other experiments stimulation was as low as 8-fold (Fig. 2.5). Decreased responsiveness correlated to passage number and reflected a higher basal level of MUC16 expression with increasing passages. The lowest concentrations of either cytokine that demonstrated a significant stimulation of MUC16 mRNA levels were 2.5 ng/ml of TNFα and 20 IU/ml of IFNγ (Fig. 2.2A and 2.2B). Therefore, these concentrations were used in subsequent experiments to determine the potential synergy between these cytokines in stimulating MUC16 expression.

2.4.3. Treatment with TNFα and IFNγ stimulated MUC16 mRNA levels in multiple cell types.

The ability of low concentrations of TNFα (2.5 ng/ml) and IFNγ (20 IU) to stimulate MUC16 mRNA expression was assessed in multiple cell types. Cells were treated for 48 h with either a vehicle control, or low cytokine doses individually or in combination (Fig. 2.3A-F). Values obtained for vehicle controls in each case were set to 1 for comparison. Treatments with the individual cytokines only modestly stimulated MUC16 gene expression (2-3-fold) in any cell context; however, combined cytokine treatment provided significantly higher stimulation in 5 of 6 cell types. The sole exception was OVCAR-3 cells, which display extremely high basal levels of MUC16 mRNA (Fig. 2.1). Higher cytokine
concentrations in combination did not further stimulate MUC16 expression in any context (Fig. 2.4). Therefore, the stimulation observed with the combined cytokine treatments appeared to be maximal in each case. The strongest stimulations were observed with cells displaying the lowest basal MUC16 levels, namely IOSE 261F, SKOv3-ip and MCF-7 (Fig. 2.3A, B and F). Modest, predominantly TNFα-dependent, stimulation was observed in the two uterine adenocarcinoma cell types, HEC50 and RL95-2 (Fig. 2.3D and E), which displayed intermediate basal levels of MUC16.

2.4.4. MUC16 protein expression and shedding were stimulated in response to treatment with a combination of TNFα and IFNγ.

Because MCF-7 cells displayed the most robust response to cytokine treatment, they were used to perform time course studies for MUC16 (CA125) protein and mRNA accumulation. MUC16 mRNA (Fig. 2.5A) and cell-associated protein (Fig. 2.5B) levels were essentially maximal within 6 days of cytokine exposure while media levels continued to accumulate throughout the 12 day time course (Fig. 2.5C). It also was found that for MCF-7 cells about 90% of the MUC16 protein produced was ultimately found in the media by 12 days of treatment (Fig. 2.5C). Consequently, the cytokine stimulation of MUC16 mRNA expression was also reflected at the level of MUC16 protein expression. We used six days of treatment to examine the actions of individual cytokines on MUC16 protein expression. A small increase in MUC16 stimulation in the cell-associated fraction resulted from treatment with IFNγ alone (*, p < 0.05). Nonetheless, much greater
stimulation was observed with TNFα or TNFα plus IFNγ in the media and total fractions indicating that TNFα was a strong driver of MUC16 production (***, p < 0.001 vehicle vs TNFα + IFNγ) (Fig. 2.6A).

The effect of cytokine treatment on cell surface MUC16 was investigated further in MCF-7 cells by immunostaining (Fig. 2.6B panels a through l). The promotion of MUC16 expression was a consistent response in 5 of the 6 cell types tested.

2.4.5. MUC16, TNFα and IFNγ are coexpressed in malignant neoplasms.

The in vivo association of cytokine expression with that of MUC16 was assessed with immunohistochemical staining of a multi-tumor human tissue microarrays. Serial sections of a human cancer tissue array were stained simultaneously using anti-MUC16 or anti-TNFα or anti-IFNγ antibody. The array included various cancers including ovary, endometrium and breast, which were the focus of this study (Table 2.2). The purpose of these studies was to determine if relative MUC16 expression correlated with TNFα or IFNγ expression, independently of tumor stage or grade. Samples were classified according to staining intensities, which represents protein expression. The array staining generally revealed that strong cytokine expression was associated with elevated MUC16 expression in many cancers. In cancers such as ovarian (Fig. 2.7A and C) and breast (Fig. 2.7B and D), there was a direct correlation between the staining intensity for both cytokines and MUC16. The correlation for endometrial cancer was not as clear (Fig. 2.7 E and F).
2.4.6. MUC16 mRNA responses to cytokines are attenuated when NFκB/p65 is knocked down by siRNA.

Given the particularly strong actions of TNFα on MUC16 expression, we sought to determine if the key downstream transcription factor, NFκB, mediated this response. To accomplish this, NFκB/p65 was knocked down via siRNA in two cell types displaying the strongest MUC16 mRNA elevation in response to cytokines, namely MCF7 and SKOv3-ip. NFκB/p65 knockdown reduced target mRNA and protein levels by 60-80% of the control (Fig. 2.8A and B and Fig. 2.9). Cytokine stimulated MUC16 mRNA levels were significantly (p < 0.001) decreased when NFκB/p65 was knocked down in both cell types (Fig. 2.8C and D). This also was the case even when TNFα or IFNγ were added individually (Fig. 2.10). Collectively, these studies demonstrate that NFκB is an important mediator for both TNFα and IFNγ responsiveness. Even though MUC16 expression in OVCAR-3 cells was not affected by cytokines (Fig. 2.3), we used siRNA-mediated knockdown to determine if NFκB mediated high level MUC16 expression in this cell context. Though we again achieved effective p65 knockdown (> 80%), no changes in MUC16 mRNA levels were noted (Fig. 2.11). Thus, the extremely high basal levels of MUC16 in this cell line appear to be due to processes independent of NFκB.
2.4.7. A conserved, consensus, NFkB binding site in the proximal

MUC16 promoter accounts for much of the cytokine responsiveness.

Knowing that the cytokine response was associated with the transcription factor NFkB, we next studied the MUC16 promoter to look for binding sites for NFkB and associated transcription factors. The start of transcription of the MUC16 gene has been predicted, but until now has not been biochemically determined. Therefore, we performed 5' RACE to determine the start site of MUC16 transcription. Two cell types were used for 5' RACE, MCF-7 and OVCAR-3. It was found that MUC16 has alternative sites of transcription; it is cell type specific and differs by a few nucleotides from the predicted start site (Fig. 2.12). We next performed a bioinformatics analysis of the region 2000 bp upstream of the transcriptional start site to determine if any consensus NFkB binding sites occurred in this region and if they were conserved between mice and humans. In the region within 200 bp from the start of transcription (Fig. 2.13A) a consensus NFkB binding site was found in addition to others such as AP-1 and Sp1. To determine if the NFkB binding site was associated with the cytokine response, we cloned the proximal 250 bp of the MUC16 promoter into a pGL3 basic luciferase vector. Transient transfection reporter assays revealed that the 250 bp promoter accounts for a large portion of the cytokine responsiveness. Furthermore, mutating the NFkB binding site destroyed the cytokine response (Fig. 2.13B and C). Thus, in addition to identifying NFkB as a key transcription factor mediating cytokine responsiveness of the MUC16 gene, we also identified
a key consensus NFkB binding element in the MUC16 promoter responsible for cytokine responsiveness.

2.4.8. Binding and recruitment of NFκB/p65 to the MUC16 promoter in vitro and in vivo.

To determine if NFκB directly interacts with the consensus NFκB, both gel shift (EMSA) and chromatin immunoprecipitation (ChIP) assays were performed. Oligonucleotides either containing (EMSA) or flanking (ChIP) the putative NFκB binding site were designed for both assays, as well as an additional oligonucleotide containing a mutated NFκB for the EMSA assay as described in Material and Methods. As expected, the wild type oligonucleotide interacted with the recombinant NFκB/p65 protein, while the mutated site showed very weak binding (Fig. 2.14A). ChIP experiments also showed an increased occupancy of the MUC16 promoters by NFκB following cytokine treatment when compared with the untreated control (Fig. 2.14B).

2.5. Discussion:

In the current study we demonstrated that the pro-inflammatory cytokines TNFα and IFNγ stimulate MUC16 expression in a variety of cellular contexts. Furthermore, we showed that elevated MUC16 expression is associated with elevated cytokine levels in several cancers of female reproductive tissues. We
identified NFκB and a key NFκB binding site in the proximal \textit{MUC16} promoter as key mediators of this response.

Stimulation of MUC16 expression by cytokines has been reported in ocular surface epithelial cell lines (Argüeso et al., 2003; Paulsen et al., 2008). In the present study we found that cytokines increase MUC16 mRNA and protein levels in a variety of epithelial cancer cell types. Knowing that cytokines stimulate expression of multiple transmembrane mucins suggests a general response potentially intended to enhance the protective functions of epithelia (Chapela et al., 2015; Dharmaraj et al., 2010). In general, the degree to which cytokines stimulated MUC16 expression was related to the basal level of MUC16 expression, i.e.; the lower the basal MUC16 level was the greater stimulation observed with cytokines. We found that maximal MUC16 expression was observed with very low concentrations of cytokines used in combination. This suggests that \textit{in vivo} low cytokine levels diffusing throughout a tumor may strongly potentiate MUC16 expression.

While a large number of studies report serum cytokine levels or increases in cytokine mRNA levels in tumor tissues, very few have measured cytokine levels in tumor tissues (Mallmann et al., 1991). These studies generally indicate that levels of TNFα and IFNγ are low in these tissues, but in the range where we observed cooperative stimulation of MUC16 expression. It is likely that cytokine concentration gradients are created, radiating from the cellular sources of their
production. Nonetheless, the ability of these cytokines to cooperatively elevate MUC16 indicates that even low amounts of cytokines are sufficient to drive this response in tumors. Moreover, these gradients may also account for heterogeneity in MUC16 expression within tumor tissues.

Cytokine stimulation of mucin expression in cancer cells likely reflects conservation of a biological response of normal mucosal epithelia. Cytokines are elevated in normal tissues in response to injury or infection to prompt the immune system to respond to the challenge. As part of this response, it appears that mucins are overproduced to protect the site from further damage or pathogenic invasion. In the case of cancer, these responses protect the mucin-expressing cancer cells from the immune system (Hollingsworth & Swanson, 2004; Roulois et al., 2013) and cytotoxic drug penetration (Lai et al., 2009; Roulois et al., 2013). MUC16 can also carry ligands for immuno-inhibitory Siglecs (Belisle et al., 2010; Tyler et al., 2012). Thereby, the mucin response to cytokines represents a “vicious cycle”. Overexpression and shedding of MUC16 is predicted to contribute to immunosuppressive activities further protecting tumor cells from host immune responses.

While many normal epithelial cells express MUC16 (Argüeso et al., 2003; Blalock et al., 2007; Davies et al., 2007; Dharmaraj et al., 2014), it is not clear if shedding or cellular retention is the major metabolic fate. We found that ~66% of the MUC16 produced by MCF-7 cells is released to the medium. The mechanism for
this release is unclear at present, but may involve cell surface proteases as is the case for other transmembrane mucins (Thathiah et al., 2003; Thathiah & Carson, 2002). This is the first study describing the distribution of MUC16 between the cell-associated and shed/secerned compartments. More work detailing the mechanism of release as well as whether this occurs in other cell types is needed to know if this reflects a general behavior of MUC16 or is specialized to particular cell types. In addition, determination of the biological functions of shed MUC16, beyond serving as a valuable serum marker, is needed. The energy expenditure in producing this huge glycoprotein (ca. 22,000 amino acids) heavily decorated with > 1x10^6 sugar residues is extraordinary. An estimate of 2 nucleoside triphosphates (NTPs) used per amino acid addition and 36 ATPs potentially generated by complete oxidative metabolism of each hexose indicates an energetic cost of > 3.6 x 10^7 NTPs to produce one MUC16 molecule. The amount of ATP an average human uses is over 200 moles/day (Voet et al., 1999); (200 moles ATP/day)(6 x 10^{23}) molecules/mole = 1200 x 10^{23} molecules ATP/day = 1.2 x 10^{26} molecules ATP/day. There are about 3.72 x 10^{13} cells in the human body (Bianconi et al., 2013); 1.2 x 10^{26} molecules ATP/day divided by 3.72 x 10^{13} cell = 0.32 x 10^{13} molecules ATP/cell per day = 3.2 x 10^{12} molecules ATP/cell per day. An estimate of the amount of the mucin MUC4 per cell is at least 10,000 (Komatsu et al., 1997), and a half life estimate for MUC16 is 6 h (Giannakouros et al., 2015). This means half of the molecules of MUC16 per cell are generated every 6 h ~ 20,000 MUC16 per day. Energy equivalents devoted to making 1 MUC16 is (3.7 x 10^7)(20,000 MUC16 per day) = 7.4 x 10^{11} NTP
equivalents/day or about 23% of the ATP produced per cell per day. Most of this is just potential since it is mostly comprised of the sugars that could have been used to make ATP, not the actual NTPs used. 22,000 amino acids/MUC16 x 2 NTPs per amino acid = 44,000 NTPs/MUC16. There are about 1 x 10(6) sugars per MUC16 each costing 1 NTP to activate and add to sugar chain ---> 1 x 10^6 NTPs in real energetic cost for the sugars + 44,000 NTPs to make the protein core = 1,044,000 real NTPs used to make 1 MUC16 x 20,000 MUC16 made per day = 2.088 x 10^{10} NTPs needed to make MUC16 per day or about 1% of the total ATP production on average. Given this tremendous metabolic investment, it seems likely that the functions associated with MUC16 and its shed fragments are important. Released MUC16 binds to the surfaces of various immune cells consistent with the aforementioned suggestion of an immunosuppressive function (Belisle et al., 2007; Gubbels et al., 2010). Nonetheless, Muc16 null mice display no overt phenotypes (Cheon et al., 2009). It is possible that MUC16 functions in these mice are not manifest until presented with an appropriate challenge. MUC16 also binds the cell surface protein mesothelin on mesothelial cells, perhaps providing a way for migrating MUC16-expressing cells to colonize ectopic sites, e.g. the peritoneum.

In this study we found that TNFα, IFNγ and MUC16 are often strongly co-expressed in endometrial, breast and ovarian cancers with cytokine staining intensity positively correlating with MUC16 staining in breast and ovarian cancers, in particular. These data are consistent with the notion that elevated cytokines in the tumor microenvironment induce the production of MUC16. We
suggest that elevated MUC16, in turn, protects the tumor against the immune system and cytotoxic drug penetration.

Our data provides important, novel information on cytokines as factors driving MUC16 expression in cancer cells and possibly in normal tissues as well. Regulation of MUC16 expression through NFκB could aid in early detection of ovarian cancer. CA125 (MUC16) levels can rise months to years prior to conventional diagnosis of ovarian cancer (Bast et al., 1985). While administration of TNFα of IFNγ may or may not be tolerable clinically, selective enhancement of NFκB and MUC16 levels in cancer cells by other methods might provide a provocative test to confirm the presence of early stage ovarian cancer in asymptomatic women with rising levels of serum CA125 (MUC16). As MUC16 is likely important for metastasis in the peritoneal cavity (Gubbels et al., 2006), anti-inflammatory strategies might slow progression of the disease. Interfering with cytokine actions in tumor cells then represents an avenue to develop therapeutic approaches to reduce MUC16 levels to increase cancer cell susceptibility to the host immune system and cytotoxic drugs.
Chapter 2: Figures and Tables

Table 2.1. Cell types used in the current study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue source</th>
<th>Type</th>
<th>Level of differentiation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOv3-ip</td>
<td>Ovary: ascites</td>
<td>Adenocarcinoma</td>
<td>Moderate</td>
<td>Hua, W., et al., 1995</td>
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<tr>
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<td>Adenocarcinoma</td>
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</tr>
<tr>
<td>HEC-50</td>
<td>Uterus: endometrium</td>
<td>Adenocarcinoma</td>
<td>Poor</td>
<td>Kassan, S., et al., 1989</td>
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</table>
Figure 2. 1. Basal MUC16 mRNA levels in various epithelial cell types.

MUC16 mRNA levels were measured via quantitative qRT-PCR relative to the mRNA levels for ACTB in the indicated cell lines as described in Materials and Methods. IOSE 261F is the cell line with the lowest basal MUC16 mRNA levels and its value was arbitrarily set to 1 for comparison. Although error bars are not evident in all cases, triplicate independent determinations were performed in each case with variation < 5% among samples. In order to express all values on the same graph due to the very high basal levels of MUC16 expressed by OVCAR-3 cells a log base 10 scale was used for the Y-axis.
Figure 2. 2. TNFα and IFNγ stimulate MUC16 mRNA levels in a dose dependent manner.

MCF-7 cells were treated with the indicated concentrations of TNFα (panel A) or IFNγ (panel B) for 48 h, RNA extracted and the levels of MUC16 mRNA relative to those of ACTB were determined by qRT-PCR as described in Materials and Methods. The boxes indicate the lowest concentrations of either cytokine that demonstrated a significant stimulation of MUC16 mRNA levels and were used in subsequent experiments to determine the potential synergy of action between these cytokines. Values for the vehicle controls were arbitrarily set to 1 in each case. Bars and error bars indicate the means +/- SD for triplicate determinations in each case. **, p < 0.01 vs. vehicle; ***, p < 0.001 vs. vehicle.
Figure 2. Cytokine treatments stimulate *MUC16* mRNA levels in various cellular contexts.

Each indicated cell line was treated for 48 h with either a vehicle control (0.1% [w/v] BSA in PBS), TNFα (2.5 ng/ml), IFNγ (20 IU/ml), or a combination of TNFα + IFNγ. RNA then was extracted and the levels of *MUC16* mRNA relative to that of *ACTB* were determined by qRT-PCR as described in Materials and Methods. Values obtained for vehicle controls in each case were set to 1 for comparison. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. ***, p < 0.001, **, p < 0.01 vehicle vs. IFNγ, TNFα or TNFα + IFNγ. Panels: A, IOSE261F; B, SKOv3-ip; C, OVCAR-3; D, RL95-2; E, HEC50 and; F, MCF-7.
Figure 2. High cytokine treatments failed to stimulate *MUC16* mRNA levels in OVCAR-3 cells.

OVCAR-3 cells were treated for 48 h with either a vehicle control (0.1% [w/v] BSA in PBS) or a combination of TNFα (10 to 25 ng/ml), IFNγ (100 to 200 IU/ml). RNA then was extracted and the levels of *MUC16* mRNA relative to that of *ACTB* were determined by qRT-PCR as described in Materials and Methods. Values obtained for vehicle controls in each case were set to 1 for comparison. No significance was obtained.
Figure 2.5. Time course of stimulation of MUC16 mRNA levels and protein production in response to combined cytokine treatment.

MCF-7 cells were treated either with vehicle (0.1% [w/v] BSA in PBS) or TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) for the indicated times. MUC16 mRNA levels were determined by qRT-PCR and amounts of cell-associated and secreted/shed MUC16 were determined by CA 125 ELISA assays as described in Materials and Methods. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. Panels: A. mRNA levels; B, cell associated MUC16; C, media MUC16.
Figure 2. 6. Individual cytokines stimulate MUC16 protein production in MCF-7 cells.

MCF-7 cells were treated for 6 days either with vehicle (0.1% [w/v] BSA in PBS), TNFα (2.5 ng/ml), IFNγ (20 IU/ml), or TNFα + IFNγ. MUC16 levels then were determined (A) in the cell associated and media fractions by CA125 ELISA and (B) by immunofluorescence staining as described in Materials and Methods. Total MUC16 production was calculated by summing the amount of CA125 reactivity in the cell-associated and secreted fractions for each sample (A). A small stimulation of MUC16 production resulted from treatments with either TNFα or IFNγ alone. Much greater stimulation was observed with the combination of TNFα and IFNγ. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. ***, p < 0.001 vehicle vs. TNFα + IFNγ.  

(B) Vehicle; panels a, e and i; TNFα alone; panels b, f and j, IFNγ alone; panels c, g and k, or TNFα + IFNγ panels d, h and i. Cells were stained with MUC16 antibody (OC125; red) and DAPI (blue) and imaged. 

Prior to immunostaining, cells were treated for six days with vehicle (0.1% [w/v] BSA in PBS; Fig. 5B panels a, e and i), TNFα (2.5 ng/ml; Fig. 5B panels b, f and j), IFNγ (20 IU; Fig. 5B panels c, g and k), or TNFα + IFNγ (Fig. 5B panels d, h and i). Cells subsequently were fixed and stained with MUC16 antibody (OC125; red) and DAPI (blue) and imaged.
Table 2. MUC16, TNFα and IFNγ are present in malignant neoplasms

<table>
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<th>TNFα Staining intensity</th>
<th>IFNγ Staining intensity</th>
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The table shows the results of a tissue array staining for MUC16, TNFα and IFNγ. Each entry represents a sample from different patient. In general, MUC16 is present in areas where TNFα and/or IFNγ are also present. The letters represent staining observed in the following portions of the sections: c=cancer, s=stroma, b=both. MUC16 staining was only observed in the cancers. The numbers 1 to 3 refer to the staining intensity. 1= low staining level and 3= high staining level. Examples of staining intensity are shown in Fig. 2.7.
A. Ovary Tumor  
\( n=14 \)

B. Breast Tumor  
\( n=19 \)

C.  
OC125 | TNF\(\alpha\) | IFN\(\gamma\)
---|---|---
1 |

D.  
OC125 | TNF\(\alpha\) | IFN\(\gamma\)
---|---|---
1 |

E. Endometrial cancer  
\( n=8 \)

F.  
OC125 | TNF\(\alpha\) | IFN\(\gamma\)
---|---|---
1 |

3 |
Figure 2. 7. MUC16, TNFα, and IFNγ are co-expressed in malignant ovarian and uterine neoplasms.

Tissue staining and staining intensity assessments were performed as described in Materials and Methods. A correlation between the intensity of MUC16 staining and TNFα and IFNγ staining was observed in ovarian cancer (A) and to a lesser degree in breast cancer (B), but not in endometrial cancer (E). Cytokine staining intensity was plotted against the staining intensity for MUC16 in each case and a correlation analysis was performed. Ovarian cancer TNFα vs MUC16: Correlation coefficient (r) = 0.8016. r squared = 0.6426, The P value is 0.0006, considered extremely significant and IFNγ vs MUC16: Correlation coefficient (r) = 0.7077. r squared = 0.5009, the P value is 0.0046, considered very significant. Breast cancer, TNFα vs MUC16: Correlation coefficient (r) = 0.4771. r squared = 0.2276, the P value is 0.0389, considered significant and IFNγ vs MUC16: Correlation coefficient (r) = 0.7705. r squared = 0.593, the P value is 0.0001, considered extremely significant. Data was plotted with a small variation to help visualization. Representative tissue microarray images show different combinations of MUC16, TNFα and IFNγ expression in different uterine neoplasms. (C) Representative staining of ovarian cancers scored as 1 or 3 for MUC16, TNFα and IFNγ with staining in both the cancer and stroma. Lower magnification, scale bars: 25 µm. Higher magnification (inset), scale bars: 10 µm. (D) Representative staining of breast cancers scored as 1 or 3 for MUC16, TNFα and IFNγ. Lower magnification, scale bars: 25 µm. Higher magnification (inset), scale bars: 10 µm. (F) Representative staining of endometrial cancers scored as 1 or 3 for MUC16, TNFα and IFNγ. Lower magnification, scale bars: 25 µm. Higher magnification (inset), scale bars: 10 µm.
Figure 2. 8. MUC16 mRNA responsiveness to cytokines is attenuated when NFκB/p65 is knocked down by siRNA.

MCF-7 (A, C) and SKOv3-ip (B, D) cells were treated for 24 h with either scrambled or NFκB siRNA at a final concentration of 50 nM. Cells then were treated for 24 h with either a vehicle control (0.1% [w/v] BSA in PBS), or a combination of TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml). RNA then was extracted and the levels of NFκB/p65 (A and B) and MUC16 (C and D) mRNA relative to that of ACTB were determined by qRT-PCR as described in Materials and Methods. Values obtained for scrambled vehicle control in each case were set to 1 for comparison. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. *** p < 0.001.
Figure 2.9. Elevation of MUC16 in response to cytokines is attenuated when NFκB/p65 is knocked down by siRNA.

MCF-7 cells were treated for 24 hrs with either scrambled or NFκB siRNA at a final concentration of 50 nM. Cells then were treated for 24 hrs with either a vehicle control (0.1% [w/v] BSA in PBS), or a combination of TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml). Cell-associated protein then was extracted and the levels of NFκB/p65 relative to that of β-actin were determined by western blot. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. *, p < 0.05, scrambled vehicle vs. NFκB/p65 siRNA-TNFα + IFNγ and ***, p < 0.001scrambled TNFα + IFNγ vs. NFκB/p65 siRNA-TNFα + IFNγ.
SKOV3-ip cells were treated for 24 h with either scrambled or MUC16 siRNA at a final concentration of 50 nM. Then, cells were treated for 24 h with high doses TNFα (25 ng/ml) or IFNγ (200 IU/ml). The levels of NFκB/p65 and MUC16 mRNA relative to that of ACTB were determined by qRT-PCR. Values obtained for scrambled vehicle control in each case were set to 1 for comparison. *** p < 0.001, scrambled TNFα or IFNγ vs. NFκB/p65 siRNA-TNFα or NFκB/p65 siRNA-IFNγ and ** p < 0.01, scrambled vehicle vs. NFκB/p65 siRNA.
Figure 2. 11. High levels of MUC16 expression are not mediated by NFκB.

OVCAR-3 cells were treated for 24 h with either scrambled or NFκB siRNA at a final concentration of 50 nM. RNA then was extracted and the mRNA levels of NFκB/p65 (A) and MUC16 (B) relative to that of ACTB were determined by qRT-PCR as described in Materials and Methods. Values obtained for scrambled control in was set to 1 for comparison. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. ***, p < 0.001, **, p < 0.01 scrambled vehicle vs. NFκB/p65 siRNA.
Figure 2. 12. Alternative transcriptional start sites of the MUC16 gene.

A. The diagram shows two alternative sites of transcription found using 5'RACE in two different cell lines MCF-7 and OVCAR-3. Sites are compared with the start of transcription predicted by the Ensembl genome browser. Diagram also shows the sites of the primers used in 5'RACE: GSP1, GSP2, GSP3. B. Image shows the bands amplified by PCR used in the 5'RACE. C. Sequence alignment of human chromosome 19 and the two cell lines MCF-7 and OVCAR-3 in which it is shown how the two cell lines start transcription at sites other than those predicted. Note that MCF-7 shows a nucleotide deletion.
A. Diagram of the proximal 250 bp MUC16 promoter indicating the position of the putative NFκB binding site. +1 refers to the 5'RACE determined start of transcription for MCF-7 cells. B. Sequence of the potential NFκB binding site and the mutated site generated for the transfection studies described in C. C. MCF-7 cells were transfected with the wild type 250 bp proximal MUC16 reporter plasmid or the mutated putative NFκB binding site and then treated with either vehicle (0.1% [w/v] BSA in PBS), or TNFα (2.5 ng/ml) + IFNγ (20 IU/ml) for 24 h before measuring luciferase activity in cell lysates cells as described in Materials and Methods. Data are reported as the values obtained relative to the vehicle-treated wild type promoter. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. ***, p < 0.001 wild type promoter vehicle vs. TNFα + IFNγ.

Figure 2. 13. A consensus NFκB-binding site accounts for much of the MUC16 gene responsiveness to cytokines.
Figure 2. 14. Binding and recruitment of NFκB/p65 to the MUC16 promoter.

A. An electrophoretic mobility shift assay (EMSA) was performed using unlabeled wild type or mutated NFκB oligos (.15 μM) alone and in combination with the recombinant NFκB/p65 protein (1 μM). Samples were incubating in 5X binding buffer for 40 min and run in a non-denaturing 12% gel followed by gel staining. Gels were imaged using the Carestream Imaging system following the manufacturer’s instructions. B. ChIP assay of NFκB/p65 binding to MUC16 promoter was done using MCF7 cells treated in stripped media containing TNFα and IFNγ to a final concentration of 2.5 ng/ml and 20 IU, respectively. Cells were cross-linked and chromatin harvested 4 h following treatment as described in Materials and Methods. Two biological replicates for chromatin extractions were performed and all ChIP assays were performed in triplicate. One μg of chromatin stock was used with 2 μg NFκB antibody as per the standard Chromatrap® ChIP protocol. Two sets of primers (labelled 1 and 2) flanking the MUC16 NFκB site were used as detailed in Materials and Methods. Fold change is above the mean of the background.
Figure 2. 15. Proposed model of MUC16 regulation by cytokines through NFκB.
Chapter 3: PPARγ modulation of cytokine-stimulated MUC16 (CA125) expression in breast and ovarian cancer cell lines

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3.1. Abstract:

CA125 has been used as a serum marker for ovarian tumors for over 30 years. Nonetheless, little is known about regulation of its expression. CA125 is an epitope carried by a portion of the large (> 3 MDa) high molecular weight, heavily glycosylated cell surface transmembrane mucin, MUC16. Transmembrane mucins are normally localized at the apical surface of simple epithelia where they lubricate and protect these tissues from pathogens and enzymatic attack. In malignancies, membrane bound mucins lose their polarized distribution and are aberrantly over-expressed. High level transmembrane mucin expression protects tumor cells from the actions of chemotherapeutic agents as well as the immune system. Previously, we reported that MUC16 expression is highly stimulated by the proinflammatory cytokines, tumor necrosis factor α (TNFα) and interferon γ (IFNγ), in breast and ovarian cancer cell lines and tissues. Elevated proinflammatory cytokine expression characterizes various cancers and is considered one of the “hallmarks of cancer”. We previously demonstrated that peroxisome proliferator-activated receptor-γ (PPARγ) agonists inhibit expression
of the transmembrane mucin, MUC1, in a variety of contexts. In this report, we show that PPARγ modulates cytokine-stimulated MUC16 in a complex manner: at low concentrations (≤ 10 µM) in combination with cytokines, rosiglitazone further stimulates MUC16 expression while at high concentrations (> 20 µM) rosiglitazone antagonizes cytokine stimulation. Rosiglitazone actions were fully reversible by the PPARγ antagonist, GW9662. Furthermore, siRNA-mediated PPARγ knockdown also prevented a large portion of high dose rosiglitazone suppression indicating that rosiglitazone inhibition is at least partially PPARγ-dependent. Collectively, these studies demonstrate that PPARγ is an important modulator of MUC16 expression. The ability to deliver high doses of PPARγ agonists to MUC16-expressing tumor may provide an avenue to reduce expression of this tumor marker and increase tumor sensitivity to killing by chemotherapeutic drugs and the immune system.

3.2. Introduction

Mucins are high molecular weight, heavily O-glycosylated glycoproteins normally found at the apical surface of epithelial cells. Mucins lubricate and protect the epithelium from pathogens, toxins and enzymatic attack (Haridas et al., 2014). Aberrantly glycosylated mucins are associated with cancer growth and metastasis (Chugh et al., 2015). MUC16 is one of the transmembrane mucins and one of the twenty-one identified human mucin genes (Strous & Dekker, 1992) MUC16 was originally identified as the antigen, CA125, overexpressed in
ovarian cancer (Bast et al., 1981). In contrast, MUC16 is absent in normal ovarian epithelium (Thériault et al., 2011). MUC16 was later found to be expressed not only by ovarian tumor cells, but also in other epithelial cell and tissue types including those of the coelom, mullerian duct, epicardium and uterus (Kabawat et al., 1983; Menczer et al., 2014; Whitehouse & Solomon, 2003). Immunohistochemical analysis reveals CA125/MUC16 in other tissues including conjunctiva, lung, breast and prostate (Nap et al., 1996; Whitehouse & Solomon, 2003). MUC16 has an average molecular weight of ~3-5 million Da (O’Brien et al., 2001; Yin & Lloyd, 2001). It is composed of a heavily O-glycosylated N-terminal domain, a tandem repeat region, a stretch of 56 SEA domains, a single transmembrane domain and a short C-terminal cytoplasmic tail (O’Brien et al., 2001; Yin & Lloyd, 2001). The N-terminal domain alone is composed of 12,068 amino acids, while the tandem repeat domain is composed of up to 60 repeats of 156 amino acids. The C-terminal domain is the smallest part composed of 284 amino acids (O’Brien et al., 2002).

Regulation of certain mucins, particularly MUC1, has been well studied (Brayman et al., 2007; Mitchell et al., 2002). Nonetheless, in spite of its important use as a tumor marker, little is known about regulation of MUC16 expression. Cytokine release follows inflammatory events and are found as products of immune cells that concentrate near tumor sites in vivo (Yamamura et al., 1993). IL-6, IL-8, IL-17, TNFα and IFNγ greatly stimulate both MUC1 and MUC16 mRNA and protein expression in human corneal epithelia (Albertsmeyer et al., 2010). Recently, we
demonstrated that MUC16 expression is stimulated by TNFα and IFNγ in a variety of tumor cell contexts, including human breast and ovarian cancer (Morgado et al., 2016). Nevertheless, there is little information about factors that suppress MUC16 expression. Transmembrane mucins protect tumor cells from the immune system and promote resistance to various chemotherapeutic agents (Hollingsworth & Swanson, 2004). Therefore, identifying agents that can suppress expression of these mucins, particularly in the presence of stimulatory cytokines, may provide new avenues to improve cancer therapy.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-driven transcription factors. There are three PPARs in mammals; PPARα, PPARβ/δ and PPARγ (Ahmadian et al., 2013). After binding to PPAR response elements, PPAR heterodimerizes with retinoid X receptor (RXR) and controls expression of genes involved in lipid metabolism, adipogenesis, and inflammation (Ahmadian et al., 2013; Barish et al., 2006). PPARs play an essential role in a variety of biological processes including adipose cell differentiation and modulation of metabolism and inflammation in immune cells (Tontonoz & Spiegelman, 2008). Thiazolidinediones (TZDs) are synthetic PPAR ligands (Rangwala & Lazar, 2004). Two TZDs, rosiglitazone and pioglitazone, are currently used for the treatment of type 2 diabetes (Richter et al., 2007; Wang et al., 2010). Both rosiglitazone and pioglitazone were discovered in the 1900s as part of the TZDs family. Soon rosiglitazone became the most effective anti-diabetic drug (Mughal et al., 2015). In 2011, due to its
cardiac impact on some patients, rosiglitazone was withdrawn from the market in the European Union (EU) and usage was restricted in the US (Mughal et al., 2015). In the US such restriction was removed in 2013 by the FDA since a trial found no risk of heart failure (Mughal et al., 2015).

The potential utility of TZDs as adjunct cancer treatments is currently unclear since a variety of studies report contradictory findings. According to a meta-analysis of clinical trials of diabetes, there is a reduction in the cancer risk for patients being treated with TZDs for more than one year (Bosetti et al., 2013; Raana & Javeed, 2015). In another study, it was found that pioglitazone use might increase the risk of cancers (Lewis JD, 2015). Nevertheless, a population based study found an overall relationship between cancer and type 2 diabetes, with type 2 diabetic patients displaying a 28% higher cancer risk, especially for liver and pancreas (Andrea Gini, 2016). These studies also found that diabetic patients with breast cancer had a lower 5-year survival rate compared to patients without diabetes (Andrea Gini, 2016). Additionally, a pilot study of women with breast cancer undergoing short term rosiglitazone treatment did not demonstrate significant effects on breast tumor growth (Yee, 2007). Rosiglitazone treatment caused an increase in serum adiponectin levels, as well as decreased insulin levels and increased insulin sensitivity (Yee, 2007). To our knowledge, there are no studies that have attempted to measure the impact of thiazolidinedione treatment on CA125/MUC16 levels in patients.
Studies from our lab indicate that both progesterone and EGF receptor ligand-stimulated human \textit{MUC1} expression is greatly suppressed by rosiglitazone in a dose-dependent manner (Dharmaraj et al., 2013; Wang et al., 2010) and cytokine-stimulated \textit{MUC1} expression is inhibited by rosiglitazone (Constantinou et al., 2015). Additionally, rosiglitazone decreases pro-inflammatory cytokine production and \textit{in vivo} studies have shown that it inhibits lung metastasis (Shen et al., 2012). Here we report that rosiglitazone regulates MUC16 expression in a complex, dose-dependent fashion.

\textbf{3.3. Materials and Methods}

\textbf{3.3.1. Cell culture}

Two well-characterized breast and ovarian cancer cell lines were used. MCF-7 (last profiled on 7/22/13 by STR profiling at Johns Hopkins Genetic Resources Core Facility), were cultured in Minimum Essential Medium (MEM) (Life Technologies; 11095098) and SKOv3-ip, were cultured in McCoy’s 5A (Thermo Scientific; SH30270.01). All media were supplemented with 10\% (v/v) fetal bovine serum (FBS) and penicillin (100 U/ml)–streptomycin (100 µg/ml) (Gibco®; 15140-122). MCF-7 cell media also were supplemented with 10 µg/ml of insulin (w/v) (Sigma-Aldrich Inc.; I9278).
3.3.2. Cell Treatments

Cells were plated in six well plates in media containing 5% (v/v) charcoal-stripped FBS (Life Technologies; 12676029) and maintained until cells reached 60-70% confluence. Cells were serum-starved for 24 h prior to treatment with tumor necrosis factor alpha (TNFα, ROCHE; 113718430), interferon gamma (IFNγ ROCHE; 11040596001), rosiglitazone (Cayman Chemical; 71740), GW9662 (SIGMA; M6191) at the following concentrations: Vehicle (0.1% [w/v] BSA in PBS), TNFα (2.5 ng/ml) plus IFNγ (20 IU), and cytokines with or without rosiglitazone (1 µM to 100 µM) and with or without GW9662 (50 µM; GW9662) as indicated in the text in media containing 10% (v/v) charcoal-stripped FBS. Cells were incubated with cytokines with and without rosiglitazone and with and without GW9662 for 48 h prior to RNA or protein extraction.

3.3.3. siRNA knockdown

PPARγ was knocked down using an human oligonucleotide PPARγ siRNA (h) (Santa Cruz; sc-29455) and a non-silencing, scrambled siRNA control: 5’-AATTCTCCGAACGTGTCACGT-3’ (Schroeder et al., 2001). PPARγ siRNA was resuspended to a final concentration of 10 µM and scrambled control to 20 µM following the manufacturer’s instructions. Oligonucleotides were transfected using Lipofectamine 2000 (Invitrogen; 11668019) according to the manufacturer’s instructions. Briefly, cells were plated in antibiotic free media in 12 well plates incubated for 24 h at 37°C in a humidified atmosphere of air: CO₂ (95:5, v/v). Oligonucleotides were transfected at a final concentration of 50 nM in Opti-MEM® Reduced Serum Medium, GlutaMAX™ Supplement (Gibco;
Six h after transfection, media was changed to regular medium with FBS, lacking antibiotics. Twenty-four h later cells were treated with or without cytokines and with or without rosiglitazone for 24 h followed by RNA extraction

3.3.4. RNA isolation and reverse transcription-PCR

TRIZOL reagent (Invitrogen; 15596-026) was used to isolate total RNA subsequent to treatment with DNAsé according to the manufacturer’s instructions (Ambion; AM1906). Reverse transcription was performed using 1 µg of total RNA in a 10 µl reaction using qScript cDNA Super mix (Quanta; 95048) incubated for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. Real time qPCR was performed using specific oligonucleotide primers as follows:

MUC16 F: 5’GCCTCTACCTTAACGGTTACAATGAA- 3’ and MUC16 R: 5’-GGTACCCCATGGCTGTTGTG -3' (Argüeso et al., 2003);

ACTB F: 5’-GATGAGATTGGCATGGCTTT-3' and

ACTB R: 5’-CACCTTCACCGGTCCAGTTT-3' (Dharmaraj et al., 2010);

and PPARγ F: 5’-AGAGATGCCATTCTGCGC-3’ and

PPARγ R: 5’-GTGGAGTAGAATGCTGGAGA-3' (Wang et al., 2010).

SYBR Green Super mix and primers were prepared according to the manufacturer’s instructions (Quanta Bioscience; 95030-216). Samples were cycled as follows: MUC16 and ACTB, (30 sec at 95°C and 30 sec at 59°C for 40 cycles); PPARγ (an initial incubation at 50°C for 2 min followed by 40 cycles of
95°C for 10 min, 95°C for 15 s and 60°C for 1 min. Relative amounts of mRNA were identified using the comparative threshold cycle method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008) and normalized to that of ACTB.

### 3.3.5. Western blot analysis

Protein was isolated using the RIPA Lysis Buffer System (Santa Cruz Biotechnology; sc-24948) following the manufacturer’s instructions. Total protein extract was separated by 12% (w/v) SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked at 4°C in TBS plus 0.1% (v/v) Tween 20 (TBST) and 3% (w/v) BSA for 2 h and probed with the NFκB/p65 (Santa Cruz Biotechnology; sc109) primary antibody (1:200) or β-actin antibody (1:10,000) in incubation buffer (3% w/v BSA) overnight at 4°C. Blots were rinsed three times for 10 min each at room temperature and incubated for 2 h at 4°C with horseradish peroxidase conjugated sheep anti-mouse IgG (Jackson Immuno research; 515-035-062) or goat anti-rabbit (Sigma; A6154-1ML) at final dilutions of 1:200,000 in blocking solution. Finally, the blots were rinsed three times for 10 min each in TBST and signal intensities were detected using the ECL system (Pierce; 32106) as described by the manufacturer. Blots were exposed to x-ray film, and signal intensities were quantitated using Image J software (NIH, Bethesda, MD).

### 3.3.6. Statistical analysis

All data is shown as the mean ± SD of triplicate determinations of independent biological samples. Statistical analyses were performed using a one-way ANOVA followed by Turkeys post-test using Graph Pad InStat software, version 3.05
(GraphPad Software, San Diego, CA). Differences were considered significant when P<0.05 by Student’s two-tailed t-test.

3.4. Results

3.4.1. Basal and cytokine stimulated MUC16 mRNA levels are suppressed by rosiglitazone in a dose dependent manner.

Our preliminary studies indicated a suppressive effect of high concentrations of rosiglitazone on cytokine-stimulated transmembrane mucin expression in a human female reproductive tract cell line (Constantinou et al., 2015). To expand on this observation, we performed rosiglitazone dose response studies on basal MUC16 mRNA levels in MCF-7 cells. Rosiglitazone exerted a suppressive effect in basal MUC16 mRNA levels in a dose dependent manner (Fig. 3.1A). Rosiglitazone’s actions on basal MUC16 mRNA levels were biphasic (Fig. 3.1.A). At low concentrations rosiglitazone was stimulatory with a maximal effect at 0.1 µM. At higher concentrations this effect was diminished returning to basal levels.

We next tested rosiglitazone in the presence of TNFα and IFNγ, proinflammatory cytokines that markedly stimulate MUC16 expression in a variety of cellular contexts (Morgado et al., 2016). At low rosiglitazone concentrations (1 - 10 µM) cytokine-stimulated cells showed a further increase in MUC16 mRNA levels (Figure 3.1B). At higher concentrations (>20 µM), decreased MUC16 mRNA levels (Fig. 3.1B) were observed. We next sought to determine to what extent these responses were mediated by PPARγ versus off-target effects.
3.4.2. *The PPARγ antagonist, GW9662, reverses rosiglitazone suppression of cytokine-stimulated MUC16 expression.*

As one approach to determine if the rosiglitazone effect on the MUC16 mRNA levels was PPARγ dependent, the PPARγ antagonist, GW9662 (Seargent *et al.*, 2004), was employed. Neither rosiglitazone nor GW9662 alone or in combination had an effect on the MUC16 mRNA levels (Figure 3.2). GW9662 in combination with cytokines slightly, but significantly, increased the MUC16 mRNA levels compared to cytokines only. As shown above, 40 µM rosiglitazone substantially suppressed the cytokine response. Inclusion of GW9662 completely reversed the rosiglitazone suppression consistent with this response being PPARγ dependent.

3.4.3. *NFκB suppression is rescued by cytokines in presence of low, but not high, rosiglitazone concentrations.*

Previous studies indicated cytokine stimulation of MUC16 expression is mediated by NFκB (Morgado *et al.*, 2016). We sought to determine if rosiglitazone exerted any of its effects on cytokine-mediated stimulation of MUC16 expression by altering NFκB expression. Cytokines alone did not change NFκB/p65 protein levels relative to untreated controls; however, rosiglitazone at either low (1 µM) or high (40 µM) concentrations substantially (>80%) reduced NFκB levels (Fig. 3.3). This reduction was largely restored by the addition of cytokines when low, but not high, rosiglitazone concentrations were used. Consequently, it appeared that at
high rosiglitazone concentrations the antagonism of cytokine stimulation of MUC16 expression could be largely explained by the reduction in NFκB levels.

3.4.4. siRNA-mediated PPARγ knockdown.

As a second test of PPARγ’s involvement in the rosiglitazone responses we used siRNA to knockdown PPARγ. In all cases, the PPARγ-directed siRNA reduced PPARγ mRNA levels by ~80% (Fig. 3.4A). Interestingly, cytokine treatment resulted in large decrease in PPARγ mRNA levels, an effect that was exacerbated by PPARγ siRNA. A similar profile was observed when 1 µM rosiglitazone was included (Fig. 3.4A). Rosiglitazone at high concentrations (40 µM) in combination with cytokines reduced PPARγ basal mRNA levels and further reduced PPARγ siRNA levels (Fig. 3.4B). After effective knocking down of PPARγ, the effect on MUC16 levels were assessed.

3.4.5. siRNA-mediated PPARγ knockdown reverses rosiglitazone effect on MUC16 mRNA levels.

MUC16 mRNA levels were reduced by cytokines in the presence of high concentrations of rosiglitazone and increased in the presence of low rosiglitazone concentration. Nevertheless, those effects were reversed when PPAR was knocked down (Figure 3.5), further indicating a PPARγ role in this process.
3.5. Discussion

CA125/MUC16 serves as a serum marker for many cancers, including those of the ovary, endometrium, pancreas, colon and breast, as well as in benign conditions such as endometriosis (Reinartz et al., 2012). In spite of the long-standing and broad use as a disease marker, very little is known about its regulation. Factors that modulate MUC16 expression could lead to misinterpretation of a disease state or the efficacy of treatment when based solely on serum CA125 levels. It is well documented that cancer cells lose polarity leading to expression of transmembrane mucins such as MUC16 over the entire tumor cell surface. Given the roles that transmembrane mucins generally play in protecting cancer cells from the uptake and actions of chemotherapeutic agents (Hollingsworth & Swanson, 2004) and the immune system (Hollingsworth & Swanson, 2004), identifying methods to reduce MUC16 expression holds promise for increasing the efficacy of existing therapies. In breast and ovarian cancer, we demonstrated that the proinflammatory cytokines greatly stimulate MUC16 mRNA and protein levels mediated by NFκB (Morgado et al., 2016). Preliminary studies indicated that supra pharmacological concentrations of rosiglitazone, a drug used to treat a subset of patients with type II diabetes, dramatically suppressed cytokine-stimulated MUC1, MUC4 and MUC16 mRNA levels (Constantinou et al., 2015), although no mechanistic information was provided.
We used both pharmacological and gene knockdown approaches to demonstrate the role of PPARγ as a mediator of the rosiglitazone suppression of MUC16 expression even when used at higher (≥ 40 µM) concentrations. Previously, rosiglitazone was shown to greatly suppress expression of another oncogenic mucin, MUC1, in multiple contexts (Constantinou et al., 2015; Dharmaraj et al., 2013; Wang et al., 2010). The current work indicates a broader applicability of PPARγ agonists in suppressing transmembrane mucin expression with the goal of sensitizing tumor cells to chemo- or immunotherapy. While we focused our more detailed mechanistic studies on MCF-7 cells, we also demonstrated the ability of rosiglitazone to suppress cytokine-activated MUC16 expression in other human tumor cell type such as SKOv3-ip (3.6), but did not have an effect on OVCAR-3 cells, the cells with high basal MUC16 mRNA levels that did not further increase its mRNA levels after cytokine treatment (Fig. 3.7). PPARγ agonists may have broad applicability as mucin suppressors.

Diabetes may influence the female reproductive tissue carcinogenesis in several ways. These diseases commonly display increased insulin and insulin-like growth factor (IGF) signaling, chronic inflammation and ovarian steroid hormone imbalances (Mughal et al., 2015). Treatment with rosiglitazone and other thiazolidinediones (TZDs) produce conflicting effects on cancers, since in some cases TZDs reduce and in others increase cancer risk (Monami et al., 2013; Tuccori et al., 2016). Rosiglitazone (100 µM) treatment of another breast cancer cell line, MDA-MB-231, sensitizes these cells to the antitumor effects of TNFα
apparently involving upregulation of proapoptotic genes (Mody et al., 2007). MUC16 expression was not examined in this study; however, MUC16 knockdown in this same cell line reduces cell growth and tumorigenicity and increases apoptosis (Lakshmanan et al., 2012). Still other studies indicate that MUC16 protects breast and ovarian cancer cells from apoptosis, including apoptosis induced by genotoxic drugs (Boivin et al., 2009; Matte et al., 2014; Reinartz et al., 2012). Thus, if TZDs or other PPARγ agonists can be delivered to such tumors at the concentrations that suppress MUC16 expression, it is likely that this would sensitize the tumors to killing. It may not be feasible to do this by standard oral administration, but local or targeted delivery of these agents may hold promise.

The present work provides the novel description of a means to reduce MUC16 expression. This inhibition is evident and impressive even in the presence of the strong and physiologically relevant stimulators, TNFα and IFNγ. Nonetheless, rosiglitazone actions are complex. The highest concentration of rosiglitazone given to patients in the treatment of type 2 diabetes is 8 mg, which corresponds to a blood level of 1.4 µM (Mody et al., 2007). At this concentration, our work indicates that rosiglitazone actually would further elevate MUC16 expression, potentially complicating the interpretation of changes in serum CA125 levels. In this scenario, a cancer patient receiving conventional therapies while also receiving PPARγ agonists might appear to not respond to the cancer treatment based on serum CA125 levels. Furthermore, the elevated levels of the protective
mucin might further challenge therapy by providing additional defense against both the immune system and the cancer therapy. On the other hand, higher doses of rosiglitazone (>40 µM) strongly suppress MUC16 expression. It might be difficult to generate levels this high unless locally administered or if more potent and selective PPARγ agonists can be identified. High doses of rosiglitazone might be toxic if used over a long term (weeks-months); however, shorter term (days) treatments perhaps coupled with more targeted delivery to the tumor might be effective in transiently lowering MUC16 expression, allowing for cytotoxic drug penetration and improved tumor killing.
Chapter 3: Figures

Figure 3. 1. Rosiglitazone effects on basal and cytokine-stimulated $MUC16$ mRNA levels is done in a dose dependent manner.

MCF-7 cells were treated with vehicle control (0.1% [w/v] BSA in PBS) or TNFα (2.5 ng/ml) plus IFNγ (20 IU) or TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) plus the indicated concentration of rosiglitazone. $MUC16$ mRNA levels were determined as described in Materials and Methods and the value obtained for the vehicle control was arbitrarily set to 1. The data represent values obtained for triplicate determinations of independent samples. *** $p< 0.001$ relative to the vehicle control or to cytokine treated; ** $p< 0.01$, 0.10 µM relative to 10, 40 and 80 µM; * $p< 0.05$, 0.10 µM relative to vehicle and 5 and 10 µM. Error bars on B are negligible and not evident in the figure.
Figure 3.2. The PPARγ antagonist, GW9662, reverses rosiglitazone suppression of cytokine-stimulated MUC16 mRNA.

MCF-7 cells were treated for 2 days with vehicle (0.1% [w/v] BSA in PBS), TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml), rosiglitazone (ROS1 40 μM) alone, TNFα plus IFNγ plus rosiglitazone, TNFα plus IFNγ plus rosiglitazone plus GW9662 (50 μM; GW), rosiglitazone plus GW9662, or GW9662 alone as indicated. MUC16 mRNA levels determined as described in Materials in Methods and the value obtained from the vehicle control was arbitrarily set to 1 for comparison. The data represent values obtained for triplicate determinations of independent samples. *** p< 0.001 relative to cytokine treated. Error bars are negligible and not evident in the figure.
Figure 3. Cytokines rescue NFκB suppression in presence of low, but not high, rosiglitazone concentrations.

MCF-7 cells were treated for 2 days with either vehicle (0.1% [w/v] BSA in PBS), TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml), rosiglitazone (1 or 40 µM as indicated) or TNFα plus IFNγ plus rosiglitazone as indicated on the figure. NFκB protein levels were measured and normalized to ACTB levels by western blotting as described in Materials and Methods. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. ** p< 0.01 relative to vehicle and cytokine treated.
Figure 3. 4. siRNA-mediated PPARγ knockdown.

MCF-7 cells were treated for 24 h with either scrambled siRNA or PPARγ siRNA at a final concentration of 50 nM as described in Materials and Methods. Cells then were treated for 24 h with either a vehicle control (0.1% [w/v] BSA in PBS), or a combination of TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) or TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) + rosi (1 or 40 µM). RNA then was extracted and the levels of PPARγ and MUC16 mRNA were determined as described in Materials and Methods. Values obtained for scrambled vehicle control in each case were set to 1 for comparison. The bars and error bars indicate the mean ± SD of triplicate independent determinations in each case. ***, p < 0.001.
Figure 3.5. Rosiglitazone effect in MUC16 mRNA levels is reversed when PPARγ is knockdown.

MCF-7 cells were treated for 24 h with either scrambled siRNA or PPARγ siRNA at a final concentration of 50 nM as described in Materials and Methods. Cells then were treated for 24 h with either a vehicle control (0.1% [w/v] BSA in PBS), or a combination of TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) or TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) + rosi (1 or 40 µM). RNA then was extracted and the levels of PPARγ and MUC16 mRNA were determined as described in Materials and Methods. Values obtained for scrambled vehicle control in each case were set to 1 for comparison. ***, p < 0.001.
Figure 3.6. Rosiglitazone dual role in cytokine-stimulated MUC16 mRNA levels in SKOv3-ip cells.

SKOv-3 ip cells were treated with vehicle control (0.1% [w/v] BSA in PBS) or TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) or TNFα (2.5 ng/ml) plus IFNγ (20 IU/ml) plus the indicated concentration of rosiglitazone. MUC16 mRNA levels were determined as described in Materials and Methods and the value obtained for the vehicle control was arbitrarily set to 1. The data represent values obtained for triplicate determinations of independent samples. *** p < 0.001, ** p < 0.01 relative to the cytokine treated.
Figure 3.7. Rosiglitazone failed to suppress or stimulate MUC16 mRNA at any concentration.

OVCAR-3 cells were treated with the indicated concentrations of rosiglitazone for 48 h, RNA extracted and the levels of MUC16 mRNA relative to those of ACTB were determined by qRT-PCR as described in Materials and Methods. Values for the vehicle controls were arbitrarily set to 1 in each case. Bars and error bars indicate the means +/- SD for triplicate determinations in each case. No statistically significant differences were found among these treatments.
Figure 3. 8. Proposed model of regulation of MUC16 by PPARγ
Chapter 4: Development of MUC16 knockout cell lines and mini-MUC16 expressing cell lines

4.1. Introduction

MUC16/CA125 is a widely used ovarian tumor marker. Nonetheless, very little is known about its functional role in cancer. The extremely large size of the glycoprotein and its extensive glycosylation add to the challenge of studying MUC16 function. It has been generally easier to study the function of smaller transmembrane mucins, such as MUC1 and MUC4. Overexpression of MUC1 and MUC4 is associated with increased metastasis of pancreatic and ovarian cancers and induces epithelial-mesenchymal transition (EMT) (Ponnusamy et al., 2010; Roy et al., 2011); however, the function of MUC16 has not been defined in these processes. To begin to address these questions, I have developed a cell line overexpressing a truncated form of MUC16 (mini-MUC16) as well as cell lines in which MUC16 expression has been knocked out via CRISPR/cas9 targeting.

In order to develop knockout cell lines, the clustered, regularly interspaced, short palindromic repeat (CRISPR) associated endonuclease Cas9 technology (Marraffini & Sontheimer, 2010) was used. The CRISPR technology consists of short repetitions of 24-47 bp repeated sequences called direct repeats (DR). Each repeat is succeeded by short unique segments of similar length sequences of DNA (spacers) generated from previous exposure to a bacterial virus (Grissa et al., 2007; Marraffini & Sontheimer, 2010; Sander & Joung, 2014).
The role of the CRISPR system in prokaryotes is to confer resistance to foreign DNA such as plasmids and phages (Barrangou et al., 2007). The role of the CRISPR spacers is to recognize and enable excision of foreign genetic elements in a similar way that RNA interference does in eukaryotes (Marraffini & Sontheimer, 2010; Sander & Joung, 2014). Of the sequenced bacterial genomes, 40% contain CRISPRs. For archaea, this number is 90% (Grissa et al., 2007).

Two teams found an easier CRISPR system that depends on the Cas9 protein (Pennisi, 2013). This protein is a nuclease that cuts both sides of the DNA’s double helix without interfering with the ability of the complex to be directed on its target DNA (Pennisi, 2013). Transcripts from the CRISPR repeats are processed into CRISPR RNAs (crRNAs). Each crRNA contains a variable sequence from the invading DNA known as the “protospacer” and part of the CRISPR repeat. Each crRNA hybridizes with a second RNA called the transactivating CRISPR RNA (tracrRNA) which, in turn, complex with the Cas9 nuclease. The protospacer part of the complex directs Cas9 to complementary target DNA sequences only if the sequence is adjacent to short proto-spacer adjacent motifs (PAMs) (Sander & Joung, 2014). Sequences not adjacent to a PAMs motif will be cleaved (Sander & Joung, 2014). In order to perform genome editing two elements need to be introduced into the system. The first is a guide RNA (gRNA) that consists of a fusion between a crRNA and a fixed tracrRNA. At the 5’ end of the gRNA, twenty nucleotides corresponding to the protospacer cRNA direct Cas9 to the target DNA site using the canonical RNA-DNA complementary
base-pairing rules. The target sites must be 5’ to a PAM sequence (Sander & Joung, 2014). Accurately spaced single strand breaks in the host DNA triggers homologous direct repair, which is less error prone than non-homologous end joining that normally follows a double strand break (Fig. 4.1) (Ran et al., 2013). When the purpose is to knockout a gene, double strand breaks (DSB) induced by Cas9 can be repaired by error-prone nonhomologous end-joining (NHEJ). Thus, when the ends of a DSB are repaired and rejoined, the result can be a random insertion/deletion (indel) mutation at site of the break/junction. Indel mutations in the coding region can lead to frameshifts and formation of premature stop codons resulting in gene knockout (Fig. 4.1) (Ran et al., 2013).

In order to better understand the functional role of MUC16 in cancer initiation and progression, in this research tools such as an over expressing MUC16 and a MUC16 knockdown cell lines were developed. These tools will facilitate the study of this large glycoprotein to better understand its functional and regulatory roles in epithelial cancers.
Diagram shows how double strand breaks (DSB) induced by Cas9 can be repaired via two mechanisms, error-prone nonhomologous end-joining (NHEJ) and Homology directed repair (HDR). The NHEJ pathway uses the endogenous DNA repair machinery. This can result in random indel mutations at the site of the break. When an indel mutation occurs within the coding region of a gene, the result can be a frameshift and the creation of an early stop codon, which can result in a gene knockout. [Reproduced from Ran et al.: Genome engineering using the CRISPR-Cas9 system. *Nature Protocols* 8, 2281–2308 (2013), With permission from Nature Publishing Group].
4.2. Materials and Methods

4.2.1. Cell culture

Two cell lines were used for these studies. MCF-7 breast cancer cells (last genomically profiled on 7/22/13 by STR profiling at Johns Hopkins Genetic Resources Core Facility) were cultured in Minimum Essential Medium (MEM) (Life Technologies; 11095098) supplemented with 10 μg/ml of insulin (v/v) (Sigma-Aldrich Inc.; I9278). SKOv3-ip ovarian cancer cells were cultured in McCoy’s 5A (Thermo Scientific; SH30270.01). Both media were supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Gibco®, 15140-122).

4.2.2. Plasmid construction

As a backbone for the MiniMUC16 (A truncated form of MUC16 containing the last two SEA domains, 54 and 55, and a partial 53 domain, the transmembrane domain and the cytoplasmic tail), the pTriEX™ 1.1 plasmid was used. The pTriEX™ vector contains a CMV enhancer and a β-actin promoter as well as neomycin resistance cassette (Adgene, the non profit repository). MiniMUC16 expressing lines were obtained and isolated after selection with G418. MiniMUC16 expression was confirmed by western blotting using an antibody that recognizes the C-terminal portion of MUC16 (4H11) (Rao et al., 2010).
The vectors, Cas9 expression (containing a Tet-inducible Cas9 promoter and a neomycin resistance cassette), MUC16 gRNA, pMD2.G and psPAX2, were purchased from the Baylor College of Medicine Cell-Based Assay Screening Service (Houston, TX). The MUC16 gRNA-1 targeting vector contained the sequence TTGGGGTCCCTTTAGGTGAT targeting the first exon of MUC16 and the MUC16 gRNA-2 vector contained the sequence ATTGTGCTGGCTCTGGCCTC also targeting the first exon of MUC16. Both sequences are respectively 820 bp and 945 bp downstream from the start of translation of MUC16.

### 4.2.3. Transient and stable transfection

Transfections were done as follows: $4 \times 10^5$ cells were plated in 6-well plate as described above but without antibiotic. The following morning cells had reached ~70% confluency and were transfected using Lipofectamine 2000 (Life Technologies; 11668019) and Opti-MEM (Life Technologies; 51985034) following the manufacturer's instructions. Six h after transfection media was replaced with media containing antibiotics as described above and cells were allowed to recover overnight. Twenty four h later media was replaced with media containing the selection antibiotic, G418 (500µg/ml).
4.2.4. CRISPR/Cas9 knockout of MUC16

4.2.4.1. Determining the geneticin (G418) concentration used for selection

To determine the optimal G418 concentration to use for selection, a killing curve was determined. On day 1, $4 \times 10^5$ target cells were plated in 6 well plates and cultured as described above. On day 2, when cells had reached approximately 80-90% confluency, culture media was replaced with fresh media containing G418 concentrations ranging from 0-1000 µg/mL. Each day cells were examined microscopically and media was replaced with fresh G418-containing every other day. The minimum concentration of G418 that resulted in complete cell death within 7 days was the concentration used for selection in subsequent experiments.

4.2.4.2. CRISPR/Cas 9 knockout of MUC16

HEK293 cells were transiently co-transfected with 1 µg of Cas9 or MUC16 gRNA expression vectors in combination with 250 ng pMD2.G, 750 ng psPAX2 and 6 µL Lipofectamine 2000 to produce lentiviral particles. After two days, conditioned media containing viruses was either spun at 1,250 rpm for 5 min or filtered through a 0.45 µm pore size filter to remove any HEK293 cells in the media. Conditioned media then was used to infect cells, or stored frozen at -20 °. In either case, 500 µl of the conditioned media containing lentivirus harboring Cas9 was used to infect MCF-7 and SKOv3-ip cells overnight in the presence of 8 µg/mL polybrene (Sigma-Aldrich; H9268). Subsequently, selection was done
using 500 µg/mL of G418. Single, clonal populations were isolated using first a 96 well plate and serial dilution. Single clones were allowed to reach confluency and then transferred to 12-well plates. Doxycycline at 2 µM then was added to selection media for 2 days to activate Cas9 expression. Cells were lysed and western blotting performed with anti-FLAG antibody. The clone with highest Cas9 expression was used for CRISPR/Cas9 targeting. Selected MCF-7 and SKOv3-ip Cas9-expressing clones then were treated for 24 h with 500 µl of MUC16 gRNA viruses in the presence of 8 µg/mL polybrene. A serial dilution was done and single clones were grown to confluency as before. Single clonal populations were tested for MUC16 expression by western blotting and immunofluorescence.

4.2.5. Western blot analysis

Single clones were grown in 12 well plates and protein was extracted using RIPA buffer (Santa Cruz; NC9165254) using the manufacturer’s instructions. Briefly, 100 µL of RIPA buffer containing PMSF (2 mM), protease inhibitor cocktail and sodium orthovanadate (1 mM). Cell lysates were mixed and incubated at 99°C for 10 min. Proteins were subjected to SDS-PAGE using a 12% (w/v) polyacrylamide gel. Proteins then were transferred to nitrocellulose membranes at 40V for 5 hours at 4°C. Blots were blocked at 4° in TBST (TBS + 0.1% [v/v] Tween-20 (Fisher Scientific; BP337500) and 3% (w/v) BSA for 2 h at room temperature. Blots then were incubated in blocking buffer with primary antibody (1: 2000) overnight at 4°C. Blots were rinsed three times for 10 min each at room temperature with TBST and incubated with secondary antibodies (1:50,000) in blocking buffer for 2 h at room temperature. Finally, the blots were rinsed three
times for 10 min each with TBST, and developed using WestDura ECL (Fisher Scientific; PI34076) as described by the manufacturer and exposed to HyBlot CL autoradiographic film (Denville Scientific Inc., Holliston, MA). Densitometry was performed and analyzed using ImageJ software (Schneider et al., 2012).

4.3. Results

4.3.1. Creation of an ovarian cancer cell line that constitutively expresses a truncated form of MUC16 (MiniMUC16)

A plasmid containing a fragment of MUC16 (Fig 4.2B) known to be active in other cells (Boivin et al., 2009; Comamala et al., 2011) was cloned into the pTriEX-1.1, a multiple expression vector (Fig. 4.3). The fragment of MUC16 (MiniMUC16) contains an N-terminal, BM40 signal sequence to facilitate protein trafficking through the secretory pathway. This was followed by SEA domains including partial number 54, and full numbers 55 and 56, the transmembrane domain, the cytoplasmic tail, and a C-terminal histidine tag for detection of the protein (Fig. 4.2). The molecular weight of the MiniMUC16 protein core is about 43 kDa not taking into consideration potential cleavage or glycosylation (Fig. 4.2). Expression of MiniMUC16 was confirmed on transiently transfected cells via western blotting. It was confirmed that MiniMUC16 was expressed in HEK 293 cells that do not normally express MUC16 (Fig. 4.4).

The ovarian cancer cell line, SKOv3-ip, was used to create a cell line stably
expressing the truncated version of MUC16 (Mini MUC16). SKOv3-ip cells have been previously determined to have relatively low basal levels of MUC16 (Morgado et al., 2016). SKOv3-ip cells were successfully transfected with MiniMUC16 and after selection with G418 stable clones were isolated. Clones were tested via western blotting using an antibody that recognizes the C-terminal portion of MUC16 (4H11) (Fig. 4.5).

4.3.2. Development of Stable breast and ovarian cancer cell lines containing the MUC16 knockout gene.

The CRISPR/Cas9 system for genome editing (Marraffini & Sontheimer, 2010; Sander & Joung, 2014) was used to knock out the MUC16 gene. CRISPR guide RNA (gRNA) design for MUC16 gene and gRNA vector construction was done by the Cell-based Assay Screening Service (C-BASS) core of Baylor College of Medicine, as described in Materials and Methods. Two MUC16 guide RNA sequences were used, gRNA 1 (referred as A) and gRNA 2 (referred as B). Both sequences are, respectively, 820 bp and 945 bp downstream from the start of MUC16 translation (both in exon 1). Two cell lines were selected for the knockout experiments, the moderately differentiated breast cancer cell line, MCF-7, and the ovarian cancer cell line, SKOv3-ip.

First, a killing curve was performed to determine the optimal concentration of the antibiotic, G418, required for selection (the minimum concentration that completely killed untransfected control cells). This was determined to be 500
µg/ml. Cells were first transfected with the Cas9 plasmid by lentiviral infection, followed by incubation with 500 µg/ml G418. Individual clones were selected via serial dilution and further screened by western blotting (Fig. 4.6). Positive Cas9 clones were transfected with either MUC16 gRNA A or B and Cas9 was induced using doxycycline. Serial dilution was used to select individual clones. Clones then were further screened via western blotting (Fig. 4.7 and 4.8) and immunostaining (Fig. 4.9 and 4.10).

I concluded I had successfully created tools that would be important in the future studies of MUC16 function.

**4.4. Discussion**

MUC16/CA125 is the only ovarian tumor marker that has survived as a clinically useful marker for three decades (Bast et al., 1981; Bast & Spriggs, 2011; Felder et al., 2014; Gipson et al., 2008; Nowak et al., 2015). Nonetheless, very little is known about the function of this mucin not only in ovarian cancer, but also in many of the other cancers in which it is overexpressed. MUC16 is a very large glycoprotein about 2-5 MDa in size and with extensive glycosylation (Felder et al., 2014; Haridas et al., 2014), which makes the study of its function challenging. The functions of lower molecular weight mucins including MUC1 and MUC4 have been successfully studied and serve as models for MUC16 functions (Agrawal et al., 1998; Baruch et al., 1999; Dharmaraj et al., 2013; Komatsu et al., 1999;
Meerzaman et al., 2001). MUC1 and MUC4 are associated with metastasis of pancreatic and ovarian cancers by inducing epithelial to mesenchymal transition (Gao et al., 2013; Zhang et al., 2016) (EMT), but it is not clear if MUC16 is associated with this or any other processes associated with cancer progression, invasion and metastasis. Additionally, mucins possess adhesive and anti-adhesive properties (Hollingsworth & Swanson, 2004). MUC16 exerts anti-adhesive properties in the endometrium and it is required to be absent to allow trophoblastic adherence (Gipson et al., 2008) to the endometrium. In general, natural killer (NK) cells form immune synapses with their target cells thus requiring cell to cell contact; having an anti-adhesive molecule in the cell surface may interfere with the tumor cell interactions with NK cells restricting the effector cell activation and lysis of tumor cells. A study has shown that MU16 expressing ovarian cancer cells decreases the interactions between ovarian cancer and NK cells (Gubbels et al., 2010). It would be interesting to determine if complete elimination of MUC16 (MUC16 KO) restores the interaction between the cancer and NK cells. Epidermal growth factor receptor (EGFR) that belongs to the ErbB family of receptor tyrosine kinases (RTK) gets activated after binding with peptide growth factors of the EGF-family of proteins, which subsequently gets auto phosphorylated leading to cell proliferation. EGFR seems to be involved in the pathogenesis and progression of different cancers (Normanno et al., 2006). There is evidence of the association of EGFR to mucins such as MUC1 (Dharmaraj et al., 2013) and to some extent MUC16 (Comamala et al., 2011). It would be interesting to further determine the association between EGFR and
MUC16 and the cell consequences of this association in cancer. Therefore, it is important to develop tools, i.e. cell lines that can be used to study the consequences of altering MUC16 levels on cell behavior.

Here I have successfully created stable cell lines in which MUC16 either is knocked out or is constitutively expressed in a truncated form (MiniMUC16). This truncated form of MUC16 produces biological responses in other contexts (Thériault et al., 2011). The cell lines developed in this study will be of use in studying MUC16 functions. Mucins, including MUC16 are known to be overexpressed in a variety of epithelial cancers (Kufe, 2009). The C-terminal part of mucins has been proven to be important for its transduction of growth and survival signals from cell surface receptors. Additionally, studies have shown that the C-terminal part of mucins can be used as potential drug targets (Raina et al., 2015). The role of MUC16 as a potential target has not been clearly determined and could be interesting to study using mini MUC16. Even though it is emerging, there is still little information regarding the role of MUC16 in cell survival, growth and metastasis and the role of its C-terminal part in these processes (Das et al., 2015). Previous studies suggest that MUC16 is involved in cell adhesion, migration and invasion, and may have immunosuppressive properties but detailed mechanisms are still not clear (Gipson et al., 2008; Gubbels et al., 2006). While the MUC16 cytoplasmic domain encompasses potential phosphorylation sites, there is minimal evidence of cell signaling involvement. Recently, a possible role for MUC16 in ovarian cancer cell epithelial–
mesenchymal transition was demonstrated, presumably through the modulation of EGFR phosphorylation (Comamala et al., 2011). MiniMUC16 will be of great use in further determining the role of MUC16 in cell signaling as well as a potential target in cancer therapies.

MUC16 overexpression occurs in multiple cancers conditions including cancers of the ovary, uterus, breast, pancreas and colon (Felder et al., 2014; Haridas et al., 2014; Wang et al., 2008), as well as non-cancerous conditions including benign ovarian neoplasms, endometriosis, pregnancy and cirrhosis. Antibody-targeted therapies depend on the accessibility and size of the N-terminal region of mucins. Nevertheless, the antibodies are not specific since they recognize both cell surface and secreted forms. Antibody targeting the portion of the cell-associated ectodomain remaining after proteolytic cleavage and release of the large ectodomain an appealing approach. MiniMUC16 can be used in this type of studies, especially because there is already a specific antibody that targets that region, 4H11. Additionally, It is well known that in order for the immune system as well as drugs to target and kill a tumor, they need to be in close proximity. MUC16 KO cells could be a good way to determine if truly MUC16 blocks the cell from drug penetration. It can be done by studying the effect of the presence vs. the absence of MUC16 in combination with different commonly used cancer drug. This study will help us better understand if cancer cells are more susceptible to drugs when MUC16 is absent. Additionally, the KO cells can help in further elucidates MUC16 function in cancer cell motility and metastasis.
In this work, I have successfully created tools to study MUC16 function. Stable cell lines in which MUC16 either has been knocked out or which constitutively expresses a truncated form of MUC16 (MiniMUC16) had been developed. Having these tools will help in the study of the role of MUC16 in cell signaling events associated with cancer progression, as well as ways in which this molecule can be used as targets in cancer therapies. Understanding how MUC16 levels affect cell behavior will help in revealing methods to control cell susceptibility to cancer drugs.
Chapter 4: Figures

Figure 4. 2. Schematic of MUC16 and Mini MUC16.

Panel A shows a schematic of MUC16 and the truncated version of MUC16 (Mini MUC16). Panel B indicates in detail the major features of the miniMUC16, including the size of its individual parts. Abbreviations: BM40, BM40 signal sequence, SEA 54, partial SEA 54 domain; SEA 55, SEA domain 55; SEA 56, SEA domain 56; TM, transmembrane domain; CT, cytoplasmic tail.
The C-terminal portion of the MUC16 gene is cloned into the mammalian vector pTri EX™ 1.1.

The diagram on the left shows the pTri EX™ 1.1 vector backbone. Key features of this plasmid include a chicken β-actin promoter, a CMV enhancer, an ampicillin resistance gene, a 6xHis N-terminal tag and a multiple cloning site. The diagram on the right shows the pTri EX™ 1.1 plasmid in which the C-terminal portion of MUC16 was inserted (Mini MUC16) behind a BM40 signal sequence. The insertion was done in the multiple cloning site in which the BM40 sequence was inserted between the Eco RV and Bgl II cloning sites and the Mini MUC16 was inserted between the Not I and Xho I cloning sites.
**Figure 4.** Mini MUC16 is expressed in HEK293 cells.

HEK293 cells were transfected with the MiniMUC16 containing plasmid, pTriEX1.1. After 48 h, protein was isolated from cells followed by western blotting with a His tag antibody. The arrow to the left indicates the migration position of the transfected mini MUC16. The bands above and below this are cross-reactive proteins evident in the non-transfected controls.
Figure 4. 5. Mini MUC16 is stably expressed in SKOv3-ip cells.

SKOv3-ip cells were transfected with the MiniMUC16 containing plasmid, pTriEX1.1. After 24-48 h cells were selected using the G418 antibiotic and single clones isolated as described in Materials and Methods. The presence of MiniMUC16 was verified by western blotting using an antibody that recognizes the C-terminal portion of MUC16 (4H11). The band is located at ~53 kDa which correlates with the expected molecular weight of MiniMUC16 (43 kDa not including glycosylation). The gel represents triplicates of a single clone.
Cells were transfected with the tetracycline-induced Cas9-FLAG plasmid by lentiviral infection, followed by selection with 500 µg/ml G418. Clones were selected via serial dilution and further stimulation with 2 µM of doxycycline to drive Cas9 expression, and screened by western blotting using an anti-FLAG antibody. The numbers represent the individual clones for each cell line. As is evident in the figure, although G418 resistant, not all clones expressed Cas 9.
Figure 4. 7. CRISPR/Cas mediated MUC16 knock out in the breast cancer MCF7 cells.

MCF-7 cells stably expressing inducible Cas9 were transfected with a lentivirus containing either a guide MUC16 RNA (gRNA 1) (clones A) or gRNA 2 (clones B) and selected as described in Materials and Methods. At least 2 serial dilutions in 96 well plates were performed to select for single clones. Clones were screened by western blotting using an antibody that recognizes the C-terminal portion of MUC16 (4H11). Three controls were used: C1, untransfected MCF-7 cells; C2, Cas9 stably expressing cells and; C3, SKOv3-ip cells stably transfected with Mini MUC16. The numbers indicate clone number and the letters A and B indicate the gRNA used, as explained in the text. Positive clones include 7A, 8B and 9B. Clones 11B and 12B showed a partial knockout. GAPDH was used as a protein loading control.
SKOv3-ip cells stably expressing inducible Cas9 were transfected with a lentivirus containing either a guide MUC16 RNA (gRNA 1) (clones A) or gRNA 2 (clones B). Clones were screened by western blotting using the MUC16 (4H11) antibody. Three controls were used: C1, SKOv3-ip cells stably transfected with Mini MUC16; C2, untransfected SKOv3-ip cells and; C3, SKOv3-ip cells stably expressing Cas9. Numbers indicate clone number and letters A and B indicate the gRNA used as explained in the text. Positive clones include 2B, 3B, 4B and 9B. Clones 8A, 1B, 10A and 12A showed a partial knockout.
Figure 4.9. Immunostaining of MUC16 knockout controls.

MCF-7 cells were immunostained with a well-used MUC16 antibody, oc125 (green), and DAPI (blue). Non-transfected MCF-7 cells in which only secondary antibody but not primary antibody were used as controls. Panel a is DAPI signal only and panel b is staining with the secondary 488-conjugated anti mouse antibody only. Panels c and d are untransfected controls while panels e and f shown Cas9-expressing cells not receiving targeting gRNA. Panels e and f were zoomed out to emphasize staining. The magnification bar represents 50 µm.
Knockout clones were immunostained with the MUC16 (oc125) antibody (green) and DAPI (blue). Non-transfected MCF-7 cells (Panels A and B) and Cas 9 stable clones (Panel C and D) were used as controls. A representative image of a knockout MUC16 clone is shown in panels E and F. The size bar represents 50 µM.
Chapter 5: Summary and Future Perspectives

5.1. Summary

In spite of the fact that the well-known ovarian tumor marker, CA125/MUC16, is widely used in the diagnosis and progression of ovarian cancer, factors regulating MUC16 expression were unknown. The current studies were designed to identify factors that regulate MUC16 expression primarily using breast and ovarian cancer as model systems.

The epithelium is a complex system that protects our body from the external environment and maintains the integrity of our organs. The family of mucin glycoproteins, comprised of 21 genes, includes MUC16, the largest transmembrane mucin. Generally, mucins protect epithelial surfaces from pathogens while promoting their lubrication. Mucins are high molecular weight glycoproteins whose function and regulation are involved in a variety of normal as well as pathogenic conditions such as cystic fibrosis, asthma and cancer. Mucin expression depends on tissue type, and normal mucin production supports protective functions. In disease states that balance can be disrupted and mucins are overproduced, changing the role of these glycoproteins to evade the immune system and treatments.
The transmembrane mucin (TM), MUC16, is overexpressed in the majority of human epithelial ovarian cancers (Bast et al., 1983; Bast & Spriggs, 2011; Kabawat et al., 1983). Furthermore, clinical development and relapse of ovarian cancer is associated with MUC16 serum levels (Bast et al., 1983; Sölétormos et al., 2016). MUC16 overexpression in ovarian cancer has been associated with a more aggressive phenotype (Thériault et al., 2011). Additionally, since MUC16 is also overexpressed in other cancers such as breast, lung and pancreas, there is evidence suggesting its usefulness as a predictive tool in combination with other markers in these cancers (Haridas et al., 2011; Wang et al., 2014; Wang et al., 2014). In the clinical setting MUC16 is considered a very useful cancer tumor biomarker (Sölétormos et al., 2016), yet very little is known about its regulation, function and biological role in these processes.

In these studies, we demonstrated that inflammation, which is associated with the release of cytokines such as TNFα and IFNγ stimulate MUC16 expression in human breast and ovarian cancers (Morgado et al., 2016). TNFα and IFNγ stimulate expression of other mucins including MUC1 (Dharmaraj et al., 2010; Lagow & Carson, 2002) and MUC4 (Chapela et al., 2015). Peroxisome proliferator activated receptor gamma (PPARγ), a ligand dependent transcription factor and a member of the fatty acid nuclear receptor superfamily, regulates adipocyte differentiation, fatty acid storage and glucose metabolism. PPARγ plays an important role in metabolic diseases such as obesity and insulin resistance, and is a target of thiazolidinediones (TZD) including antidiabetic
drugs such as rosiglitazone. Not long ago, the important role of PPARγ in immune response by inhibiting expression of inflammatory cytokines has been identified (Hammarstedt et al., 2005). Rosiglitazone has been known to reduce mucin expression such as is the case for MUC1 (Dharmaraj et al., 2013; Wang et al., 2010). Here, I found that rosiglitazone’s effects on MUC16 regulation is complex and dose dependent. Its influence on MUC16 regulation also is associated with the presence of the pro-inflammatory cytokines, TNFα and IFNγ. Additionally, tools to study the function of MUC16 were developed in this work. In this part of the project, the interest was to develop useful tools to be used in the study of MUC16 function. There are many studies describing the utility and limitations of MUC16 as a tumor biomarker (Bast et al., 2009; Bast et al., 1983; Bast & Spriggs, 2011; Bast et al., 2012; Bast et al., 1998; Buller et al., 1992; Felder et al., 2014; Gossner et al., 2006; Gubbels et al., 2010). Nonetheless, there are very few studies focused on its function (Comamala et al., 2011; Giannakouros et al., 2015; Matte et al., 2014). Herein, I established a cell line stably overexpressing the C-terminal region of MUC16 (MiniMUC16) which displays biological activity in other contexts (Thériault et al., 2011), and a MUC16 knockout cell line using CRISPR/Cas9 technology. These cell lines will be useful in the study of signaling events associated with MUC16 cytoplasmic domain in tumor progression and invasion. Additionally MUC16 can be used in targeted therapy or to answer questions regarding the consequences of completely eliminating MUC16 in a cell in terms of drug penetration and immune response.
5.1.1. The major findings of the cytokine studies are as follows:

1. Basal MUC16 mRNA levels vary considerably among different epithelial cell lines. In order to determine if the cell lines to be used in these studies express MUC16, basal mRNA levels were measured via quantitative qRT-PCR. A normal ovarian cell line (IOSE 261F), ovarian cancer cell lines (SKOv3-ip and OVCAR3), endometrial cancer (RL95 and HEC50) and breast cancer (MCF-7) cell lines were used. The normal ovarian cell IOSE 261F contains the lowest basal MUC16 mRNA levels, followed by MCF-7 and SKOv3-ip. The poorly differentiated OVCAR3 cells showed extremely high basal MUC16 mRNA levels. I concluded that MUC16 mRNA levels differ in each cell type and did not seem to be related to level of differentiation.

2. TNFα and IFNγ stimulate MUC16 expression in a dose-dependent manner. MCF-7 cells with low basal MUC16 mRNA levels were treated with increasing concentrations of either TNFα or IFNγ. Both cytokines independently stimulated MUC16, but the lowest concentrations of either cytokine that demonstrated a significant stimulation of MUC16 mRNA levels were 2.5 ng/ml of TNFα and 20 IU/ml of IFNγ. I concluded that low combined levels of cytokines are sufficient to induce a cellular response into making MUC16. This is an important observation because in a tumor environment a variety of cytokines are present and probably at various concentrations.
3. **TNFα and IFNγ stimulate MUC16 mRNA levels in multiple cell contexts.** To determine the effect of cytokines on MUC16 mRNA levels, the low concentrations determined previously were used to treat the panel of cell lines mentioned in 1. I found that in most cases combinations of cytokines stimulate MUC16 expression. In some cells the response was additive, in others, such as MCF-7, the response was highly synergistic, and in cells with high basal MUC16 mRNA levels there was no stimulation. I concluded that cytokines stimulate MUC16 gene expression and the extent of the stimulation is cell type specific.

4. **Cytokines stimulate MUC16 protein expression and shedding.** Using MCF-7 cells I determined the time course for MUC16 protein and mRNA accumulation. MUC16 mRNA and cell-associated protein levels reached their maxima within 6 days of cytokine exposure while shed levels measured in the media continued to accumulate throughout the 12 day period. Additionally, about 90% of the MUC16 protein produced was ultimately found in the media by 12 days of treatment. Day 6, the day of maximal MUC16 production, was used to assess the actions of individual cytokines on MUC16 protein expression. It was found that similar to mRNA, protein levels increased with the presence of individual cytokines, and further increased with combined cytokine treatment. I concluded that the majority of MUC16, at least in the cells tested, is shed, which justifies its use as a tumor biomarker.
5. MUC16, TNFα and IFNγ are co-expressed in malignant cancers.

To determine if relative MUC16 expression correlated with TNFα or IFNγ expression in vivo, immunostaining of a multi-tumor human tissue microarray was performed. Serial sections of a human cancer tissue array were stained simultaneously for anti-MUC16 and anti-TNFα or anti-IFNγ. To parallel the in vitro data, the array included cancers of the ovary, endometrium and breast. Samples were classified according to staining intensities. The array staining generally revealed that strong cytokine expression was associated with elevated MUC16 expression. In ovarian and breast cancer, there was a direct correlation between the staining intensity for both cytokines and MUC16. The correlation was not as clear for endometrial cancer. I concluded that this further strength the notion that an inflammatory response associated with cytokine appears to promote MUC16 expression.


Due to the strong stimulation of MUC16 expression by TNFα, we considered if the key transcription factor, NFκB, played a role in this response. In order to achieve this, NFκB/p65 was knocked down in MCF-7 and SKOv3-ip cells, the cells that showed the greatest cytokine stimulation of MUC16. In both cell types, the stimulation of MUC16 observed in response to individual or combined cytokines were significantly reduced when NFκB/p65 was knocked down. These studies demonstrated that NFκB is a key mediator of cytokine responsiveness.
7. A consensus NFκB binding site in the proximal MUC16 promoter accounted for much of the cytokine responsiveness. After learning that NFκB is an important mediator of the cytokine responsiveness, an analysis of the MUC16 promoter revealed a consensus NFκB binding site 200 bp upstream of the start of transcription, which I had determined via 5' RACE. This region of the promoter demonstrated a substantial (10-fold) increase in promoter activity in response to cytokine treatment. Mutation of the putative NFκB/p65 binding element completely destroyed cytokine responsiveness. Thus, in addition to identifying NFκB as a key regulator of MUC16 gene transcription, I also identified an important cytokine response element in the MUC16 promoter.

5.1.2. The major findings of the PPARγ studies are as follows:

1. Rosiglitazone suppresses basal and cytokine-stimulated MUC16 mRNA levels in a dose dependent manner. In order to determine the influence of rosiglitazone on basal MUC16 mRNA levels, the cells that showed the greatest response to cytokine treatment, MCF-7, were treated with concentrations of rosiglitazone ranging from 0.01 µM to 80 µM. A slight stimulation of MUC16 was observed at low concentrations, but as the rosiglitazone concentration increased, the levels of MUC16 decreased. Next, the effect of rosiglitazone on cytokine-stimulated MUC16 expression was examined and found to be biphasic. At
pharmacologically relevant concentrations, rosiglitazone in combination with cytokines further increased *MUC16* mRNA levels. At higher concentrations, rosiglitazone suppressed cytokine stimulated *MUC16* expression. Thus, rosiglitazone actions are quite different at low and high concentrations.

2. **GW9662, a PPARγ antagonist, reverses rosiglitazone suppression of cytokine-stimulated MUC16 expression.** As one approach to determine if rosiglitazone suppression was PPARγ dependent, a PPARγ antagonist, GW9662, was used to determine if it could reverse the effects of rosiglitazone. GW9662 by itself had no effect on *MUC16* mRNA levels; however, GW9662 not only enhanced cytokine stimulation, but also reversed suppression observed at high rosiglitazone concentrations. These observations were consistent with PPARγ as the mediator of rosiglitazone actions.

3. **NFκB suppression is rescued by cytokines in presence of low, but not high, rosiglitazone concentrations.** With the knowledge that rosiglitazone modulated cytokine-stimulated *MUC16* expression, I considered that rosiglitazone might produce its effects, in part, by modulating NFκB levels. Both low and high concentrations of rosiglitazone reduced NFκB/p65 protein levels by ~80%. In the presence of cytokines, low concentrations of rosiglitazone (1 µM) restored NFκB levels to >50% of the control values. Importantly, this rescue was not observed at high rosiglitazone concentrations. I concluded that the partial
restoration of NFκB observed at low rosiglitazone concentrations was sufficient to fully support cytokine-driven MUC16 expression; however, the greatly reduced NFκB levels observed at high rosiglitazone concentrations were not sufficient to support cytokine-driven MUC16 expression and could largely account for rosiglitazone suppression.

5.1.3. The major findings of my efforts to develop tools to study MUC16 function are as follows:

1. An ovarian cancer cell line was created that constitutively expresses a truncated form of MUC16 (MiniMUC16). First, a construct containing the C-terminal part of MUC16 was made using a fragment of the MUC16 gene known to be biologically active in other contexts (Thériault et al., 2011). This construct, referred to as “MiniMUC16” contains a small portion of the ectodomain adjacent to the putative proteolytic cleavage site of MUC16, the transmembrane domain and the short cytoplasmic tail. MiniMUC16 was first transiently and then stably expressed in SKOv3-ip cells, known to have low MUC16 expression. Clones were isolated and screened for the presence of MiniMUC16 using an antibody that reacts with the C-terminal portion of MUC16 (Dharma Rao et al., 2010). Having a MUC16 overexpressing cell line opens endless opportunities to study MUC16 function. Special focus can be given to the C-terminal portion of MUC16, which includes the part of the ectodomain that remains attached to the cell after cleavage, and the cytoplasmic tail. Studying their relevance in cell signaling
processes such as the activation of genes including EGFR, MAPK, JNK (Kharbanda et al., 2014; Vasseur et al., 2015) would be of interest. In addition, β-catenin degradation has recently been found to be influenced by MUC16 (Giannakouros et al., 2015).

2. Development of breast and ovarian cancer MUC16 knockout cell lines.

The CRISPR/Cas9 system for genome editing (Marraffini & Sontheimer, 2010; Sander & Joung, 2014) was used to knockout MUC16 in MCF-7 and SKOv3-ip cell lines, breast and ovarian cancer cell types, respectively. These cell lines were chosen since they express low basal levels of MUC16, which are greatly stimulated by cytokines, allowing us to study the functions of MUC16 in response to cytokines. MCF-7 and SKOv3-ip clones stably expressing Cas9 were isolated and subsequently used to knockout MUC16. A series of clones were isolated and several clones in which MUC16 expression was ablated were obtained. Having a cell line in which MUC16 is absent can be of great use in further studies of MUC16 role in various processes including tumor resistance to chemotherapeutic drugs, resistance to apoptosis, drug metabolism, and epithelial-mesenchymal transition. In breast cancer, overexpression of MUC1 is involved in drug resistance since silencing the gene reverses the effect (Jonckheere et al., 2014). Other mucins, including MUC16 may play similar roles in drug resistance and should be examined, in this regard.
5.2. Future Perspectives

CA125/MUC16 is a well-established ovarian tumor marker, but a relatively understudied mucin with regard to both regulation of its expression as well as its function. Studies of this extremely large, complex mucin have recently begun to appear; however, much remains to be discovered.

Prior to my studies, there was very little information about MUC16 regulation by cytokines (Argüeso et al., 2003; Paulsen et al., 2008), especially in ovarian and breast cancer. OVCAR-3 cells have a very high basal level of MUC16 mRNA and I found that neither cytokines nor rosiglitazone affected expression further at any concentration used. Furthermore, a knockdown of NFκB knockdown did not reduce expression of OVCAR-3 MUC16 mRNA levels. It is not clear why MUC16 levels are so extremely high in this cell line. While gene duplication accounts for overexpression of other mucins, the MUC16 gene is rarely duplicated in ovarian cancer. Understanding the factors influencing the very high levels of MUC16 expression observed in OVCAR-3 cells might provide insight into drivers of other cancer-associated processes in ovarian cancer. Therefore, I propose future experiments should be done to explore the mechanisms driving MUC16 expression in OVCAR-3 cells.

This work is the first to address the distribution of MUC16 between the cell-associated and secreted/shed fractions. My work revealed that in MCF-7 cells,
the vast majority of MUC16 produced is destined to be released/shed. It is not clear if this is generally the case, or if it differs from cell to cell. Additional work examining MUC16 release/shedding in different cell types should be performed to address this. The availability of sensitive, commercially available ELISAs to CA125 makes this a straightforward endeavor. In addition, work examining the mechanism of MUC16 release is needed. By analogy to other transmembrane mucins, MUC16 is presumably released from the cell surface via the actions of possibly cytoplasmic phosphorylation followed by proteolytic cleavage, with the influence of cell surface proteases or “sheddases”. Good tools and approaches are available to identify the enzymes responsible for MUC16 release (Blalock et al., 2008). Candidate sheddases include proteases such as MMP-7, MMP-9 and bacterial metalloprotease (ZmpC) as found in the ocular epithelium (Blalock et al., 2008; Govindarajan & Gipson, 2010; Govindarajan et al., 2012) as well as TACE/ADAM17 and MT1-MMP which are demonstrated to mediate MUC1 shedding (Thathiah et al., 2003).

Additionally, it is important to determine if shed MUC16 has functions beyond simply serving as a reliable tumor marker. When released, MUC16 binds to various surfaces of immune cells consistent with its immunosuppressive role (Tyler et al., 2012). Additionally MUC16 binding to mesothelin may provide a way for cancer cells to migrate to ectopic sites (Shimizu et al., 2012). Also, I propose that MUC16 perhaps plays a role to stabilize anchoring of the secreted mucins, and might also be a mediator in the interaction with other proteins, which would
be interesting to discover.

The peroxisome proliferator activated receptor gamma (PPARγ) is a target of intense research because ligands such as the thiazolidinediones (TZDs) associated with this receptor are potent insulin sensitizers used to treat type 2 diabetes. PPARγ is highly expressed in adipose tissue and plays a role in glucose and lipid metabolism. Additionally, reports have shown that PPARγ exhibits antitumorigenic functions (Janani & Ranjitha Kumari, 2015). Several studies have shown that PPARγ represses lung, breast, colon and prostate cancer (Dong, 2013; Hazra et al., 2008; Ogino et al., 2009; Santha et al., 2015).

The TZD rosiglitazone reduces mucin expression such as MUC1 (Dharmaraj et al., 2013; Wang et al., 2010). In this work rosiglitazone proved to play a complex dual role in the regulation of MUC16. Low doses of rosiglitazone in combination with cytokines further stimulated MUC16 expression. On the other hand, cytokine-stimulated MUC16 mRNA levels were reduced by high doses of rosiglitazone. Further research is required regarding this effect, including in vivo assays to determine localization of MUC16 in relationship to PPARγ in cancer tissues. Additionally, it would be interesting to determine if there is a correlation on MUC16 serum levels of normal vs. cancer patients that have undergone treatment with rosiglitazone. My speculation would be that diabetic-cancer patients that are long users of rosiglitazone would have low MUC16 serum levels compared to normal patients. On the other hand, diabetic-cancer patients that have recently been given rosiglitazone would have high MUC16 serum levels
compared to normal. I would propose that rosiglitazone could be a promising drug in the treatment of small tumors if it can be locally deliver to the specific tumor at high concentrations for a short period of time.

Knock out and over expressing cell lines developed during this project offer important tools to study MUC16 function. Certain transmembrane mucins are known to be multifunctional, providing both surface barrier and signaling functions, the latter through its cytoplasmic tail or through EGF-like domains found near their membrane-spanning region in the ectodomain (Gipson et al., 2014). The truncated version of MUC16 (MiniMUC16) provides a useful tool to assess potential MUC16 roles in cell signaling. Other TMs such as MUC1 play a role by complexing with and activating ERBB2. MUC16 role in a process like this is unknown and would be useful to discover. The MUC16 cytoplasmic tail contains three potential tyrosine phosphorylation sites (Hattrup & Gendler, 2008), but the role they play in signaling, if any, is not understood. Furthermore, MUC16 appears to protect cells from apoptosis induced by certain genotoxic drugs (Boivin et al., 2009). It would be valuable to expand these studies to other families of drugs.

The availability of MUC16 knockout cell lines also will be useful in studies of susceptibility to cancer drugs. MUC16 is often overexpressed in epithelial cancers. This overexpression protects cancer cells in several ways, including protection from cell killing by blocking the interaction between the tumor and NK cells; presentation of immunosuppressive ligands, i.e. SIGLEC ligands; and
inhibition of uptake of chemotherapeutic drugs. One hypothesis is that the absence of MUC16 would make cancer cells easy to recognize and eliminate by the immune system. This can be tested by possibly co-culturing MUC16 expressing and MUC16 KO cells with immune cells such as monocytes. My prediction would be that the KO cells would die in this environment while the MUC16 expressing cells would survive. Additionally, the resistance to chemotherapeutic drugs can also be tested using the MUC16 KO cells. I propose that MUC16 expressing and MUC16 KO cells can be subject to a variety of commonly used anti cancer drugs. My prediction is MUC16 expressing cells would be resistant to killing by drugs, while the MUC16 KO cells would be more easily killed by the anti cancer drugs.

Additionally, it has been recently demonstrated that knockdown of MUC16 shows a decrease of barrier function causing dye penetration, bacterial invasion, and disruption of tight junctions in a telomerase transformed human corneal-limbal epithelial (HCLE) cell line (Gipson et al., 2014). It would be interesting to test if similar effects are seeing in cancer epithelium. To test this I propose to repeat some of these experiments using the MUC16 KO cells. My hypothesis is that by completely eliminating MUC16 there would be a greater effect in the decrease of barrier function, increase in bacterial invasion and possibly a greater disruption of the tight junctions. Information from this study would be very useful in looking for ways to eliminate cancer cells.
The discovery of MUC16 regulation by proinflammatory cytokines and PPARγ coupled with the understanding of some of the mechanism involved promises to be an exciting first step in the developing of novel therapeutic approaches to increase cancer cell susceptibility to cytotoxic drugs, treat cancer or limit its progression. Information gained from this research coupled with the future directions proposed in this chapter may have a significant positive impact on human health.
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Apendix A: Preliminary data of potential role of MUC16 in Epithelial-Masenchymal transition (EMT) and cell migration

**A.1. Introduction**

Mucins and Epithelial-Mesenchymal Transition.

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells undergo changes to acquire a mesenchymal phenotype, such as enhancement in migratory potential, invasiveness, increase in apoptosis and an increase in extracellular matrix (ECM) component expression (Kalluri & Weinberg, 2009). EMT is completed when the basement membrane is degraded and mesenchymal cells migrate away from their original epithelial layer (Kalluri & Weinberg, 2009).

EMT is important during development, especially during gastrulation and neural crest migration (Shao *et al.*, 2009). EMT also plays a role in cancer: many cancer cells lose their epithelial phenotype and acquire a mesenchymal one, and invasion and metastasis is initiated (Shao *et al.*, 2009). During EMT transcription factors such as Snail1 and Slug repress E-cadherin and enhance vimentin and N-cadherin expression (Roy *et al.*, 2011). As a result, β-catenin, an E-cadherin-binding protein, is translocated to the nucleus where it potentiates EMT signaling events (Hugo *et al.*, 2007). Down regulation of E-cadherin in cancer cells is a critical step in cancer progression and invasion (Shao *et al.*, 2009). MUC1 and MUC4 induce EMT in some cancers, including pancreatic and ovarian cancers (Ponnusamy *et al.*, 2010; Roy *et al.*, 2011). In ovarian cancer, MUC4 overexpression induces a mesenchymal phenotype and enhances expression of
EMT markers (Ponnusamy et al., 2010). MUC4 activates the FAK pathway stimulating the expression of N-cadherin (Ponnusamy et al., 2010). To date, few studies have been done to assess the role of MUC16 in EMT and cancer metastasis (Comamala et al., 2011), and this possibility can be explored using the MiniMUC16 and MUC16 KO constructs made in this research.

**A.2. Rationale**

Other transmembrane mucins, such as MUC1 and MUC4, are associated with metastasis of pancreatic and ovarian cancers by inducing EMT (Boivin et al., 2009; Ponnusamy et al., 2010; Roy et al., 2011). In contrast, one report indicates that loss of MUC16 induces EMT (Comamala et al., 2011); however, these studies did not clearly discriminate between EMT vs. enhanced cell motility. It also is not clear if this is a common MUC16 activity or the mechanism by which MUC16 controls this biological response. The goal would be to determine if MUC16 modulates EMT, and elucidate a possible mechanism.

**A.3. Proposed Hypothesis**

I propose that similar to MUC1 and MUC4, MUC16 will induce EMT. Expression of the specific transcription factors associated with EMT induction such as Twist, Snail, and Slug, will be altered by the manipulation of MUC16 levels. Specifically, expression of these transcription factors will decrease if MUC16 is knocked out and will increase if MUC16 is overexpressed. While this hypothesis is in apparent conflict with previous studies (Comamala et al., 2011), preliminary results support this hypothesis.
A.4. Preliminary Results

Mucins are normally restricted to the apical surface, but lose this polarized characteristic in tumor cells. This results in disruption of cell-cell and cell-matrix adhesion and the potential for mucin cytoplasmic tails to interact with additional cytoplasmic signaling proteins that are normally sequestered. I found that MUC16 mRNA and protein levels are increased by cytokine treatments in multiple cellular contexts. I also observed that after treating MCF-7 cells with low doses of combined cytokines (TNFα and IFNγ) for six days, the cells changed their morphology from a cobblestone appearance typical of epithelia to an elongated mesenchymal phenotype (Fig. 1D). There is little or no change in cell morphology with TNFα or IFNγ alone (Fig. 1B-C). Also, more cells undergo some type of cell death with the combined treatments, evident in these images by the reduced cell density. This morphological change is fully reversible upon cytokine withdrawal (Fig. 1H).

After observing this response I tested for EMT marker expression along with that of MUC16 by immunostaining MCF-7 cells after a six-day treatment with the combined cytokines. I previously noted that there is no detectable MUC16 expression in the vehicle control (0.1% [w/v] BSA in PBS), little MUC16 with individual cytokines, but a large increase in MUC16 with combined cytokine treatment. Again, I observed no MUC16 or the intermediate filament protein vimentin, an important marker of EMT, in the vehicle control, but high MUC16 and vimentin with combined cytokine treatment (Fig. 2). There is also a decrease
of E-cadherin, loss of E-cadherin is considered to be a fundamental event in EMT, and an increase of N-cadherin, high levels are normally expressed in mesenchymal cells, in cytokine treated cells. qRT PCR analysis also shows a decrease of \textit{E-cadherin} and an increase in both \textit{N-cadherin} and \textit{Twist}. Collectively, these results are consistent with EMT when MCF-7 cells are treated with combined cytokines accompanied by elevated MUC16 expression.

Also, to determine if MUC16 over-expression was due to low confluency of cells caused by cytokine treatment, MCF-7 cells at low confluency and without cytokine treatment were immunostained with MUC16, vimentin and N-cadherin (EMT markers). Cells stained positive for all MUC16 and EMT markers (Fig. 3).

This result indicates that MUC16 and well established EMT markers are present when cells are at low density, characterized by loss of cell polarity and cell-cell adhesion. Loss of cell polarity and cell-cell adhesion is a normal process for wound healing and embryogenesis, but also happens during cancer metastasis.

This result still does not directly test the role MUC16 plays in EMT and at this point I cannot state that MUC16 induction drives EMT. Nonetheless, these studies establish a system to test MUC16’s role in this process. MiniMUC16 was effectively cloned and expressed in mammalian cells, as well as a MUC16 KO system to test this and other functions of MUC16.
Figure A. 1. Morphologic change after treatment with cytokines is reversible.

MCF-7 cells were treated for six days with either a vehicle control (0.1% [w/v] BSA in PBS), TNFα (2.5 ng/ml), IFNγ (20 IU), or TNFα + IFNγ. A morphological change from a cobblestone appearance typical of epithelia to an elongated phenotype typical of mesenchyme was observed with combined (Panel D), but not individual cytokine treatments (panels A and B). This change is fully reversible following cytokine removal for six days (day 12), compare panels D and H. Magnification bar 50 µm.
Figure A. 2. MUC16 and vimentin levels increase after treatment with cytokines.

MCF-7 cells were treated for six days with either vehicle control (panels A-D) (0.1% [w/v] BSA in PBS) or TNFα + IFNγ (panels E to H). Cells were immunostained with OC125 antibody, MUC16 (green); anti-vimentin (red); and DAPI (blue nuclear stain). There is no detectable MUC16 or vimentin in the vehicle control (panels B and C), but there are high levels of MUC16 (panel F) and vimentin (panel G) in the cytokine treated cells. Magnification bar 50 µm.
Figure A. 3. MCF-7 cells at low confluency and no cytokine treatment stain positive for MUC16, vimentin and E-cadherin.

MCF-7 cells were plated at low confluency, followed by immunolabeling with MUC16 (OC125) and N-cadherin (green) antibodies; anti-vimentin (red); and DAPI (blue nuclear stain). Single cells stained positive for MUC16 (panel F) and both EMT markers, N-cadherin (panel B) and vimentin (panels C and G). Magnification bar 50 μm.
Apendix B: Co-author manuscripts

Constantinou PE, **Morgado M** and Carson DD. (2015). “Transmembrane Mucin Expression and Function in Embryo Implantation and Placentation”. In: Geisert DR and Bazer WF, eds. Regulation of Implantation and Establishment of Pregnancy in Mammals: Tribute to 45 Year Anniversary of Roger V Short's "Maternal Recognition of Pregnancy". (Cham: Springer International Publishing), pp. 51-68.

**Contribution:** My contribution includes all the required experiments to generate figure 4.2, such as treatment of cells, isolation of RNA and performance of qRTTPCR, as well editing of the manuscript.


**Contribution:** I performed most of the RNA isolation and all the real time qRTTPCR assays required to generate figures 3D, 4A, 4B, 4E, 4F. I also wrote a section of materials and methods and edited the manuscript.


**Contribution:** I isolated all the RNA and performed qRTTPCR in order to generate data for figures: 1C and 2B, as well as writing of a section of materials and methods and editing of the manuscript.


**Contribution:** All required experiments to generate figures: 1, 2, 3 and 6 i.e. cleaning of RNA, making of cDNA, performing of qRTTPCR and immunostaining, in addition to making the figure and writing of the materials and methods section and editing of manuscript.

**Contribution:** Performed RNA isolation and qRT-PCR to generate data for figures: 1A and 2A
Transmembrane mucin expression and function in embryo implantation and placentation

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Abstract

Transmembrane mucins (TMs) are extremely large, complex glycoproteins that line the apical surfaces of simple epithelia including those of the female reproductive tract. TMs provide a physical barrier consistent with their role as part of the innate immune system. This barrier function must be overcome in the context of embryo implantation to permit blastocyst attachment. Three major TMs have been identified in uterine epithelia of multiple species: MUC1, MUC4, and MUC16. MUC1 has been found in all species studied to date, whereas expression of MUC4 and MUC16 have been less well studied and may be species specific. The strategies for removing mucins to permit embryo attachment also vary in a species-specific way and include both hormonal suppression of TM gene expression and membrane clearance via cell surface proteases. Studies emerging from the cancer literature indicate that TMs can modulate a surprisingly wide variety of signal transduction processes. Furthermore, various cell surface proteins have been identified that bind either the oligosaccharide or protein motifs of TMs suggesting that these molecules may support cell attachment in some contexts, including trophoblast interactions with cells of the immune system. The intimate association of TMs at sites of embryo–maternal interaction and the varied functions these complex molecules can play make them key players in embryo implantation and placentation processes.

Keywords

MUC1
MUC16
Embryo implantation
Placentation

4.1. Introduction

Embryo attachment to the uterine wall is a critical and highly regulated process in mammals. Embryo attachment is only allowed during a defined period of the estrous cycle, often called the “window of receptivity.” While it was appreciated for many years that such a receptive window existed in many species, the molecular basis underlying receptivity remained unclear. Many investigations identified cell
surface and extracellular matrix proteins capable of supporting embryo attachment in vitro with provocative correlations to their expression patterns in vivo, including certain integrins and heparan sulfate proteoglycan binding proteins [reviewed in Carson et al. (2000)]. Nonetheless, it also had long been recognized that the apical surface of uterine epithelia is covered with a thick glycocalyx that changes in complex ways during the cycle and in preparation for embryo attachment (Schlafke and Enders 1975). Yet, the identity of the molecules carrying these carbohydrates remained elusive. MUC1 was the first major molecule identified that was capable of carrying the large amount of oligosaccharides that could account for the major changes in carbohydrate composition at the uterine luminal surface. Subsequently, important functions of MUC1 and related glycoproteins were revealed as well as many aspects of the processes that control their expression. This review focuses on the role transmembrane mucins play in human implantation and placentation but discusses key, relevant findings in other species.

4.2. Implantation and Placentation

Following fertilization, the mammalian embryo, covered in a glycoprotein coat called the zona pellucida, passes through the oviduct to arrive at the apical surface of the uterine epithelium. Shortly after arrival in the uterus, the zona pellucida is lost and the embryo subsequently becomes attachment competent. Attachment competency includes expression of various adhesion-promoting proteins on the external surface of the trophectoderm surrounding the embryo. In parallel and coordinated by steroid hormone influences provided by corpora lutea, the uterus differentiates to a state in which it can support embryo attachment and implantation, the “receptive” state. This state is transient lasting for a period of hours to days in species like mice and humans, respectively (Cha and Dey 2014). If the embryo fails to attach during this period, the uterus converts to a refractory state where it will no longer support embryo attachment. When attachment occurs, the embryo may begin further development including formation of the placenta. In noninvasive species like pigs and sheep, placentation occurs essentially in the uterine lumen, while in invasive species like rodents and humans, the trophoblast penetrates the uterine epithelium and placentation occurs within the decidual tissue of the endometrium (Chavatte-Palmer and Guillomot 2007; Cha et al. 2012). In humans, this process is particularly invasive with the trophoblast reaching the maternal arteries and displacing endothelial cells to establish contact with the maternal blood supply (Fisher 2004). Failure to reach the maternal arteries results in preeclampsia, a disease occurring in 3–5% of all human pregnancies with potentially life-threatening consequences to both the fetus and mother (Fisher 2004). The placenta is surrounded by a dense accumulation of maternal NK cells as well as regulatory T cells (Jennings et al. 1986; Alijotas-Reig et al. 2014). Thus, suppression of the potential maternal immune response to the allogeneic placenta is crucial.

4.3. Transmembrane Mucins (TMs): Expression and Functions

4.3.1. Structure

TMs are a subset of the family of high molecular weight, heavily glycosylated mucin glycoproteins. The hallmarks of mucins are their large size; the occurrence of multiple tandem repeat peptide motifs largely composed of serine, threonine, and proline; and the dense substitution of the tandem repeat motifs with O-linked oligosaccharides. Mucins may be either secreted or retained at the plasma membrane via membrane-spanning domains. The major TMs are MUC1, MUC4, and MUC16 (Fig. 4.1). Each is a type I membrane glycoprotein in which the vast majority of the structure is composed of the tandem repeat-containing ectodomain. The extremely large size and highly extended structures of the ectodomains account for many of the functions attributed to TMs. All three TMs undergo proteolytic cleavage and remain associated at the cell surface as heterodimers. Each has a single membrane-spanning sequence and a short cytoplasmic domain. Although still very much larger than conventional cell surface receptors,
MUC1 is the smallest of these three TMs. MUC1’s ectodomain is relatively simple being composed primarily of a series of tandem repeat motifs of 20–21 amino acids. Allelic polymorphism generates species of different sizes with the differences being in the number of tandem repeats (Gendler et al. 1990). Other forms of MUC1 lacking the cytoplasmic tail or ectodomain are generated by alternative splicing and are expressed by uterine tissues and uterine epithelial cell lines but usually constitute a minor amount of the total (Julian and Carson 2002; Hey et al. 2003). MUC1 has the largest cytoplasmic domain of the three TMs being approximately 70 amino acids in length. Consistent with this, MUC1’s cytoplasmic tail has been implicated in more intracellular signaling processes than the other TMs (see below).

**Fig. 4.1**

Structural representations of TMs. (a) MUC1, MUC4, MUC16 (not drawn to scale). Abbreviations: **VNTR** variable number of tandem repeats, **SEA** sea urchin sperm protein–enterokinase–agrin, **TMD** transmembrane domain, **CT** cytoplasmic tail, **CysD** Cys-rich domain, **NIDO** nidogen homology sequence, **AMOP** adhesion-associated domain, **vWD** von Willebrand factor D domain, **EGF** epidermal growth factor-like regions. (b) Size comparison of MUC1, extending 200–500 nm from the cell surface, and epidermal growth factor receptor (EGFR), 50 nm from the cell surface (drawn to scale).

MUC4’s ectodomain is substantially larger and more complex than that of MUC1. This structure is dominated by a large tandem repeat domain as well as a nidogen-like (NIDO) domain, an adhesion-associated in MUC4 and other protein (AMOP) domain, von Willebrand factor D (vWD) domain, and epidermal growth factor-like (EGF) domains. MUC4 splice variants also have been reported, although not in uterine tissues (Choudhury et al. 2000; Moniaux et al. 2000). MUC16 is the largest of the three
and is the largest cell surface glycoprotein known. The ectodomain has a massive (12,000 amino acid) O-glycosylated domain, a tandem repeat domain consisting of more than 60 repeats of 156 amino acids, and Sperm–Enterokinase–Agrin (SEA) domains (O’Brien et al. 2001; Yin and Lloyd 2001; Gipson et al. 2014). Indirect evidence for the occurrence of MUC16 splice variants has been suggested [discussed in Haridas et al. (2014)]; however, mRNA splice variants have not been rigorously identified. Fragments of MUC16 released from the cell surface are the CA 125 antigens commonly used as a serum marker for certain cancers, notably ovarian and endometrial (Patsner and Yim 2013; Baser et al. 2014; Felder et al. 2014), as well as endometriosis (Spaczynski and Duleba 2003).

4.3.2. Functions

Not surprisingly, most of the functions attributed to TMs are accounted for by their massive ectodomains. Their heavy glycosylation makes them highly hygroscopic providing lubrication for mucosal surfaces. Their large size, high concentration at apical cell surfaces, extended structures, and generally antiadhesive nature make them excellent barrier molecules and a key part of the innate immune system (Hilkens et al. 1992; Voynow and Rubin 2009). In the context of embryo implantation, this barrier function is problematic since TMs are quite effective in inhibiting cell adhesion (Wesseling et al. 1995; Komatsu et al. 1997), including embryo attachment (DeSouza et al. 1999). Muc1, Muc4, and Muc16 (rodent nomenclature) are expressed by rodent uterine epithelia under most conditions (DeSouza et al. 1998; Idris and Carraway 1999, 2000; Wang et al. 2008). In rodents and certain other species, Muc1 is lost during the receptive phase providing access for embryo attachment to the uterine epithelium (DeSouza et al. 1998). Nonetheless, neither Muc1 nor Muc16 null mice display an implantation phenotype in vivo although Muc1 null uterine epithelia are constitutively “receptive” in vitro (DeSouza et al. 1999). In their roles as inhibitors of embryo attachment, loss of TMs would not be expected to inhibit implantation but rather would be expected to promote this process. Nonetheless, embryo transfer experiments have shown that uteri of Muc1 null mice are not chronically receptive but rather display the same window of receptivity as their wild-type counterparts (DeSouza et al. 1999). These observations led to the conclusion that factors in addition to loss of Muc1 expression are required to permit embryo attachment in vivo. These factors could include increased expression of various growth factors or expression of adhesion-promoting receptors; however, other TMs, e.g., Muc4 and Muc16, also may need to be lost during this process.

4.4. Control of TM Expression

4.4.1. Cytokines

TMs are almost exclusively expressed by simple epithelia throughout the body with low-level expression in some hematopoietic cells and activated T cells (Agrawal et al. 1998; Kruger et al. 2000). In most tissues, a substantial basal level of expression occurs; however, proinflammatory cytokines substantially elevate TM expression in many contexts, including epithelial cell lines derived from reproductive tract tissues (Lagow and Carson 2002; Thathiah et al. 2004; O’Connor et al. 2005; Dharmaraj et al. 2010; Kasimanickam et al. 2014; Chapela and Carson in press; Morgado M, Constantinou P and Carson DD, unpublished studies). These actions are mediated by the transcription factors Nuclear Factor κ B (NFκB) and Signal Transducers and Activators of Transcription (STATs) (Lagow and Carson 2002; Dharmaraj et al. 2010). Conversely, a class of transcriptional corepressors of cytokine actions, protein inhibitors of activated STATs (PIASs), suppresses MUC1 gene expression in response to cytokines as well as progesterone (see below) in several cellular contexts and may serve as a feedback control on this system (Brayman et al. 2007). Cytokine responsiveness is likely to represent a system to elevate the barrier functions of epithelia when challenged by infection or irritants. Proinflammatory cytokine levels also change in dynamic ways in uterine tissues of various species during early stages of pregnancy and may
4.4.2. Steroid Hormones

Uterine TM expression is also strongly influenced by steroid hormones with estrogen elevating expression and progesterone antagonizing estrogen action, in this regard, in rodents (Surveyor et al. 1995; DeSouza et al. 1998; McNeer et al. 1998). While estrogen stimulates TM expression in vivo, estrogen receptor does not appear to directly regulate TM gene expression (Zhou et al. 1998; Brayman et al. 2006). Rather, it appears that estrogen effects on MUC1 expression are mediated via factors produced by other uterine cell types, e.g., stroma, in response to estrogen. In contrast, progesterone receptor directly binds to the MUC1 promoter region and regulates MUC1 gene expression in an isoform-specific fashion. Progesterone receptor B is a stimulator of MUC1 gene expression, whereas progesterone receptor A antagonizes the activity of the B isoform (Brayman et al. 2006). This isoform-specific response largely accounts for the apparently conflicting observations that progesterone inhibits TM expression in some species, e.g., rodents, while apparently elevating TM expression in other species, i.e., rabbits and humans (Hey et al. 1994; Hoffman et al. 1998).

4.4.3. PPARs and Trophoblastic Expression of MUC1

Studies in mice revealed two interesting features of Muc1 expression: (1) that another transcriptional coregulator, Peroxisome Proliferator-Activated Receptor-γ (PPARγ), stimulates Muc1 expression and (2) Muc1 is expressed by placental trophoblast (Shalom-Barak et al. 2004). PPARγ is activated by various natural ligands including certain polyunsaturated fatty acids and prostaglandin J2, as well as the synthetic thiazolidinediones, including rosiglitazone and pioglitazone. Investigation of PPARγ actions in human cell lines revealed an opposite response to that observed in mice, namely, inhibition with regard to both progesterone- (Wang et al. 2010) and EGF-simulated (Dharmaraj et al. 2013) MUC1 expression. The human MUC1 gene has a 21 bp insertion in the PPARγ-responsive region which appears to account for the differences in responsiveness between species. In addition to other actions, PPARγ and its agonists have anti-inflammatory actions (Kapadia et al. 2008). In this regard, PPARγ activators can inhibit cytokine-stimulated expression of all three TMs (Fig. 4.2). Therefore, it appears that TM expression can be regulated coordinately offering opportunities for broad therapeutic control. PPARγ activators have therapeutic value in placental dysfunction, including mitigation of symptoms associated with preeclampsia (McCarthy et al. 2011; Kadam et al. 2015). Whether this has relevance to reduction of trophoblast MUC1 expression is unclear.

Fig. 4.2

Coordinate regulation of TMs by cytokines and rosiglitazone. MCF7 cells were incubated for 48 h with vehicle control (V), rosiglitazone (100 μM; R), TNFα (25 ng/ml) plus IFNγ (200 IU) (T + I), or TNFα plus IFNγ plus rosiglitazone (T + I + R). RNA was extracted from triplicate independent samples in each case for qRT-PCR analyses of (a) MUC1, (b) MUC4, and (c) MUC16 mRNA relative to that of β-actin. *** p < 0.001 V vs. T + I and T + I + R, R vs. T + I and T + I + R.
Detection of Muc1 in murine placenta via cDNA microarray analyses was surprising since this tissue does not contain simple epithelia that would be the normal sites of Muc1 expression (Shalom-Barak et al. 2004). Nonetheless, several studies of human trophoblast and trophoblastic cell lines have confirmed that human trophoblasts not only express MUC1, but also that MUC1 is elevated in trophoblast of preeclamptic placentae and suppresses trophoblast invasion in vitro (Shyu et al. 2008, 2011). It is possible that the suppression of invasion is due to MUC1 physically inhibiting the interactions with the extracellular matrix through which trophoblast must invade; however, it also is possible that MUC1 interactions with specific cell surface receptors mediate aspects of this inhibitory response (see below).

4.4.4. Sheddases

In rabbits, MUC1 is removed locally at sites of embryo attachment in vivo (Hoffman et al. 1998). Human blastocysts also trigger clearing of MUC1 at their attachment sites on layers of human uterine epithelia cell lines in vitro (Meseguer et al. 2001). Thus, it appears that TM-removing activities exist that are either produced by blastocysts or activated in uterine epithelia by blastocysts. Conditioned media from human blastocysts do not stimulate MUC1 release by uterine epithelial cell lines (Thathiah A and Carson DD, unpublished studies) implying that these activities reside in the uterine epithelia themselves. Fragments of TMs lacking their cytoplasmic tails are released into serum in certain disease states (Spaczynski and Duleba 2003; Patsner and Yim 2013; Baser et al. 2014; Felder et al. 2014), as well as into female reproductive tract secretions (de Bruijn et al. 1986; Martinez et al. 1994; Andersch-Bjorkman et al. 2007). As mentioned above, mRNA slice variants can generate secreted forms of TMs lacking transmembrane and cytoplasmic domains; however, most of these fragments appear to be the result of ectodomain release or shedding from the cell surface via the action of cell surface proteases or “sheddases.” Two sheddases that release MUC1 ectodomains have been identified in uterine epithelia, namely, TACE/ADAM17 and MT1-MMP (Ando and Kusano 1992; Thathiah et al. 2003). Sheddase activity can be controlled in various ways including through conversion from a proenzyme, transport to the cell surface from intracellular locales, and protein kinase C activation (Edwards et al. 2008). In addition, TNFα stimulates MUC1 shedding in uterine epithelial cell lines (Thathiah et al. 2004). In addition to the controls over TM gene expression mentioned above, TM shedding offers another point of intervention to control the levels of cell surface TMs either to enhance protective functions of the endometrium or to reduce TM expression to promote embryo attachment.

4.5. Transmembrane Mucin Binding Proteins

Multiple proteins bind to TMs. This includes proteins that bind to oligosaccharide as well as protein motifs (Table 4.1). In the case of the former, these proteins often also can bind to other proteins or lipids carrying the same carbohydrate structures. Protein–oligosaccharide binding usually displays substantially
lower affinity constants than protein–protein interactions, e.g., growth factor–growth factor receptor; however, TMs provide multiple binding sites for these proteins which can greatly increase the avidity of these interactions. In some of cases described below, binding may serve to aggregate or cross-link glycoproteins at the cell surface (galectins). In other cases, the interactions may support cell adhesion (mesothelin, selectins) or lead to intracellular signal transduction (Siglecs, β-catenin).

Table 4.1
Transmembrane mucin binding proteins

<table>
<thead>
<tr>
<th>Mucin binding proteins</th>
<th>Binding specificity</th>
<th>Biological activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galectins</td>
<td>N-acetyllactosamine (Galβ1-4GlcNAc)</td>
<td>Increase barrier function of TM</td>
<td>Hirabayashi et al. (2002), Jeschke et al. (2013), Panjwani (2014)</td>
</tr>
<tr>
<td></td>
<td>Lacto-N-biose (Galβ1-3GlcNAc)</td>
<td>Promote anti-inflammatory effects [Gal-1, Gal-3]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reepithelialization associated with wound repair [Gal-7]</td>
<td></td>
</tr>
<tr>
<td>Selectins</td>
<td>NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc (sLeα)</td>
<td>Potential to tether blastocysts to uterine epithelia</td>
<td>Hemmerich et al. (1995), Leppanen et al. (2000), Nimrichter et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>NeuAcα2-3Galβ1-4GlcNAcn1-3 [Galβ1-4 (Fucα1-3)Glc-NAcβ1-3]2Galβ1-14GlcβCer [E-Selectin]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Core-2-based O-linked sLeα on Thr residue on P-selectin glycoprotein ligand-1 near TyrSO(3) [P-selectin]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-6-SO4 [L-selectin]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neu5Acaα2-3Galβ1-4Glc [Siglecs-7,9]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neu5Acaα2-6Galβ1-4Glc [Siglecs-7,9]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NeuAcaα2-8NeuAcaα2-3Gal [Siglec 7]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesothelin</td>
<td>N-linked glycosylation on MUC16</td>
<td>Supports MUC16-dependent cell adhesion and mesothelial colonization</td>
<td>Gubbels et al. (2006)</td>
</tr>
</tbody>
</table>

4.5.1. Galectins
Galectins are a family of small (14–39 kDa), soluble, β-galactoside binding proteins expressed that occur both intracellularly and on the cell surface (Jeschke et al. 2013). Galectins do not have canonical signal sequences or transmembrane domains and reach the cell surface by nonclassical pathways. While the presence of β-galactose is required for galectin recognition, other aspects of oligosaccharide structure, e.g., sialylation, presence of lactosamine repeats, create differences in recognition by different galectins changing binding affinities up to 100-fold (Stowell et al. 2008; Zhuo et al. 2008). Multiple galectins
have been detected in human endometrium and placental tissues with a wider variety found in placental tissues (Jeschke et al. 2013).

Several galectins can bind to TMs, including MUC1, MUC4, and MUC16 (Bancalari et al. 1989; Seelenmeyer et al. 2003; Yu et al. 2007; Argueso et al. 2009; Senapati et al. 2011). Functional consequences of these interactions are suggested to include enhancement of the barrier function of TMs to activation of signal transduction cascades. A series of studies have implicated important functions for galectins in embryo implantation and placentation, notably galectin-1 [Jeschke et al. (2013) for review]. In addition, elevated galectin-7 expression is associated with uterine repair after menstruation (Evans et al. 2014), miscarriage (Menkhorst et al. 2014b), and preeclampsia (Menkhorst et al. 2014a). Many of these observations relate to the proposed role for galectin-7 in reepithelialization associated with wound repair (Panjwani 2014).

4.5.2. Selectins

Selectins are a family of three cell adhesion-promoting transmembrane proteins (E-, L-, and P-) that bind complex oligosaccharide structures (Rosen and Bertozzi 1994). Originally discovered as mediators of lymphocyte homing responses, it since has been recognized that selectins are expressed in many other contexts, including by human blastocysts (Genbacev et al. 2003). In the endometrium, MUC1 carries selectin ligands throughout the cycle (Carson et al. 2006). Thus, it is possible that MUC1 and other selectin ligand-bearing TMs initially tether blastocysts to the epithelial cell surface at early stages of the implantation process. MUC16 also binds E- and L-selectin in pancreatic cancer cells (Chen et al. 2012); however, it is not clear if MUC4 or MUC16 carry selectin ligands in the uterus or placenta.

4.5.3. Siglecs

Siglecs (sialic acid-binding immunoglobulin-like lectins) are a family of 14 mammalian cell surface proteins primarily expressed in cells of the immune system (Pillai et al. 2012; Macauley et al. 2014). Structurally, they are type I transmembrane glycoproteins with an oligosaccharide-binding ectodomain, a single transmembrane domain, and an intracellular domain that mediates signal transduction in most Siglecs. The intracellular domain usually contains either an ITIM (immunoreceptor tyrosine-based inhibitory motifs) or ITAM (immunoreceptor tyrosine-based activation motif) that functions to modulate protein phosphorylation-dependent signaling events. In the case of ITIM-containing Siglecs, ligand binding triggers phosphorylation of the ITIM creating a binding site for and activation of SHP phosphatases. Different Siglecs preferentially bind oligosaccharides with distinct structures, although all contain sialic acids. In humans, a unique ITIM-containing Siglec, Siglec-6, is expressed by trophoblast in a pattern complementary to the pattern of expression observed for its ligands (Brinkman-Van der Linden et al. 2007). Thus, Siglecs expressed by immune cells abundantly expressed in decidua as well as trophoblast Siglec-6 are likely to engage their complementary ligands in uteroplacental tissue. The identity of the molecules carrying Siglec ligands in uteroplacental tissue is unknown, but TMs may be among them. All three TMs can carry Siglec ligands in some contexts (Brinkman-Van der Linden and Varki 2000; Swanson et al. 2007; Belisle et al. 2010; Tanida et al. 2013; Kiwamoto et al. 2015). Trophoblast TM binding to inhibitory Siglecs could potentially restrict trophoblast invasion as well as attenuate immune responses to the allogeneic placenta (Redzovic et al. 2013). Consistent with this notion, Muc1 null mice display greatly enhanced placental resorption (Croy et al. 1997). In addition, MUC16 binds to NK cells of pregnant women, presumably mediated by Siglec-9 on NK cells (Belisle et al. 2010; Tyler et al. 2012). With regard to trophoblast invasion, overexpression of MUC1 and Siglec-6 are associated with preeclampsia (Shyu et al. 2011); however, trophoblast TMs have not been formally demonstrated to carry Siglec-6 ligands.
4.5.4. Mesothelin

Mesothelin is a protein that recognizes the MUC16 ectodomain (Kaneko et al. 2009). Binding again is oligosaccharide dependent but requires N-linked, rather than O-linked, mucin-type oligosaccharides (Gubbels et al. 2006). Since mesothelin is retained at the cell surface via a glycosylphosphatidylinositol anchor binding does not result in any known signal transduction events. Rather, it is believed that mesothelin, primarily expressed by peritoneal mesothelial cells and certain cancers, supports MUC16-dependent cell adhesion and tissue colonization (Pastan and Hassan 2014). Mesothelin does not appear to be expressed by normal endometrium or placenta and is unlikely to play a role in embryo implantation or placentation.

4.5.5. TM Binding Signaling Proteins

The largest body of evidence for TM involvement in signal transduction processes relates to MUC1 [reviewed in Carson (2008), Kufe (2013)]. The MUC1 cytoplasmic domain is the largest of the TMs and appears to be able to support multiple interactions including phosphorylation, interaction with apoptosis modulators, and direct binding to β-catenin and several transcription factors. In almost all cases, these interactions have been demonstrated in cancer cells. Cancer cells not only lose apical restriction of TMs but also can accumulate substantial levels in intracellular locales which may account for many of these interactions (Hollingsworth and Swanson 2004; Bafna et al. 2010). None of these interactions have been demonstrated in normal endometrial or placental tissues or cells. Both MUC1 and MUC4 interact with members of the ERBB family and are suggested to promote responsiveness to EGF family members (Schroeder et al. 2001; Kozloski et al. 2010). ERBB family members are expressed by uterine epithelia, although interactions with MUC1 or MUC4 have not been demonstrated (Berchuck et al. 1989; McBean et al. 1997). It is possible that the apical restriction of TMs sequesters them from ERBBs under most circumstances. Both MUC1 and EGFR are expressed by trophoblast and could conceivably interact at these surfaces; however, MUC1:EGFR interactions have not been demonstrated in trophoblast either. TMs also are implicated in promoting epithelial-to-mesenchymal transition (Comamala et al. 2011; Ponnusamy et al. 2013), although the relevance of this response to implantation is not clear.

4.6. Summary and Future Directions

TMs have emerged as major components of the apical surface of uterine epithelia. In addition to their general roles as hydrating agents and barriers to infection, TMs represent barriers to embryo attachment in the endometrium. In this regard, TMs contribute importantly to the creation of the non-receptive uterine state. Proinflammatory cytokines generally elevate TM expression in many contexts which is suggested to be part of an innate protective response. Different species have developed different strategies to remove TMs to permit blastocyst attachment. These strategies include hormonal downregulation of TM expression as well as local removal of TMs at blastocyst attachment sites through the action of cell surface proteases called sheddases. MUC1 also is expressed by trophoblast and elevated expression is associated with disease states, including preeclampsia. The discovery that TMs can carry ligands for and bind to various oligosaccharide-binding proteins found in the endometrium and placenta opens the possibility that TMs may play additional, adhesion-promoting roles in reproduction. These potential roles include promotion of certain cell–cell interactions as well as attenuation of the maternal immune response. Future work should focus on formally testing these exciting new roles for TMs as well as determining if control of TM expression and/or interference with TM–TM binding protein interactions can serve as points of therapeutic intervention in disease states and to enhance fertility.
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A 3D in vitro model of patient-derived prostate cancer xenograft for controlled interrogation of in vivo tumor-stromal interactions

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A R T I C L E I N F O

Article history:
Received 25 June 2015
Received in revised form 20 October 2015
Accepted 26 October 2015
Available online 9 November 2015

Keywords:
Patient-derived xenograft
Prostate cancer
Three-dimensional
Co-culture
Osteoblasts
Hydrogel
Model

A B S T R A C T

Patient-derived xenograft (PDX) models better represent human cancer than traditional cell lines. However, the complex in vivo environment makes it challenging to employ PDX models to investigate tumor-stromal interactions, such as those that mediate prostate cancer (PCa) bone metastasis. Thus, we engineered a defined three-dimensional (3D) hydrogel system capable of supporting the co-culture of PCa PDX cells and osteoblastic cells to recapitulate the PCa-osteoblast unit within the bone metastatic microenvironment in vitro. Our 3D model not only maintained cell viability but also preserved the typical osteogenic phenotype of PCa PDX cells. Additionally, co-culture cellularity was maintained over that of either cell type cultured alone, suggesting that the PCa-osteoblast cross-talk supports PCa progression in bone, as is hypothesized to occur in patients with prostatic bone metastasis. Strikingly, osteoblastic cells co-cultured with PCa PDX tumoroids organized around the tumoroids, closely mimicking the architecture of PCa metastases in bone. Finally, tumor-stromal signaling mediated by the fibroblast growth factor axis tightly paralleled that in the in vivo counterpart. Together, these findings indicate that this 3D PCa PDX model recapitulates important pathological properties of PCa bone metastasis, and validate the use of this model for controlled and systematic interrogation of complex in vivo tumor-stromal interactions.

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1. Introduction

Given the heavy investment in understanding cancer over the past several decades, the high drug attrition rates in oncologic drug development are disappointing [1]. The poor translation of seemingly promising preclinical findings to clinical success is partly due to the heavy reliance on cancer cell lines as tumor models for preclinical studies [2]. Recently, patient-derived xenograft (PDX) models have emerged as better surrogates of human cancer. PDX models, developed through serial propagation of patient tumor tissue in murine hosts, closely resemble the parental tumor in histology, gene expression profiles, preserved heterogeneity, and drug response [3–7]. However, use of PDX models in vitro mechanistic studies is hampered by their poor adaptation to traditional two-dimensional tissue culture and the potential for two-dimensional culture to induce undesired adaptations [6,8]. Improved methods for in vitro PDX culture have been developed that rely on three-dimensional (3D) cell culture strategies, such as spheroid culture, or encapsulation of tumor cells within naturally derived gels such as Matrigel or collagen [9–12]. However, while spheroid cultures enable primary tumor tissue culture, the lack of a surrounding matrix prohibits control over spatial positioning of multiple cell types. Similarly, the often ill-defined and variable composition of naturally derived gels makes consistent re-creation of the engineered in vitro tumor microenvironment challenging.

In current paradigms, cancer is viewed as a complex manifestation of aberrant interactions between tumor cells and the
surrounding stromal compartment. Accordingly, increasing emphasis is being placed on understanding the role of the host cells in the tumor microenvironment in cancer progression and metastasis and in design of therapies [13]. In prostate cancer (PCa), stromal-epithelial signaling dictates tumor cell behavior and metastasis [14–16]. Metastasizing PCa cells predominantly target bone, and the characteristic osteoblastic lesions that form reflect the functional dependency of PCa on the bone microenvironment for disease progression. Therefore, targeting the bone stroma and disrupting the PCa-stromal cross-talk can be an effective therapeutic strategy for treating or preventing bone metastases [17].

In our recent integrated clinical and preclinical studies using the MDA PCa 118b PDX model, we demonstrated that blockade of fibroblast growth factor receptor (FGFR)-dependent PCa-stromal interactions in the bone microenvironment could be an effective therapeutic strategy for a subset of PCa patients [17]. In light of these findings, we wished to further investigate the specific stromal targets and affected pathways to guide biomarker development and patient selection. However, in vitro platforms to support such mechanistic studies of PCa PDX-stromal interactions had to be created.

In the interdisciplinary study reported here, we addressed this need by developing and validating an in vitro PCa PDX model that accurately reflects PCa-stromal interactions in bone for controlled mechanistic studies. We demonstrate for the first time, to our knowledge, an engineered tumor microenvironment consisting of clinically relevant PDX tumor cells and osteoblastic cells, co-encapsulated within a well-defined 3D hyaluronan (HA) hydrogel. Osteoblasts play a key role in promoting PCa progression in bone [18]. Ubiquitous in the extracellular matrix of connective tissues, HA is a building block for the fabrication of hydrogel matrices designed to mimic HA-rich tissues such as the bone marrow, where bone metastatic PCa cells reside [19,20]. Indeed, we previously reported the first demonstration of using a 3D scaffold-based approach to culture PDX cells in vitro in unmodified HA hydrogels [21]. Prior to development of our 3D hydrogel approach, it was difficult to culture PCa PDX cells in vitro for experimentation given their poor viability on tissue culture plastic. In this work, to enable the culture of osteoblastic cells with PCa PDX cells, HA was specifically modified with integrin-binding peptides and cross-linked with matrix metalloproteinase (MMP)-degradable peptides. Plain, unmodified HA hydrogels are not capable of supporting the attachment and spreading of encapsulated osteoblastic cells. Incorporation of these peptides is an approach that was previously developed to enable cell-mediated remodeling of synthetic 3D hydrogels for tissue regeneration applications [22–24]. This PDX co-culture model maintains key phenotypic tumor markers; recapitulates the in vivo structural arrangement of osteoblasts with respect to tumor cells; mimics many elements of the previously observed FGFR-mediated tumor-stromal cross-talk for PDX tumor cells grown in bone, including cross-talk involving FGFR1 and fibroblast growth factor 9 (FGF9) [17,25]; and offers a robust platform for in vitro drug evaluation. This model addresses the critical unmet need in PCa research and drug discovery for platforms that support the controlled interrogation of complex in vivo tumor-stromal interactions as discrete units in vitro.

2. Materials and methods

2.1. Study design

The primary objective of this study was to develop an auxiliary model to the in vivo PCa PDX model that supports controlled mechanistic studies of tumor-stromal interactions in vitro. We cultured PCa PDX cells and osteoblastic cells within a well-defined 3D hydrogel matrix and characterized the tumor architecture, viability, phenotype, as well as the biochemical interaction between the two cell populations. These findings were then validated against the corresponding PCa PDX in vivo model [17]. We also evaluated whether [1] standard molecular biology tools (such as gene knockdown) can be employed to manipulate cell–cell interactions for mechanistic investigations and [2] the effect of dovitinib in vivo [17] can be at least in part, reflected in this in vitro PCa PDX model.

2.2. Synthesis and characterization of thiolated HA

Sulhydryl groups were incorporated in HA (620 kDa, Genzyme, Cambridge, MA) by reacting HA with a disulfide-containing dihydrazide compound, followed by reduction with dithiothreitol, using a previously reported method [20,26]. The degree of modification (35–43%) in thiolated HA was measured [20,26] by 1H nuclear magnetic resonance, and the lyophilized product was stored at −20 °C under argon prior to use.

2.3. Synthesis of acrylated peptides

Cell-adhesive peptide GRGDS (GenScript USA Inc., Piscataway, NJ) with C-terminal amiation was reacted with acrylate-PEG-SVA (3400 g/mol, Laysan Bio Inc., Arab, AL) at a molar ratio of 1:2.1 in HEPBS buffer (20 mM HEPBS [Santa Cruz Biotechnology Inc., Dallas, TX], 100 mM NaCl, 2 mM CaCl₂, and 2 mM MgCl₂) adjusted to pH 8.0 using 0.1 N NaOH [27]. The reaction was allowed to run overnight at 4 °C on a shaker protected from light and then dialyzed for 2 days against ultrapure water using a 3500 Da MWCO dialysis membrane ( Spectrum Laboratories Inc., Rancho Dominguez, CA) before lyophilization for an additional 2 days. MMP-degradable peptide, KGGGPQG(IWGQGK (GenScript USA Inc.) with N-terminal acetylation, herein referred to as PQ (1 marks the MMP-cleavable site), was reacted with acrylate-PEG-SVA at a molar ratio of 1:2.5 using the same protocol. Conjugation of acrylate-PEG to the peptides was verified by high-performance liquid chromatography (Vydak C18 218TP54 column, Varian Prostar solvent delivery module and UV–vis detector) and MALDI-TOF (Bruker AutoFlex II). The lyophilized solids were stored at −20 °C prior to use.

2.4. MDA PCa 118b PCa PDX in vivo propagation and processing

The MDA PCa 118b PDX [25] was routinely maintained as subcutaneous tumors in C57BL/6ScSn (Charles River). Propagation of tumors in mice was conducted under approval by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. Following harvest, the tumors were processed for hydrogel encapsulation as previously described [21]. To obtain sections of MDA PCa 118b PDX in bone for histologic characterization, tumor-bearing femurs were prepared and processed as previously described [25].

2.5. Cell culture

MC 3T3-E1 (ATCC, Manassas, VA) cells were maintained in alpha-MEM (Life Technologies, Grand Island, NY) containing 10% (v/v) FBS and in the presence of 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C and 5% CO₂.

2.6. Preparation of cell-laden hydrogel constructs

To prepare the MDA PCa 118b-only constructs, following our previously established protocol [21], tumor cell aggregates that formed in suspension were mixed with thiolated HA dissolved in PBS to 10 mg/mL. Each MDA PCa 118b-only construct was prepared...
with a theoretical encapsulation density of 300,000 cells [21]. Next, acrylate-PEG-GRGDS (73.7 mg/mL) and acrylate-PEG-PQ-PEG-acrylate (37.0 mg/mL) dissolved in PBS were added to the mixture at a volume ratio of 4:1:1 (thiolated SH: acrylate-PEG-GRGDS: acrylate-PEG-PQ-PEG-acrylate). Final concentrations of GRGDS and PQ in each resulting 42 μL hydrogel construct were 3 mM and 0.73 mM, respectively. The tumor cell—hydrogel mixture was then pipetted into custom-made PDMS molds as previously described [21] and allowed to cross-link for 1 h before immersion in cell culture medium and incubation overnight. Cell-laden hydrogel constructs were transferred into 12-well plates the next day. Medium was exchanged every 2 days. MC 3T3-E1 mono-culture constructs were prepared in a similar manner with encapsulation density of 100,000 cells per construct. To prepare the co-culture constructs, a theoretical encapsulation density of 300,000 tumor cells and actual density of 100,000 MC 3T3-E1 cells per hydrogel construct was used. A 1:1 (v/v) mixture of DMEM/F-12 and alpha-MEM medium supplemented with 2% (v/v) FBS was used for all studies.

2.7. Immunocytochemistry

As described previously [21], cell-hydrogel constructs in 12-well plates were washed with PBS, fixed with 4% (v/v) para-formaldehyde for 10 min at room temperature, and processed for immunostaining as described in Supplementary materials.

2.8. Cell viability and growth

Cell viability was evaluated using the LIVE/DEAD viability/cytotoxicity kit (Life Technologies) per the manufacturer’s instructions. At the designated time point, cell-hydrogel constructs were incubated in 400 μL of 2 μM calcein-AM and 4 μM ethidium homodimer in PBS for 40 min at 37 °C on a shaker before confocal imaging. To assess growth, the Quant-iT PicoGreen dsDNA quantification assay (Life Technologies) was used per the manufacturer’s instructions as described in Supplementary materials.

2.9. Quantitative PCR

At the designated time point, cell-hydrogel constructs were collected into microcentrifuge tubes. Then 500 μL of Trizol (Life Technologies) was added, and an 18-gauge needle and 1 mL syringe were used to mechanically dissociate the hydrogel constructs. Samples were then stored at −80 °C and processed for RNA isolation, cDNA synthesis, and real-time PCR per the manufacturer’s instructions (Supplementary materials).

2.10. Dovitinib studies

MDA PCa 118b-only, MC 3T3-E1-only, and co-culture constructs were prepared and cultured for 4 days. Dovitinib (Novartis, Pharma AG) was dissolved in fresh medium (0, 500, or 1000 nM) and added to the constructs. Cells were incubated in dovitinib-containing medium for an additional 4 days with one medium change. At the designated time point, constructs were collected and stored at −80 °C before the dsDNA quantification assay was run or processed for RNA isolation. DNA content was quantified in a repeat experiment under identical conditions. Within each study, measured DNA contents from each group (n = 4) were normalized against the average DNA content of the MC 3T3-E1-only untreated group, and values from the two studies were combined (n = 8) and averaged.

2.11. Fgfr1-knockdown MC 3T3-E1 studies

Fgfr1-knockdown MC 3T3-E1 cells and the corresponding controls were prepared using the ON-TARGETplus Mouse Fgfr1 (14182) siRNA—SMARTpool and ON-TARGETplus nontargeting Pool (GE Dharmacon, Lafayette, CO) in the presence of HiPerFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturers’ protocols. MC 3T3-E1 cells were incubated with 30 nM of siRNA and the HiPerFect transfection reagent for 3 days before hydrogel encapsulation either alone or with the MDA PCa 118b cells. At each designated time point, constructs were collected and stored at −80 °C before the dsDNA quantification assay was run or processed for RNA isolation.

2.12. Statistical methods

Data are expressed as mean ± standard deviation. Differences between paired data were tested using Student’s t test. For the dovitinib study (measured DNA content), one-way ANOVA and then Student’s t test were used. For the Fgfr1-knockdown MC 3T3-E1 study (measured DNA content), ANOVA was performed at each time point to investigate whether cellularity differed between Fgfr1-knockdown MC 3T3-E1 cells and scrambled controls and between Fgfr1-knockdown MC 3T3-E1 cells and scrambled controls co-cultured with MDA PCa 118b cells. Differences were considered significant at p < 0.05.

3. Results

3.1. PDX-derived PCa cells and osteoblastic cells co-encapsulated in 3D hydrogel mimic in vivo tumor organization

With the goal of modeling the PCa cell—osteoblast interaction in vitro, we co-encapsulated MDA PCa 118b PDX-derived PCa tumoroids with MC 3T3-E1 osteoblastic cells within an engineered 3D hydrogel containing integrin-binding and MMP-degradable peptide sequences (Fig. 1). MDA PCa 118b is a PCa xenograft model that we previously established from bone metastases in a patient with castration-resistant PCa [25]. The use of mouse-derived MC 3T3-E1 cells enabled the facile separation of gene expression trends from tumor and stroma in our co-culture system. In the absence of either the integrin-binding or MMP-degradable peptide, MC 3T3-E1 cells do not spread when encapsulated in hydrogels. Given the poor adherence of PDX-derived PCa cells to tissue culture plastic and their resulting tendency to form aggregates in suspension, we pre-generated tumoroids highly enriched for human PCa cells and depleted of mouse fibroblasts [21] prior to hydrogel encapsulation. Within this hydrogel matrix, the MC 3T3-E1 cells began to spread 1 day after encapsulation and remained spread through 6 days in culture, while the PCa tumoroids remained largely as compact multicellular aggregates (Fig. 2A). When MC 3T3-E1 cells were co-cultured with MDA PCa 118b tumoroids, the MC 3T3-E1 cells also began to spread 1 day after encapsulation, and these cells extended their osteoblastic processes towards and around the tumoroids over time, with this effect most evident at day 6 in culture (Fig. 2A). To better visualize this interaction, representative hydrogel constructs were immunostained for epithelial cell adhesion molecule (EpCAM) and vimentin to distinguish the epithelial tumor cells from the mesenchymal osteoblastic cells (Fig. 2B and C). As shown in Fig. 2C, while the two cell populations were largely separate at day 1 in co-culture, progressive physical association of the MC 3T3-E1 cells and MDA PCa 118b tumoroids was observed over time, culminating in envelopment of the tumoroids by osteoblastic processes at day 6 (Fig. 2C). Quantification of the association of vimentin+ cells with PCa...
tumoroids, in both 118b monoculture and co-culture, confirmed this rapid physical association (Fig S1). Comparison of the resulting structural organization in 3D co-culture (Fig. 2D) with the structural organization of MDA PCa 118b PDXs grown intrafemorally (Fig. 2E) indicated that our 3D co-culture model closely recapitulated the architectural arrangement of the two cell types in vivo, i.e., a tumor mass surrounded by osteoblasts on the periphery within a mineralized matrix.

3.2. PDX-derived PCa cells co-cultured with osteoblastic cells in 3D hydrogel retain proliferative capacity

Given that MDA PCa 118b cells historically exhibit poor viability in culture [25], we next determined if our 3D hydrogel system was capable of supporting the viability of encapsulated cells over time. Both in mono-culture and in co-culture, the MDA PCa 118b and MC 3T3-E1 cells remained viable for at least 6 days in culture (Fig. 3A). To determine whether our 3D PCa co-culture model maintains tumor proliferative potential and exhibits growth characteristics necessary for drug testing, especially testing of drugs that target actively dividing cells, we evaluated the growth profile of the cultures by measuring the tumoroid size distribution over time, as well as by measuring DNA content (as an indirect indicator of cell number). Whether in mono- or co-culture, we observed an increase in tumoroid size over time (Fig. S2). Notably, the DNA content of the MDA PCa 118b-only cultures, the DNA content of the MC 3T3-E1-only cultures, and the sum of these two values were consistently lower than the DNA content of the actual co-cultures (Fig. 3B), indicating a greater-than-additive effect of the PCa cell–osteoblastic cell interaction. Further, to confirm that the tumor cells remained proliferative in our hydrogel system, we immunostained for Ki-67. Expression of Ki-67 was detected in the MDA PCa 118b cells at all time points (Fig. 3C).

3.3. PDX-derived PCa cells co-cultured with osteoblastic cells in 3D hydrogel retain the characteristics of the tumor of origin

A unique feature of MDA PCa 118b cells is their ability to trigger robust osteoblastic reactions when implanted either orthotopically in bone or ectopically as subcutaneous tumors [25]. We next investigated if the PDX cells retained their osteogenic capacity when grown in our 3D hydrogels. Co-culture of MDA PCa 118b cells with MC 3T3-E1 osteoblastic cells increased transcript levels of osteoblast-enriched markers osteocalcin, bone sialoprotein, and alkaline phosphatase (ALP) in the MC 3T3-E1 cells (Fig. 3D), indicating that the MDA PCa 118b cells retained their native bone-forming phenotype. Further, we determined whether the hydrogel-encapsulated MDA PCa 118b cells expressed FGFR1 and FGF9, both of which are highly expressed by these cells in vivo (Fig. 3E and F). Immunostaining of the hydrogel constructs for FGFR1 and FGF9 revealed that the MDA PCa 118b cells expressed both phenotypic markers in our 3D hydrogel system (Fig. 3E and F).

3.4. PDX-derived PCa cells cultured in 3D hydrogel induce stromal Fgfr1 expression as observed in vivo

To discriminate gene expression in our human tumor-mouse osteoblast co-culture model, we used human- and mouse-specific PCR primers to probe for several signaling components in the FGF axis: FGFR1–4, FGF2, FGF9, and FGFR substrate 2a. MDA PCa 118b tumor cells in co-culture had gene expression similar to that of MDA PCa 118b cells in mono-culture (Fig. S3). In contrast, MC 3T3-E1 cells co-cultured with MDA PCa 118b cells had significantly increased transcript levels of Fgfr1 (p = 0.001) and Fgfr1–Illc isoform (p = 0.006) (Fig. 4A), a finding strikingly similar to the changes seen in vivo [17]. Additionally, in MC 3T3-E1 cells co-cultured with MDA PCa 118b cells, we observed a slight increase in Fgf2 (p = 0.057) and decrease in Fgfr4 (p = 0.098) (Fig. 4A). Expression levels of other FGF signaling components in the MC 3T3-E1 cells are shown in
Together, these results indicated that our 3D PCa PDX co-culture model closely recapitulates the FGFR-mediated cross-talk between PCa cells and osteoblasts in vivo.

3.5. Cross-talk between PDX-derived PCa cells and osteoblastic cells is at least partially mediated by FGFR1

To better understand the complex network of tumor-stromal interactions in vivo, we investigated the role of osteoblast FGFR1 in promoting tumor growth by knocking down this receptor in the MC 3T3-E1 cells (Fig. 4B). We observed that at day 6, the cellularity of co-cultures of MDA PCa 118b cells and Fgfr1-knockdown MC 3T3-E1 cells was significantly lower (p = 0.034) than the cellularity of co-cultures of MDA PCa 118b cells and MC 3T3-E1 cells transfected with control siRNA but still greater than the sum of the cellularity values for the mono-cultures (Fig. 4C). This greater-than-additive effect was observed despite suppressed transcript levels of Fgfr1 at day 6 (Fig. 4B). This observed decrease in cellularity of the co-cultures of MDA PCa 118b cells and Fgfr1-knockdown MC 3T3-E1 cells as compared to the controls corroborates with our previous in vivo study, where FGFR1 was found to be a significant mediator of the PCa cell-bone cell interaction [17].

3.6. FGFR inhibitor dovitinib decreases the cellularity of co-cultures of PDX-derived PCa cells and osteoblastic cells

Given that our previous study suggested that dovitinib, an FGFR inhibitor, mediated an anti-tumor effect in the in vivo MDA PCa
118b PDX model partly by blocking the PCa cell--bone cell interaction [17], we next sought to evaluate the effect of dovitinib in our 3D co-culture model. We found that while dovitinib at 1000 nM did not reduce the cellularity of MDA PCa 118b-only and MC 3T3-E1-only mono-cultures as compared to the untreated controls, dovitinib did significantly reduce the cellularity of the co-cultures by 26%, compared to the untreated controls (p = 0.014) (Fig. 4D). We also investigated the biochemical changes in the dovitinib-treated cells by probing for FGFR1 and Fgfr1 transcript levels using species-specific primers. No reduction in either mouse or human transcripts was observed with increasing dovitinib concentrations (Fig. 4E). This contrasts with our previous in vivo findings that FGFR1 and Fgfr1 transcript levels were reduced in both the tumor and bone stroma of tumor-bearing bones in dovitinib-treated animals [17]. Given that FGFR blockade with dovitinib was associated with an improvement in bone quality in our previous in vivo study [17], we probed for transcript levels of a well-established marker of osteoblastic activity, ALP, in dovitinib-treated MC 3T3-E1 cells. We found that ALP levels increased with increasing dovitinib concentrations (Fig. 4F). Taken together, these findings suggest that our co-culture model recapitulates two key responses to dovitinib seen in vivo, i.e., reduction in the size of the tumor microenvironment and osteogenesis.

4. Discussion

Increasing recognition of the dependence of cancer cells on their stromal environment has shifted the focus of researchers toward co-targeting tumor and stroma [14]. For PCa, a highly microenvironment-driven cancer, few preclinical models reflect the predominantly bone-forming or osteogenic phenotype of the disease [28]. Using the MDA PCa 118b PDX model [25], we previously found that use of dovitinib to interfere with the FGFR-mediated stromal-epithelial interaction in bone is a promising co-targeting strategy [17]. In this follow-up study, we asked if we could develop an in vitro PCa PDX model that recapitulates the molecular mechanisms governing the PCa cell--stromal cell interaction and allows the investigator to efficiently control and manipulate the cancer cell microenvironment. Leveraging our ability to generate tumor cell-enriched PDX-derived PCa tumoroids in vitro, we co-encapsulated PCa tumoroids derived from MDA PCa 118b PDXs with MC 3T3-E1 osteoblastic cells within a 3D hydrogel. This approach yielded a striking in vivo-like re-creation of the spatial organization of tumor cells with osteoblasts in bone, maintained cell viability and proliferative capacity, and remarkably recapitulated the FGFR-mediated PCa cell--stromal cell cross-talk observed in vivo.

Alternative culture systems such as spheroid culture or basement membrane extracts have been reported as feasible systems for primary tumor cell culture ex vivo [9,11,29] but inherently provide the investigator with little control over the in vitro cancer cell micro-environment. With matrix design considerations such as biological activity and tunable properties (structural, mechanical, and composition), we previously showed that HA matrices are highly supportive of PDX culture in vitro, able to maintain long-term cell viability with retention of phenotype [21]. However, HA-only hydrogels poorly support mesenchymal cell culture. To co-culture osteoblastic cells with PCa PDX cells, we specifically tailored HA hydrogels to include covalently bound integrin-binding and MMP-degradable peptidic modules, the combination of which enables precise manipulation of cell--cell and cell--matrix interactions and permitted our novel PCa cell-osteoblastic cell co-culture.

Compared to bone metastases from other cancers in which osteoblasts are few or absent, PCa bone metastases are characterized by a significant presence of osteoblasts adjacent to PCa cells as revealed by histopathological analysis [18]. Indeed, when MDA PCa 118b PDXs are implanted intrafemorally, robust osteoblastic reactions mimicking characteristics of the human tumor of origin are observed, with osteoblasts present in the bone matrix near the injected tumor cells [25]. Similarly, ectopic bone formation is observed around subcutaneously grown MDA PCa 118b PDX tumors [25]. These observations indicate that close proximity of PCa cells to osteoblasts is a unique characteristic of PCa, and the strong osteoinductive capacity of the MDA PCa 118b PDXs underscores the well-established dependency of PCa on osteoblasts in PCa osteoblastic metastasis [18]. In our model, MC 3T3-E1 cells extended cellular processes towards co-encapsulated PDX tumoroids and often enveloped the tumoroids completely, mimicking the spatial organization of MDA PCa 118b PDXs with osteoblasts in vivo. Our 3D model will thus enable future detailed examination of PCa cell--osteoblastic cell contact mechanisms and their impact on tumor progression.
Osteogenesis driven by MDA PCa 118b PDXs in vivo has been attributed to secreted paracrine factors, such as FGF9 and bone morphogenetic protein (BMP)-4, that induce osteoblast proliferation and differentiation [25,28,30]. The observed increase in transcript levels of osteoblast markers (osteocalcin, bone sialoprotein, and ALP) in the co-cultured MC 3T3-E1 cells suggests that MDA PCa 118b cells in our model retained their inherent ability to induce bone formation. Additionally, the greater-than-additive effect of co-culture on cellularity and maintenance of proliferative capacity in the MDA PCa 118b cells is likely reflective of the co-stimulatory PCa-osteoblast relationship, which involves several growth factors, including FGFs, BMP-2, and insulin-like growth factor [17,18,25,30,32], further supporting the notion that the PCa-osteoblast cross-talk is recapitulated in our in vitro model. This is an important feature of our model as PDXs typically cannot be propagated outside the murine host.

Among the targeted therapies in preclinical and clinical development for recalcitrant advanced PCa, FGF signaling blockade has emerged as a rational therapeutic strategy [33,34]. Examples include demonstrated osteoblast proliferation in response to FGF9 produced by MDA PCa 118b cells and overexpression of FGFR1 in PCa, especially in MDA PCa 118b cells [17,25]. In this study, we established that hydrogel-encapsulated MDA PCa 118b cells maintained their expression of FGFR1 and FGF9 when cultured in vitro, indicating that our hydrogel system preserves salient features of the FGF axis in these tumor cells necessary for accurate in vitro modeling of FGF signaling. Furthermore, we showed that our co-culture model strikingly recreated the intercellular cross-talk and stromal changes that occur when MDA PCa 118b cells are grown in bone. Using an orthotopic mouse model of MDA PCa 118b, we previously found that levels of stromal Fgfr1 and Fgf2 were increased and the level of Fgfr4 was decreased in tumor-bearing femurs compared to the contralateral sham-injected femurs [17]. While the exact molecular mechanisms governing the induction of Fgfr1 in osteoblasts and the implications of such Fgfr1 induction are unknown, these changes observed in vivo and in our co-culture model fit into the current paradigm describing the PCa-osteoblast relationship, in which FGFR1 and FGF2 are key mediators of the interaction and fuel both PCa and osteoblast growth in a co-stimulatory relationship [33].

**Fig. 4.** Manipulation of FGFR-mediated biochemical cross-talk between PCa and osteoblastic cells in co-culture. (A) Transcripts encoding FGF signaling components in MC 3T3-E1 cells, relative to GAPDH. N = 4. Differences in levels of Fgfr1, Fgfr1-IIIc, Fgf4, and Fgf2 transcripts at day 6 (D6) were observed between MC 3T3-E1 cells in mono-cultures (OB) and MC 3T3-E1 cells co-cultured (CO) with MDA PCa 118b cells. *p < 0.05. (B) Transcripts encoding Fgfr1 in the MC 3T3-E1 cells (relative to GAPDH) under various conditions. N = 3. *p < 0.05. Fgfr1-knockdown MC 3T3-E1 cells (OB-KD) and scrambled controls (OB-CONT) were co-cultured with MDA PCa 118b cells (CO-KD and CO-CONT, respectively). (C) DNA contents of the cultures in (B) and the other cultures indicated in the figure were measured over time. N = 4. Average DNA content of CO-KD cultures was lower than that of the CO-CONT cultures at D6. Average DNA content of CO-KD cultures was higher than the sum of the DNA content of mono-cultures (PCa + OB-KD), similar to what was observed with the CO-CONT cultures (compared to summed PCa + OB-CONT values), despite suppressed levels of Fgfr1 at D6, as shown in (B). (D) Normalized DNA content of dovitinib-treated mono-cultures (PCa and OB) and co-cultures (CO). *p < 0.05. Results shown are a combination of two identically performed studies. N = 8. (E) Transcripts encoding human FGFR1 and mouse Fgfr1 (relative to GAPDH) in co-cultures in the presence of increasing dovitinib concentration. N = 3. *p < 0.05. Transcript levels of FGFR1 increased with increasing dovitinib concentrations.
To confirm that our 3D PCa PDX in vitro model permits rapid and controlled examination of specific tumor-stromal interactions within the complex in vivo tumor microenvironment, we performed a proof-of-concept experiment in which we knockeddown Fgf1 in the MC 3T3-E1 cells to investigate the contribution of osteoblast Fgf1 to tumor growth. The suppressed cellularity in co-cultures with Fgf1-knockdown MC 3T3-E1 cells indicates that Fgf1 plays a vital role in the PCa-osteoblast interaction, as suggested in our previous study [17]. Fgf1 manipulation did not entirely abrogate the increased cellularity in co-culture, likely because of subtotal knockdown in the MC 3T3-E1 cells and the certain presence of multiple other redundant cooperative pathways that moderate the PCa-stromal interaction [18].

In our previous study, dovitinib, a receptor tyrosine kinase inhibitor of FGF and vascular endothelial growth factor receptor, exhibited remarkable clinical efficacy in a subset of patients with castration-resistant PCa and bone metastases [17]. The antitumor effect was achieved indirectly by co-targeting the stromal compartment in bone, highlighting the therapeutic potential of disrupting the neoplastic epithelial–stromal interaction in PCa bone metastases. In contrast to the decrease in co-culture cellularity, the lack of response to dovitinib treatment in the PCa-only and osteoblastic cell-only monocultures suggests that our model reflects the tumor-stromal targeting ability of dovitinib in vivo [17]. Furthermore, the increase in ALP transcript levels observed in our co-cultures suggests that FGFR signaling blockade promotes osteoblastic differentiation, which may account for the improvement of bone quality observed in vivo [17].

Given that dovitinib-induced changes in Fgf1 transcript levels were absent in our 3D co-culture, we propose that other cells in the tumor microenvironment contributed to the suppressive effect of dovitinib on Fgf1 transcript levels previously seen in vivo. Notably, the observed reduction in stromal Fgf1 in vivo was based on a collective measure of Fgf1 levels in all stromal cells in bone given that entire mouse femurs were analyzed [17]. Several cell types within bone express Fgfr1 [36]; indeed, we previously demonstrated that the antitumor effect of dovitinib could be ascribed partially to the antiangiogenic activity of the drug, given that endothelial cells also express Fgfr1 [17]. Current work in our laboratories seeks to increase the cellular complexity of our 3D hydrogel co-culture models to include elements of the vasculature and innate immune system. Although the current model is limited by the use of mouse-derived osteoblastic cells to model the PCa-osteoblast interaction, studies are under way to develop a human-only system. Our modular system will facilitate mechanistic studies that systematically dissect the complex effect of dovitinib in vivo by investigating the role of different stromal cell types on overall drug efficacy.

In conclusion, using a bottom-up approach with the capacity for engineered complexity, we established an in vitro auxiliary to an established murine-host PCa PDX model. Our 3D modular hydrogel system enables rapid, controlled mechanistic studies aimed at deconvoluting complex in vivo tumor–stroma interactions. This study demonstrates remarkable consistency in tumor architecture, phenotype, and tumor-stromal signaling between the patient’s tumor, the corresponding PDX model, and our in vitro PDX model. Future studies may benefit from this translational approach of employing high fidelity PDX cells in controlled 3D in vitro systems to support rapid interrogation of complex in vivo and clinical findings.

Conflict of interest

All authors confirm that there are no conflicting interests.

Acknowledgments

We thank Dr. Xiinqiao Jia from the University of Delaware for sharing her expertise in HA hydrogels. This research was supported in part by National Institutes of Health grants P01 CA098912, R01 CA180279, and Prostate SPORE grant 5P50 CA140388; the Cancer Prevention & Research Institute of Texas grant RP11055; and the David H. Koch Center for Applied Research in Genitourinary Cancers at The University of Texas MD Anderson Cancer Center. E.L.S.F. acknowledges funding support from the National University of Singapore-Overseas Graduate Scholarship.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.10.059.

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p62/SQSTM1 is required for cell survival of apoptosis-resistant bone metastatic prostate cancer cell lines

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Abstract

Background—Bone marrow stromal cell (BMSC) paracrine factor(s) can induce apoptosis in bone metastatic prostate cancer (PCa) cell lines. However, the PCa cells that escape BMSC-induced apoptosis can upregulate cytoprotective autophagy.

Methods—C4-2, C4-2B, MDA PCa 2a, MDA PCa 2b, VCaP, PC3, or DU145 PCa cell lines were grown in BMSC conditioned medium and analyzed for mRNA and/or protein accumulation of p62 (also known as Sequestome-1/SQSTM1), Microtubule-associated Protein 1 Light Chain 3B (LC3B), or Lysosomal-associated Membrane Protein 1 (LAMP1) using quantitative polymerase chain reaction (QPCR), western blot, or immunofluorescence. Small interfering RNA (siRNA) was used to determine if p62 is necessary PCa cell survival.

Results—BMSC paracrine signaling upregulated p62 mRNA and protein in a subset of the PCa cell lines. The PCa cell lines that were insensitive to BMSC-induced apoptosis and autophagy induction had elevated basal p62 mRNA and protein. In the BMSC-insensitive PCa cell lines, siRNA knockdown of p62 was cytotoxic and immunostaining showed peri-nuclear clustering of autolysosomes. However, in the BMSC-sensitive PCa cell lines, p62 siRNA knockdown was not appreciably cytotoxic and did not affect autolysosome subcellular localization.

Conclusions—A pattern emerges wherein the BMSC-sensitive PCa cell lines are known to be osteoblastic and express the androgen receptor, while the BMSC-insensitive PCa cell lines are characteristically osteolytic and do not express the androgen receptor. Furthermore, BMSC-insensitive PCa may have evolved a dependency on p62 for cell survival that could be exploited to target and kill these apoptosis-resistant PCa cells in the bone.

Keywords
p62/SQSTM1; autophagy; prostate cancer; bone paracrine factors; bone marrow

Introduction

To maintain homeostasis, cells can utilize autophagy to clear, degrade, and recycle cytoplasmic biomolecules (1,2). During autophagy, cytoplasmic contents are enveloped into
a double membrane vesicle called the autophagosome. The autophagosome fuses with the lysosome, forming the autolysosome, wherein hydrolases degrade the vesicle cargo. The degraded products are transported back into the cytoplasm for reuse by the cell (1,2). Microtubule-associated Protein 1 Light Chain 3 (LC3) is an autophagosome membrane protein that commonly serves as an autophagy induction and flux marker (3). When autophagy is induced, free cytoplasmic LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form membrane-bound LC3 (LC3-II) (4). LC3-II is located on the outer and inner autophagosome double membrane (5). During autophagic flux, outer LC3-II is proteolytically cleaved from the membrane to replenish free LC3 and inner membrane LC3-II is degraded in the autolysosome (3,6,7).

The selective engulfment of cytoplasmic contents into autophagosomes requires auxiliary proteins, such as p62 (also known as Sequestome-1/SQSTM1) (2,8), a multifunctional, multi-domain scaffolding protein that serves as a hub for several signaling pathways, including autophagy-mediated protein degradation, Nuclear Factor Kappa-B (NF-κB) activation, and Nuclear Factor Erythroid 2-Related Factor 2 (NRF2) activation (8,9). In autophagy, p62 sequesters K63-linked polyubiquitinated proteins into the autophagosome through its ubiquitin-association (UBA) and LC3-interacting (LIR) domains (8,10-12). As a consequence, p62 also is degraded in the autolysosome (10,11). In transgenic mice or human specimens, p62 overaccumulation is associated with degenerative diseases, including neurodegeneration (13), liver cirrhosis (14-16), and cardiac myopathy (17). p62 overaccumulation can also promote tumor cell survival. Although the mechanisms of p62 function in the different pathologies remain unclear, possible mechanisms of p62-mediated tumorigenesis involve genetic instability, NF-κB-mediated inflammatory response, or NRF2-mediated antioxidant signaling. For example, p62 overexpression in autophagy-deficient cells leads to genetic instability and increases tumorigenesis in mouse allografts (18). In addition, in K-ras driven lung (19) and pancreatic (20) tumors, p62 is elevated and contributes to constitutive NF-κB activation by binding to and activating TNF receptor-associated factor 6 (TRAF6). Furthermore, in liver cancer, overaccumulated p62 binds to Kelch-Like ECH-Associated Protein (KEAP1), disrupting KEAP1-NRF2 interaction and allowing NRF2 transactivation of antioxidant and detoxifying genes (21). Notably, knockdown of p62 in mice reduces tumor growth (19,22). Indeed, p62 was found to be elevated in prostate (23), breast (24-26), pancreatic (20), lung (19,27), and liver (21) cancers.

Over 80% of PCa mortality is attributed to bone metastasis (28) and, thus, it is crucial to understand the molecular mechanisms that enable PCa survival in the bone. Through this study, we uncovered the effect of bone marrow stromal cell (BMSC) paracrine factors on p62 mRNA expression and protein accumulation in bone metastatic PCa cells. Furthermore, we discovered that subtypes of PCa cell lines show differential autophagy induction, p62 accumulation, and p62-mediated cell survival in response to BMSC paracrine signaling. We conclude that paracrine factors in the bone microenvironment contribute to PCa cell survival and adaptation in the bone through a mechanism involving p62 regulation and propose that p62 may be a valuable biomarker and rational target for apoptosis-resistant bone metastatic PCa cells.

Materials and Methods

Cell Culture

PCa cell lines (C4-2, C4-2B, DU145, MDA PCa 2a, MDA PCa 2b, PC3, VCaP) and bone marrow stromal cell lines (HS-5, HS-27a) were grown in a 37°C, 5.0% (v/v) CO₂ growth chamber. C4-2, C4-2B, DU145, and PC3 cell lines were cultured in T-medium (Gibco/Invitrogen) supplemented with 5% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals), 0.4
mM L-glutamine (L-glut) (Gibco/Invitrogen), and 10 U/ml penicillin G sodium and 10 mg/ml streptomycin sulfate (pen-strep) (Gibco/Invitrogen). MDA PCa 2a and MDA PCa 2b were cultured in BRFF-HPC1 medium (AthenaES; 0403) supplemented with 20% (v/v) FBS, 0.4 mM L-glut, and pen-strep. VCaP, HS-5, and HS-27a cell lines were cultured in low glucose DMEM medium (Gibco/Invitrogen) supplemented with 10% FBS, 0.4 mM L-glut, and pen-strep.

**Conditioned Medium Treatment**

To obtain bone marrow stromal cell conditioned medium, culture medium was removed from HS-5 or HS-27a cultured cells and replaced with fresh T-medium supplemented with 5% FBS, L-glut, pen-strep. After 3 days incubation, the conditioned T-medium was collected and spun at 1400 rpm for 3 minutes to remove cell debris. The conditioned media were stored at -80°C. T-medium supplemented with 5% FBS, L-glut, pen-strep served as the control growth medium.

**Drug and siRNA Treatments**

Cells were treated with chloroquine diphosphate aqueous solution (Invitrogen; P36235). Cells were transfected with a pool of three unique 27-mer p62/SQSTM1 siRNA duplexes (Origene; SR305865) using siTran 1.0 transfection reagent (Origene; TT300001). Western blot analysis and/or immunostaining were used to confirm loss of p62 protein.

**Western Blot Analysis and Antibodies**

Protein was isolated from cells using NP40 lysis buffer (0.5% NP-40 (Sigma; NP40S), 50 mM Tris (pH 7.5), 150 mM NaCl, 3 mM MgCl2, 1× protease inhibitors (Roche; 0505489001). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific; 23225). For western blot analysis, equal protein concentrations were loaded onto and separated in 17% (w/v) sodium dodecyl sulfate polyacrylamide gel (40% acrylamide/bis-acrylamide solution; Bio-Rad; 161-0148). Proteins were transferred from the gel to 0.45 μm pore size nitrocellulose membrane (Bio-Rad; 162-0094) and total protein visualized using Ponceau S (Sigma; P7170). The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich; A7906) in 1× TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20). Primary and secondary antibodies were diluted in 3% BSA/1× TBST. Protein blot bands were visualized using Pierce ECL Western Blotting Substrate (Thermo Scientific; 32106) and imaged using the Fujifilm LAS-4000 imager (Fuji). Densitometry: Densitometry of western blot bands was performed using Image J software (http://rsbweb.nih.gov/ij/). To determine relative protein levels across treatments and/or cell lines, densitometric values for LC3B or p62 were first normalized to β-actin or Ponceau S stain loading controls and then normalized to a control treatment (set at 1.0). When comparing across cell lines, total protein (i.e. Ponceau S stain) was used as the loading control. **Primary antibodies:** LC3B (Novus Biologicals; NB600-1384), p62/SQSTM1 (Abnova; H00008878-M01), β-actin (Abcam; ab8226). **Secondary antibodies:** sheep anti-mouse (Jackson ImmunoResearch Laboratories; 515-035-062), goat anti-rabbit (Sigma-Aldrich; A6154).

**RNA Extraction and Quantitative Polymerase Chain Reaction (QPCR)**

To examine mRNA expression, cells were washed twice with 1× PBS and total RNA was extracted from the cells using Trizol reagent according to the manufacturer's instructions (Invitrogen; 15596-026) RNA was DNase-treated (Ambion; AM1906) for 30 minutes at 37°C and, following, cDNA was made using Quanta Biosciences qScript cDNA Super mix (VWR; 95048). The QPCR reaction was prepared using Quanta Bioscience SYBR Green Super mix (VWR; 95030-214). Primers specific for p62 were used and data was normalized.

*Prostate. Author manuscript; available in PMC 2015 February 01.*
to the \( \beta\)-actin transcript levels. Relative mRNA levels were calculated using the \( 2^{\Delta\Delta CT} \) method. \( \beta\)-Actin primers: Forward: GAT GAG ATT GGC ATG GCT TT; Reverse: CAC CTT CAC CGG TCC AGT TT.

**Cell Viability**

Calcein AM and ethidium homodimer 1 (EthD-1) fluorescent probes were used to stain live and dead cells, respectively, according to manufacturer's instructions (Molecular Probes; L3224). Briefly, 2 \( \mu\)M calcein AM and 4 \( \mu\)M EthD-1 were added directly to cells in culture. Following incubation, the cells were trypsinized and transferred to a 96-well tissue culture plate. Cell fluorescence was measured using the Infinite® M200 microplate reader (Tecan). Fluorescent cells also were imaged in the 96-well plate at 4× magnification (sufficient to capture 10,000 cells in a microscopy field) and counted by using Image J software (http://rsbweb.nih.gov/ij/). Cell viability was determined using the calculation live cells/(live + dead cells).

**Immunofluorescence**

Cells were fixed and permeabilized with 100% methanol at -20°C for at least 30 minutes. Cells were blocked with 2.5% BSA in 1× phosphate buffered saline (PBS) at room temperature for at least 30 minutes. Antibodies were diluted in 2.5% BSA in 1× PBS. Cells were incubated in primary antibody at 4°C overnight, washed with 1× PBS, and then incubated in fluorescently labeled secondary antibody at 4°C in the dark, overnight. Primary antibodies: LC3B (Novus Biologicals; NB600-1384), p62/SQSTM1 (Abnova; H00008878-M01), LAMP1 (University of Iowa Developmental Studies Hybridoma Bank; H4A3). Fluorescently labeled secondary antibodies: Alexafluor 488, goat anti-mouse (Invitrogen; A11001), Alexafluor 568, goat anti-rabbit (Invitrogen; A11061).

**Microscopy**

Images were taken and processed using the Eclipse TE300 inverted microscope (Nikon) and NIS Elements software (Nikon) (40× magnification, scale bar = 100 \( \mu\)m). Alexafluor 488 and 568 fluorescence was detected using the FITC and Texas Red filters, respectively.

**Statistics**

Statistical significance was determined using unpaired student t test.

**Results**

**HS-5 paracrine factor(s) can induce PCa autophagy**

We previously reported that the hematopoietic HS-5 bone marrow stromal cell line, but not the HS-27a structural bone marrow stromal cell line, secretes a milieu of soluble factor(s) that induce LC3B-II protein accumulation in C4-2 and C4-2B bone metastatic PCa cell lines (29). We expanded our analysis to determine if LC3B-II upregulation is a common response of bone metastatic PCa cell lines (Table I, (30-42)) to HS-5 secreted factor(s). Thus, we grew MDA PCa 2a, MDA PCa 2b and VCaP PCa cell lines for several days in control growth medium or in HS-5 or HS-27a bone marrow stromal cell (BMSC) conditioned medium and used western blot analysis to determine LC3B-II protein levels. As previously observed for the C4-2 and C4-2B cell lines (29), HS-5 conditioned medium induced greater LC3B-II accumulation in MDA PCa 2a, MDA PCa 2b and VCaP cells than did control growth medium or HS-27a BMSC conditioned medium (Fig. 1A).
LC3B-II accumulation can indicate autophagy induction or, alternatively, inhibition of autophagic flux (3). Using the lysotomotropic inhibitor, chloroquine, we previously showed that autophagic flux is maintained in C4-2 and C4-2B cells in the presence of HS-5 conditioned medium (29). To further confirm that autophagic flux remains functional in PCa cells grown in HS-5 conditioned medium, we also treated MDA PCa 2a and MDA PCa 2b cell lines with 40 μM chloroquine in the presence of HS-5 BMSC conditioned medium for two days. As observed for C4-2 and C4-2B cell lines (Fig. 1B, (29)), chloroquine increased LC3B-II accumulation over the vehicle control in MDA PCa 2a and MDA PCa 2b cells (Fig. 1B). Thus, HS-5 BMSC paracrine factor(s) do not inhibit autophagic flux in the bone metastatic PCa cell lines analyzed, but rather, induce PCa cell autophagy.

**HS-5 paracrine factor(s) can upregulate PCa p62 mRNA and protein**

While characterizing HS-5-induced autophagy response in C4-2, C4-2B, MDA PCa 2a, MDA PCa 2b, and VCaP cell lines, we discovered a steady-state increase in p62 protein that occurred within one day of growth in HS-5 BMSC conditioned medium (Fig. 1A). For example, HS5 conditioned medium induced a 1.7- to 4.2-fold increase in p62 protein over control medium (Fig. 1A). Because p62 sequesters ubiquitinated proteins into the autophagosome for degradation, it also is degraded through autophagy (10,11). Therefore, our results were unexpected given that HS-5 conditioned medium induces autophagy and does not block autophagy flux in PCa cells (Fig. 1, (29)). Furthermore, chloroquine increased p62 protein in the PCa cell lines grown in control medium or HS-5 or HS-27a BMSC conditioned medium (Fig. 1B), indicating that p62 is properly degraded through autophagy under these growth conditions. Therefore we investigated the effect of HS-5 conditioned medium on p62 mRNA levels in the PCa cell lines. Quantitative polymerase chain reaction (QPCR) revealed that within one day, HS-5 conditioned medium upregulated p62 mRNA levels (Fig. 1C). Thus, our results suggest (post)transcriptional regulation of PCa p62 by HS-5-secreted factor(s) and likely do not indicate a defect in autophagic flux.

**DU145 and PC3 cell lines show no significant autophagy or p62 induction when exposed to HS-5 paracrine factor(s)**

HS-5 BMSC conditioned medium induces apoptosis in C4-2, C4-2B, and VCaP cells (data not shown, (43)). Autophagy, which we propose is cytoprotective in this context, is induced in the surviving subpopulation ((29), data not shown). The DU145 and PC3 bone metastatic PCa cell lines are less sensitive to HS-5-induced apoptosis (43). Therefore, we predicted that DU145 and PC3 cells can escape HS-5-induced apoptosis by upregulating cytoprotective autophagy more efficiently than the apoptosis-sensitive PCa cell lines. To test our hypothesis, we compared the HS-5-mediated autophagy response of the DU145 and PC3 cells with that of the C4-2 cell line. C4-2, DU145, and PC3 cells were grown in control or BMSC conditioned medium for two days and analyzed for LC3B protein accumulation. Relative to control growth medium or HS-27a BMSC conditioned medium, HS-5 BMSC conditioned medium led to a greater induction of LC3B-I and/or LC3B-II accumulation in C4-2 cells than in DU145 or PC3 cells (Fig. 2A). For example, relative to control growth medium, HS-5 conditioned medium increased LC3B-I and LC3B-II levels 4.1- and 5-fold, respectively, in C4-2 cells, but had comparatively less effect on LC3B-I or LC3B-II protein levels in PC3 cells (Fig. 2A). Furthermore, while DU145 cells showed an increase in LC3-I when grown in HS-5 conditioned medium over control growth medium, we could not detect LC3B-II by western blot (Fig. 2A). Thus, HS-5 paracrine factor(s) have a more profound effect on autophagy induction in C4-2 cells than in DU145 or PC3 cells.

Because we found that DU145 cells do not accumulate LC3B-II when grown in control growth medium or BMSC conditioned medium (Fig. 2A), we concluded that canonical autophagy is not the common mechanism that mediates survival of DU145 and PC3 cells.
exposed to HS-5 paracrine factor(s). However, p62 also promotes cell survival both
dependently and independently of autophagy (9) and is induced by HS-5 conditioned
medium in multiple PCa cell lines (Fig. 1A). Therefore, we compared the effect of HS-5
BMSC conditioned medium on p62 mRNA and protein accumulation in C4-2, DU145 and
PC3 cell lines. Relative to control growth medium, after two days growth in HS-5
conditioned medium, C4-2 cells showed a 3.9- and 2.5-fold increase in p62 mRNA and
protein, respectively (Fig. 2A & B). However, DU145 and PC3 cells showed no
considerable increase in p62 mRNA or protein accumulation when grown in HS-5
conditioned medium (Fig. 2A & B). Taken together, our data reveal that, unlike the C4-2,
C4-2B, MDA PCa 2a, MDA PCa 2b, or VCaP cell lines, the DU145 and PC3 cell lines show
no notable autophagy or p62 induction in response to HS5-secreted paracrine factor(s).

**DU145 and PC3 cell lines have high basal p62 levels**
While we did not detect a considerable effect of HS-5 conditioned medium on autophagy
induction or p62 mRNA and protein accumulation in DU145 or PC3 cell lines (Fig. 2A &
B), we did observe that DU145 and PC3 cells have comparatively higher basal p62 mRNA
and/or protein levels than the C4-2, C4-2B, MDA PCa 2a, MDA PCa 2b, or VCaP cell lines
(Fig. 2A, B, & C). For example, when normalized to C4-2 basal p62 mRNA (Fig. 2B) or
protein (Fig. 2A), p62 mRNA or protein levels were 11- or 5.5-fold and 2.8- or 3.4-fold
greater in DU145 and PC3 cells, respectively.

**Down regulation of p62 is cytotoxic for DU145 and PC3 cell lines**
Given that both DU145 and PC3 cell lines have reduced sensitivity to HS-5-induced
apoptosis (43) and show comparatively high basal p62 mRNA and protein levels (Fig. 2),
we explored the possible role of p62 in DU145 and PC3 cell survival. C4-2, DU145 and
PC3 cells were grown in control growth medium and transiently transfected with 40 nM p62
siRNA to reduce p62 protein accumulation (Fig. 3A). Three days following treatment, cells
were analyzed for the presence of live and dead cells by measuring calcein AM and
ethidium homodimer 1 (EthD-1) fluorescence, respectively (graph not shown). Relative to
control siRNA, down regulation of p62 significantly reduced the calcein AM fluorescence
(p-value = 0.003) and increased EthD-1 fluorescence (p-value = 0.014) in PC3 cells. Thus,
p62 mediates PC3 cell survival. However, p62 siRNA did not significantly affect calcein
AM (p-value = 0.29) or EthD-1 (p-value = 0.31) fluorescence in C4-2 cells, implying that
p62 is not necessary for C4-2 cell survival in normal under control growth conditions.

In our experiments, 40 nM p62 siRNA was relatively less effective at reducing p62 protein
accumulation in DU145 cells (Fig. 3A) and did not have an effect on DU145 cell survival
(calcein AM, p-value = 0.49; EthD-1, p-value = 0.17; graph not shown). However, transient
transfection of 60 nM p62 siRNA was sufficient to reduce p62 protein accumulation to
nearly undetectable levels in DU145 cells (Fig. 3A). Therefore, we transfected C4-2, C4-2B,
MDA PCa 2A, MDA PCa 2B, DU145, and PC3 cells grown in control growth medium with
60 nM p62 siRNA and after two days we measured cell viability by counting calcein AM
and EthD-1 fluorescent cells. In control growth media, down regulation of p62 protein
significantly reduced the cell viability of DU145 (p-value ≤ 0.0005) and PC3 (p-value ≤
0.0005) cells, but had less effect on C4-2 (p-value ≤ 0.05), C4-2B, MDA PCa 2a, or MDA
PCa 2b cell viability (Fig. 3B, Supplemental Fig. 1).

When p62 protein was reduced under control growth conditions, C4-2, C4-2B, MDA PCa
2a, and MDA PCa 2b cells did not show as significant of a reduction in cell viability as the
DU145 or PC3 cells (Fig. 3B, Supplemental Fig. 1). However, given that HS-5 BMSC
conditioned medium upregulated p62 mRNA and protein in C4-2, C4-2B, MDA PCa 2a,
and MDA PCa 2b cells (Fig. 1 & 2), we reasoned that p62 may have a cytoprotective
function in the presence of HS5 conditioned medium. Surprisingly, siRNA-mediated knockdown of p62 did not reduce the cell viability of C4-2 or C4-2B cells grown in HS-5 conditioned medium (Fig. 3B). However, transient transfection of 60 nM p62 siRNA into DU145 and PC3 cells grown in HS-5 conditioned medium for two days significantly reduced the cell viability (DU145, p-value ≤ 0.0005 ; PC3, p-value ≤ 0.005) (Fig. 3B). Interestingly, we were unable to appreciably reduce p62 protein levels using siRNA in MDA PCa 2a or MDA PCa 2b cells grown in HS-5 conditioned medium (Supplemental Fig. 1). Taken together, these data demonstrate that that loss of p62 protein is more significantly cytotoxic for DU145 and PC3 cells than for C4-2, C4-2B, MDA PCa 2a, or MDA PCa 2b cell lines grown in control or HS-5 BMSC conditioned growth media.

Down regulation of p62 alters autolysosomal subcellular localization in DU145 and PC3 cell lines

To investigate the effect of the loss of p62 protein on autolysosome accumulation in individual cells, we performed co-immunostaining for LC3B and p62 or the lysosomal membrane protein, LAMP1, in C4-2, C4-2B, DU145, and PC3 cells transiently transfected with 60 nM p62 siRNA and grown in control growth medium or HS-5 BMSC conditioned medium for two days. In C4-2 and C4-2B cells grown in control growth medium or HS-5 conditioned medium, discrete co-localized LC3B and LAMP1 structures (autolysosomes) were found distributed throughout the cytoplasm and around the nucleus in cells transfected with control siRNA or p62 siRNA (Fig. 4A & B). However, in PC3 cells, the subcellular co-localization of LC3B and LAMP1 changed from cytoplasmic distribution to peri-nuclear clustering when p62 was down regulated (Fig. 4A & B, arrows). LC3B puncta did not accumulate in DU145 cells (data not shown), presumably because DU145 cells do not accumulate LC3B-II (Fig. 2A). However, as observed for PC3 cells, p62 siRNA led to peri-nuclear clustering of LAMP1 in DU145 cells grown in control growth medium or HS-5 conditioned medium (Fig. 4A & B, arrows). Thus, the loss of p62 has a similar effect on lysosome and autolysosome subcellular localization in DU145 and PC3 cell lines in grown in either control growth medium or HS-5 BMSC conditioned medium, but p62 siRNA does not affect autolysosome subcellular localization in C4-2 or C4-2B cells.

Discussion

Sensitivity to HS-5 secreted factors correlates with PCa androgen receptor expression and bone-forming tumor type

An intriguing similarity emerges for the PCa cell lines which we discovered can respond to HS-5 BMSC-induced apoptosis, autophagy, or p62 accumulation in contrast to the PCa cell lines that did not show a significant apoptosis, autophagy, or p62 induction response to HS-5 paracrine factor(s) (Fig. 5). The PCa cell lines (C4-2, C4-2B, MDA PCa 2a, MDA PCa 2b, and VCaP) that were sensitive to HS-5 conditioned medium express the androgen receptor (41) and form primarily osteoblastic lesions in mice (33,42,44), while the HS-5-insensitive PCa cells lines (DU145 and PC3) do not express the androgen receptor (41) and form primarily osteolytic lesions (37). It is feasible that the osteolytic PCa cell types either secrete factor(s) that neutralize the bone cell paracrine signals that induce apoptosis, autophagy, or p62 accumulation, or alternatively lack internal pathways that allow them to respond to these signals. Another possibility is that the androgen receptor can directly or indirectly mediate the induction of cytoprotective autophagy and p62 upregulation in PCa cells in response to BMSC-secreted apoptosis-inducing factor(s). Experiments are underway to determine the mechanistic role of the androgen receptor in HS-5-induced autophagy and p62 upregulation and to determine if key autophagy regulators are transcriptional targets of the androgen receptor.
**p62 is required for the cell survival of HS-5-insensitive cell lines**

If the androgen receptor indeed mediates autophagy induction in response to HS-5 BMSC paracrine factors, then not surprisingly, DU145 and PC3 PCa cells, which lack androgen receptor expression, were non-responsive to HS-5-induced autophagy. Interestingly, the PC3 cells accumulated more basal LC3B-II than did the HS-5-sensitive PCa cell lines (Fig. 2 & 3, data not shown), implying that high basal autophagy protects PC3 cells from HS-5-induced apoptosis. While PC3 cells may have higher basal autophagy, DU145 cells do not carry out canonical autophagy (45). For example, we did not detect LC3B-II (Fig. 2 & 3) or LC3B-decorated autophagosomes or autolysosomes (data not shown) in the DU145 cells. Furthermore, Ouyang and colleagues recently reported that ATG5, which promotes autophagosome formation, is mutated and non-functional in DU145 cells (45). Yet, both DU145 and PC3 cell lines are insensitive to HS-5-induced apoptosis (43), suggesting that these cell lines share an androgen receptor-independent – and possibly autophagy-independent – survival mechanism. Our data reveals that one such shared alternative cell survival mechanism requires p62 (Fig. 5). Because p62 is a multifunctional protein, there are several candidate cell survival pathways that may be compromised in DU145 and PC3 cells when p62 expression is reduced. For example, in addition to mediating autophagic degradation of cytotoxic polyubiquitinated protein aggregates, p62 also promotes cell survival through NF-κB activation and NRF2 antioxidant signaling (8,9). It will be important to determine the regulation and activity of the various p62-mediated cell survival pathways when p62 is knocked down in DU145 and PC3 PCa cell lines, as these pathways may serve as biomarkers or therapeutic targets.

**PCa cell lines with high basal p62 may have evolved a cell survival requirement for p62**

We initially predicted that the steady-state upregulation of p62 mRNA and protein levels in HS-5-sensitive PCa cell lines exposed to HS-5 paracrine signaling reflected a cytoprotective response. However, in our experiments, p62 did not appear necessary for the survival of HS-5-sensitive PCa cell lines under normal growth conditions or in the presence of HS-5 paracrine factor(s) (Fig. 3, Supplemental Fig. 1, data not shown). Our results imply that under normal growth conditions or in the presence of HS-5 paracrine factors, the HS-5-sensitive PCa cell lines, which do not normally accumulate high levels of p62, have cell survival pathways that can compensate for the loss of p62.

In contrast, under normal growth conditions or in the presence of HS-5 BMSC paracrine factors DU145 and PC3 cells showed high basal levels of p62 mRNA and protein and required p62 for cell survival (Fig. 2 & 3). Thus, we propose that the DU145 and PC3 PCa cell lines have evolved a cell survival dependency on p62 and that constitutive, long-term p62 elevation may be important for PCa adaptation to the bone microenvironment (Fig. 5).

Defective autophagy flux accounts for, in part, the high basal p62 protein accumulation in DU145 cells (45). However, we also saw an increase in basal p62 mRNA levels in both the DU145 and PC3 cells (Fig. 2), suggesting enhanced transcriptional activity and/or mRNA stability. Furthermore, HS-5-induced p62 accumulation occurred at the level of mRNA in the HS-5-sensitive PCa cell lines (Fig. 2). Transcription factors and microRNAs shown to regulate p62 expression or mRNA stability include NF-κB (20), NRF2 (46), Farnesoid Receptor X (47), Prostate Derived Ets Factor (26), or miR-17/20/93/106 (48)). One or more of these regulators of p62 mRNA levels may mediate HS-5-induced p62 expression in the HS-5-sensitive cell lines and these regulators may have altered activity in the DU145 and PC3 cell lines. Future investigation into the mechanistic regulation of p62 expression in the PCa cells lines will provide strategies to target p62 function in cancer cells.
Autolysosome peri-nuclear clustering is associated with enhanced autophagy induction and flux

In DU145 and PC3 cells we observed that lysosomes and autolysosomes, respectively, are normally distributed throughout the cytoplasm and around the nucleus (Fig. 4). However, p62 knockdown caused peri-nuclear clustering of (auto)lysosomes in DU145 and PC3 cells. Other laboratories have reported that autolysosome clustering is associated with enhanced autophagy induction and flux in response to viral infection (49) or hypertonic stress (50). In accordance, we detected peri-nuclear clustering of (auto)lysosomes in DU145 and PC3 cells that had avoided p62 siRNA-mediated cell death at the time of analysis and thus the (auto)lysosome peri-nuclear clustering could have indicated a cell survival response. Alternatively, p62 siRNA induced (auto)lysosome clustering in DU145 and PC3 cells could be an indicator of impending cell death, as this altered subcellular localization of autolysosomes was not detected in C4-2 cells which are comparatively insensitive to p62 siRNA-mediated cell death. The mechanism of the p62-siRNA-induced (auto)lysosome clustering is unclear, but likely involves regulation of trafficking along microtubules (49,50).

Conclusion
p62 may be a rational target for bone metastatic PCa

p62 is overexpressed in multiple cancer types, including PCa, and can correlate with disease progression (19-21,23-27). In line with these findings, our data suggests that the bone microenvironment can contribute to the upregulation of p62 mRNA and protein in metastatic PCa cells and it will be important to determine p62 expression in patient bone metastases. Notably, DU145 and PC3 cell lines represent a PCa cell subtype that would be resistant to androgen deprivation or anti-androgen therapies due to lack of androgen receptor expression. Importantly, our data suggest that treatment resistant PCa tumors could be identified by p62 expression and subsequently killed by targeting p62 expression and/or the function of p62 in one or more pathways, including autophagy, NF-κB activation, or NRF2 signaling. In line with our reasoning, knockdown of p62 accumulation in bone marrow stromal cells attenuated, among several signaling cascades, NF-κB signaling and cytokine secretion and concomitantly attenuated bone marrow stromal cell paracrine support of multiple myeloma cell proliferation (51). Thus, it is intriguing to speculate that targeting p62 expression and/or function in bone metastatic PCa cells would not only lead to PCa cell death, but could disrupt potential p62-mediated, tumor-promoting cross talk between PCa and bone cells in the metastatic microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank all of the members of the labs of Drs. Mary C. Farach-Carson, Daniel Carson, Cimona V. Hinton, and Nora Navone and all of the members of the Prostate Cancer P01 group for scientific discussion and/or technical support. This work was supported by NIH Funding support from NIH/NCI K01 CA160602, NIH/NCI F32 CA128296, and NIH/NCI P01 CA098912.

References


Figure 1. HS-5 BMSC paracrine factor(s) can induce autophagy and p62 mRNA and protein accumulation in PCa cell lines

(A) MDA PCa 2a, MDA PCa 2b, and VCaP PCa cell lines were grown in control growth medium or in conditioned medium (CM) from HS-5 or HS-27a BMSCs for the time points indicated. LC3B and p62 protein levels were detected using western blot and relative protein levels calculated as described in materials and methods. HS-5 CM upregulated LC3B-II and p62 protein in PCa cell lines. (B) C4-2B, MDA PCa 2a and MDA PCa 2b PCa cell lines were grown in control growth medium or HS-5 or HS-27a CM in the absence or presence of 40 μM chloroquine (CQ) for two days. CQ led to LC3B-II and p62 accumulation in each growth medium. (C) C4-2, C4-2B, MDA PCa 2a, and MDA PCA 2b PCA cell lines were grown in control growth medium or HS-5 or HS-27a CM for one day. p62 mRNA levels were determined using QPCR and relative fold mRNA levels graphed. Error bars represent standard deviation of two or three biological replicates. P-value, * ≤ 0.05, ** ≤ 0.005. HS-5 conditioned medium upregulated p62 mRNA in the PCa cell lines.
Figure 2. DU145 and PC3 PCa cell lines are less responsive to HS-5-induced accumulation of LC3B-II or p62 and have comparatively high basal p62 accumulation

(A) C4-2, DU145, or PC3 cell lines were grown in control growth medium or HS-5 or HS-27a BMSC conditioned medium (CM) for two days and analyzed by western blot for LC3B and p62. Relative protein levels were determined as described in materials and methods. HS-5 CM upregulated LC3B and p62 protein accumulation in C4-2 cells to a greater extent than in DU145 or PC3 cell lines. However, basal LC3B-II and/or p62 levels were higher in DU145 or PC3 cells than in C4-2 cells. (B) C4-2, DU145, or PC3 cell lines were grown in control growth medium or HS-5 or HS-27a CM for two days. *p62 mRNA levels were determined using QPCR and relative fold mRNA levels graphed. Error bars represent standard deviation of three biological replicates. P-value, * ≤ 0.05, ** ≤ 0.005, *** ≤ 0.0005. HS-5 CM upregulated p62 mRNA in C4-2 cells, but not in DU145 or PC3 cell lines. However, PC3 and DU145 cells showed higher basal p62 mRNA than C4-2 cells. (C) PCa cell lines were grown in control growth medium for two days. DU145 and PC3 cell lines had comparatively high p62 protein levels.
Figure 3. Down regulation of p62 mRNA is cytotoxic for DU145 and PC3 PCa cell lines
(A) (Left) C4-2, PC3, or DU145 cells were grown in control growth medium in the presence of 40 nM non-target control siRNA or 40 nM p62 siRNA for three days. LC3B and p62 protein levels were detected using western blot and relative protein levels determined as describe in materials and methods. Two biological replicates are shown for each treatment. Transfection of 40 nM p62 siRNA was sufficient to reduce p62 protein accumulation to almost undetectable levels in C4-2 and PC3 cells lines, but not in DU145 cells. (Right) DU145 cells grown in control growth medium or HS-5 conditioned medium (CM) were transiently transfected with 60 nM non-target control siRNA or 60 nM p62 siRNA for two and three days. Transfection of 60 nM p62 siRNA was sufficient to reduced p62 protein to almost undetectable levels in DU145 cells. (B) C4-2, C4-2B, PC3 and DU145 cells grown in PCa control growth medium or HS-5 CM were transfected with 60 nM non-target control siRNA or 60 nM p62 siRNA for two days. Live and dead cells were stained with calcein AM and ethidium homodimer 1 (EthD-1) fluorescent dyes, respectively, and live and dead cells were counted to determine cell viability. The error bars represent standard deviation of three biological replicates. P-value, * < 0.05, ** < 0.005, *** <0.0005. p62 siRNA reduced cell viability more significantly in DU145 and PC3 cells than in C4-2 or C4-2B cells.
Figure 4. Loss of p62 alters autolysosome subcellular localization
C4-2, C4-2B, PC3, and DU145 PCa cells were grown in (A) control growth medium or (B) HS-5 conditioned medium (CM) and treated with 60 nM non-target control siRNA or 60 nM p62 siRNA. After two days, cells were fixed and co-immunostained for p62 (FITC) and LC3B (Texas Red) or LC3B (Texas Red) and LAMP1 (FITC). Immunofluorescent cells were imaged at 40× magnification, scale bar = 100 μm. Shown are matched images of cells co-immunostained for p62 and LC3B and a merged image of cells co-immunostained for LC3B and LAMP1. p62 siRNA led to loss of total p62 protein in C42, C4-2B, and PC3 cells and loss of p62 puncta in DU145 cells. p62 siRNA caused peri-nuclear clustering of autolysosome and lysosomes, respectively, in PC3 and DU145 cells (arrow, representative cell). However, in C4-2 or C4-2B cells, p62 siRNA did not alter subcellular localization of LC3B or LAMP1 when p62 protein levels were reduced (arrow, representative cell).
Based on our data we propose the following model. (1) Prostate cancer (PCa) cells that are androgen receptor positive (AR+) and form largely osteoblastic (OB) lesions represent a PCa subtype that is sensitive to bone marrow stromal cell (BMSC) paracrine induction of PCa apoptosis and induction of autophagy and p62 accumulation in the surviving cell population. PCa cells that are androgen receptor negative (AR-) and form largely osteolytic (OL) lesions represent a PCa cell subtype that has high basal p62 levels and is insensitive to BMSC paracrine-induced PCa apoptosis, autophagy, or p62 upregulation. (2) Loss of p62 (flat arrowhead) is significantly cytotoxic (thick, solid arrow) for BMSC-insensitive PCa cells, indicating that these cells require p62 for survival. Likewise, BMSC-sensitive PCa cells that would escape BMSC-induced apoptosis and colonize the bone would evolve (thin, dashed arrow) into p62-dependent cells due to long-term paracrine induction of p62 accumulation.
Table 1

Cell Line List

Listed are the prostate cancer (above darker line) and bone marrow stromal (below darker line) cell lines used in this study. Cell line phenotypes are described (30-42).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Human Origin</th>
<th>Cell Line Establishment</th>
<th>Tumor Forming Phenotype in Mice</th>
<th>AR Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4-2</td>
<td>Lymph node metastasis, 50-year-old Caucasian male.</td>
<td>LNCaP subline isolated from C4 tumor xenograft in castrated mouse. Tumors from lymph node &amp; bone.</td>
<td>Intrafemoral injections; osteoblastic lesions.</td>
<td>Yes</td>
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<tr>
<td>C4-2B</td>
<td>Lymph node metastasis, 50-year-old Caucasian male.</td>
<td>LNCaP subline isolated from C4-2 tumor xenograft in castrated mouse. Tumors isolated from bone.</td>
<td>Intrafemoral injections; osteoblastic lesions.</td>
<td>Yes</td>
</tr>
<tr>
<td>MDA PCa 2a &amp; 2b</td>
<td>Bone metastasis, 63-year-old African American male.</td>
<td>2a &amp; 2b are distinct clones from same specimen. 2b cells generate tumors at a higher rate than 2a cells in vivo.</td>
<td>Intrafemoral injection of 2b cells; osteoblastic lesions.</td>
<td>Yes</td>
</tr>
<tr>
<td>VCaP</td>
<td>Vertebral bone metastasis, 59-year-old Caucasian male.</td>
<td>Passaged as mouse xenograft then cultured in vitro.</td>
<td>Intratibial injections; osteoblastic lesions.</td>
<td>Yes</td>
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<tr>
<td>DU145</td>
<td>Brain metastasis, 69-year-old Caucasian male.</td>
<td>Passaged in vitro</td>
<td>Injection into human bone xenograft; osteolytic lesions.</td>
<td>No</td>
</tr>
<tr>
<td>PC3</td>
<td>Bone metastasis, 62-year-old Caucasian male.</td>
<td>Passaged as mouse xenograft then cultured in vitro.</td>
<td>Injection into human bone xenograft; osteolytic lesions.</td>
<td>No</td>
</tr>
<tr>
<td>HS-5</td>
<td>Normal bone marrow, 30 year-old Caucasian male.</td>
<td>Bone marrow cells immortalized using LXSN-16 E6E7 retrovirus.</td>
<td>Small fibroblastic cells that secrete higher levels of growth factors than HS27a cells &amp; support hematopoiesis in cell culture.</td>
<td></td>
</tr>
<tr>
<td>HS-27a</td>
<td>Normal bone marrow, 30 year-old Caucasian male.</td>
<td>Bone marrow cells immortalized using LXSN-16 E6E7 retrovirus.</td>
<td>Large flattened epithelioid-like cells that support cobblestone formation of hematopoietic cells in cell culture.</td>
<td></td>
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</table>
Expression of the transmembrane mucins, MUC1, MUC4 and MUC16, in normal endometrium and in endometriosis

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Submitted on October 31, 2013; resubmitted on May 13, 2014; accepted on May 20, 2014

STUDY QUESTION: Are the transmembrane mucins, MUC1, MUC4 and MUC16, differentially expressed in endometriosis compared with normal endometrium?

SUMMARY ANSWER: This study revealed that transmembrane mucin expression does not vary significantly in normal endometrium during the menstrual cycle and is not altered in endometriosis relative to the epithelial marker, cytokeratin-18 (KRT18).

WHAT IS KNOWN ALREADY: Increased serum levels of the transmembrane mucin fragments MUC1, MUC4 and MUC16 that normally dominate the apical surface of simple epithelia are found in several pathological conditions, including endometriosis. Altered mucin expression in gynecologic diseases may promote infertility or endometrial pathologies.

STUDY DESIGN, SIZE, DURATION: This was a laboratory-based study of samples from 12 endometriosis patients as well as non-endometriosis control samples obtained from 31 patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Total RNA was isolated from endometrial biopsies of ectopic and eutopic endometrium from women with endometriosis and control patients from different stages of the menstrual cycle. Quantitative (q)-RT–PCR analyses were performed for the mucins, MUC1, MUC4 and MUC16, relative to the epithelial marker, cytokeratin-18 (KRT18), or β-actin (ACTB). Frozen sections from endometrial biopsies of proliferative and mid-secretory stage women with endometriosis were immunostained for MUC1, MUC4 and MUC16.

MAIN RESULTS AND THE ROLE OF CHANCE: qRT–PCR analyses of MUC1 and MUC16 mRNA revealed that these mucins do not vary significantly during the menstrual cycle nor are they altered in endometriosis relative to the epithelial marker, KRT18. MUC4 mRNA is expressed at very low levels relative to MUC1 and MUC16 under all conditions. There was little difference in MUC1 and MUC16 expression between eutopic endometrial and ectopic endometriotic tissues. MUC4 expression also was not significantly higher in the ectopic endometriotic tissues. Immunostaining for all three mucins reveals robust expression of MUC1 and MUC16 at the apical surfaces of endometrial epithelia, but little to no staining for MUC4.

LIMITATIONS, REASONS FOR CAUTION: qRT–PCR analysis was the main method used for mucin detection. Additional studies with stage III–IV endometriotic tissue would be useful to determine if changes in MUC1 and MUC16 expression occur, or if MUC4 expression increases, at later stages of endometriosis.

WIDER IMPLICATIONS OF THE FINDINGS: We report a comprehensive comparative profile of the major transmembrane mucins, MUC1, MUC4 and MUC16, relative to the epithelial marker, KRT18, in normal cycling endometrium and in endometriosis, and indicate constitutive expression. Previous studies have profiled the expression of individual mucins relative to β-actin and indicate accumulation in the luteal phase. Thus, these differences in interpretation appear to reflect the increased epithelial content of endometrium during the luteal phase.
**STUDY FUNDING:** This study was supported by: NIH R01 HD29963 to D.D.C.; NIH U54 HD007495 to S.M.H.; and NIH R01 HD067721 to S.L.Y. and B.A.L. The authors have no competing interests to declare.

**Key words:** mucins / endometrium / endometriosis / cytokeratin-18

**Introduction**

The transmembrane mucins, MUC1, MUC4 and MUC16, belong to a family of very large, heavily glycosylated proteins that are characterized by a variable number of tandem repeat (VNTR) motifs. These mucins are expressed on the apical surface of nearly all simple epithelial tissues as well as various tumor cells and serve to keep epithelial surfaces lubricated and hydrated, protecting them from pathogens and environmental challenges (Hattrup and Gendler, 2008). Uterine membrane-tethered mucins play a key role in embryo implantation, a process facilitated by the loss or reduction of the mucin barrier (Braga and Gendler, 1993; Brayman et al., 2004; Dharmaraj et al., 2009). Disregulated mucin expression is associated with various disease states (Hollingsworth and Swanson, 2004; Rose and Voynow, 2006; Vlad et al., 2006; Margarit et al., 2010; Chang et al., 2011; He et al., 2011) and high levels of transmembrane mucin expression reduce drug efficacy and protect cells from apoptosis (Hollingsworth and Swanson, 2004; Ren et al., 2004). Transmembrane mucin expression is hormonally regulated during the estrous/menstrual cycle in many species (Hey 2004). Transmembrane mucin expression is hormonally regulated during the estrous/menstrual cycle in many species (Hey et al., 1994; Surveyor et al., 1995; Hild-Petito et al., 1996; Hoffman et al., 1998; Julian et al., 2005; Gipson et al., 2008) and altered expression of membrane-tethered mucins is associated with infertility (Koscinski et al., 2006; Margarit et al., 2010). In addition, increased serum levels of transmembrane mucin fragments are found in gynecological pathologies such as endometrial cancer and endometriosis (He et al., 2011; Szubert et al., 2012). Endometriosis is a benign chronic inflammatory disorder associated with an increased risk of progression to ovarian cancer and impaired fecundity (Lessey et al., 2013). The Center for Disease Control reports that 11.8% of all women (≈17 million in the USA alone) between the ages of 15 and 44 have endometriosis. Identifying approaches to control transmembrane mucin expression provides novel opportunities to enhance embryo implantation success, improve protective functions of the female reproductive tract and potentially reduce endometriotic spread and/or enhance the efficacy of existing therapies.

MUC1 expression has been extensively studied in human endometrium (Apelin et al., 1994, 1998; DeLoia et al., 1998; Horne et al., 2005; Wang et al., 2008); however, there is limited information available on MUC4 and MUC16 expression in human uterine tissues. Studies from Gipson and colleagues demonstrate MUC16 (CA-125) expression at the apical surface of luminal epithelia with little change during the menstrual cycle (Gipson et al., 2008), in contrast to changing patterns observed for MUC1 (Hey et al., 1994). Recently, serological studies indicated that CA 125 levels are useful markers of endometriosis (Patrelli et al., 2011; Socolov et al., 2011; Szubert et al., 2012). Genetic polymorphisms in MUC4 are associated with endometriosis in certain populations (Chang et al., 2011), but little information on protein and mRNA expression is available. An isolated report has shown MUC4 expression by immunostaining of secretory stage samples from women in the secretory stage of the cycle and undergoing in vitro fertilization (IVF) (Koscinski et al., 2006). However, careful comparative studies of the expression of these three major membrane-tethered mucins have not been performed in the cycling endometrium and endometriosis.

In the current study, we report a comprehensive expression profile of MUC1, MUC4 and MUC16 in normal endometrium during the menstrual cycle and in eutopic and ectopic endometrium of women with and without endometriosis. Mucin expression studies so far have been performed relative to the housekeeping gene, β-actin (ACTB). Here, we use the epithelial-specific markers, cytokeratin-18 mRNA (KRT18) or pan-cytokeratin antibodies, as controls for changes in the content of the uterine epithelial population that occur during the menstrual cycle (Moll, 1983).

**Materials and Methods**

**Sample collection**

All patient samples were collected according to the Institutional Review Board for Baylor College of Medicine and affiliated hospitals, Greenville Hospital System and University of North Carolina School of Medicine and processed at Rice University with approval from the Institutional Review Board at Rice University. Sample collection was performed as described previously (Hawkins et al., 2011; Plante et al., 2012). All tissue samples were collected with patient consent and approvals from the Institutional Review Board for Baylor College of Medicine and affiliated hospitals, Greenville Hospital System and University of North Carolina School of Medicine and processed at Rice University with approval from the Institutional Review Board at Rice University. Tissue collection was performed as described previously (Hawkins et al., 2011; Plante et al., 2012). Normal endometrium tissue samples from S.L.Y. (Plante et al., 2012) were obtained from a tissue library, with participants’ cycles ranging from 25 to 35 days. Samples were randomized to a specific proliferative cycle day or a specific day post-luteinizing hormone (LH) surge. Endometrial histology and dating was performed according to Noyes criteria (Noyes et al., 1950). Matched samples of endometrium and endometriosis obtained were not LH timed but confirmed histologically according to Noyes criteria (Noyes et al., 1950). Samples were categorized as proliferative (n = 6), early secretory (n = 4), mid-secretory (n = 6) and late secretory (n = 4), and stage-matched eutopic and ectopic endometrial samples (total n = 6; early secretory, stages I-II, n = 1; secretory, stage II, n = 3; proliferative, stage II, n = 1; mid-secretory, stage III, n = 1).

For the purpose of immunohistochemistry, samples from normal secretory endometrium, and proliferative and mid-secretory endometriosis grade stage II were used. These samples obtained from S.M.H. (Hawkins et al., 2011) were from women with regular menstrual cycles, who were free from hormones for at least 30 days before surgery. Menstrual cycle phase was determined by patient-provided information and confirmed by pathology according to standard criteria (Noyes et al., 1950). Samples were categorized as non-endometriosis proliferative (n = 5), non-endometriosis secretory (n = 6) and endometriotic samples (total n = 6; proliferative, n = 2; secretory, n = 2; interval phase, n = 1; inactive phase, n = 1).

**RNA isolation, DNAse treatment and qRT–PCR**

Total RNA was isolated as described (Hawkins et al., 2011; Plante et al., 2012). RNA was diluted with nuclease free water to a volume of 40 μl and...
DNAse treated (Ambion, Austin, TX, USA; AM1906) according to the manufacturer’s instructions. Reverse transcriptase reactions were performed using 1 μg of total RNA in a 20 μl reaction using qScript cDNA Super mix (Quanta; 95048) incubated for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. Real-time PCR was performed using the primer sequences shown in Table I and SYBR Green Super mix (Quanta Bioscience). Samples were cycled for 30 s at 95 °C, 30 s at 59 °C and 30 s for 72 °C for 40 cycles for MUC1, MUC16, KRT18 and ACTB. For MUC4, and for accompanying KRT18, samples were cycled for 30 s at 95 °C, 15 s at 58 °C and 15 s for 72 °C for 40 cycles. The relative amounts of mucin mRNA were identified using the comparative threshold cycle method and normalized to that of either KRT18 or ACTB. Primer sets not previously described were designed using Primer-BLAST (NCBI) and were validated according to protocols in the BioRad CFX96 manual.

**Immunofluorescence analysis**

Frozen sections from mid-secretory stage of cycling endometrium were thawed at room temperature for 20 min, rehydrated with 1 x PBS for 10 min and fixed with 4% (wt/vol) paraformaldehyde for 10 min. Slides were washed three times for 10 min each with 1 x PBS, blocked for 1 h with 1% (wt/vol) BSA in PBS and washed three times with 1 x PBS. Primary antibody was added and incubated overnight at the indicated dilutions: rabbit polyclonal antibody to wide spectrum cytokeratin, 1:50 (Abcam; ab9377); mouse monoclonal anti-MUC4, 1:250 (kindly provided by Dr Robert Bast, MD Anderson Cancer Center). Mouse and rabbit IgG (Jackson ImmunoResearch Laboratories) at 250 ng each as well as an IgG control (rabbit polyclonal antibody to wide spectrum cytokeratin, 1:50 (Abcam; ab9377); mouse monoclonal anti-MUC4, 1:250 (kindly provided by Dr Robert Bast, MD Anderson Cancer Center). Mouse and rabbit IgG controls (Jackson ImmunoResearch Laboratories) at 250 ng each as well as a secondary antibody only were used as controls. Slides were washed three times for 10 min each with 1 x PBS, then incubated with 1:400 dilution of secondary antibody, Alexa-fluor 647 goat anti-mouse (Invitrogen; A21235) or Alexa-fluor 488 goat anti-rabbit (Invitrogen; A11034) in 1% (wt/vol) BSA in PBS for 1 h in the dark, and washed three times for 10 min with 1 x PBS. Samples were mounted with Prolong-Antifade with DAPI as per manufacturer’s instructions (Invitrogen) and subjected to analyses by confocal microscopy (LSM710).

Tissue samples from proliferative stage endometriosis were embedded in O.C.T. and stored at −80 °C. Tissue blocks were incubated at −20 °C overnight prior to sectioning in a Leica cryotome. Ten micron tissue sections were then processed as described above. Tissue sections were imaged using a Nikon A1-RsI confocal microscope.

The pancreatic adenocarcinoma cell line, HPAF was plated in an 8-well chamber slide in RPMI + 10% (vol/vol) FBS until confluent. Cells were washed three times for 10 min each with 1 x PBS, blocked for 1 h with 1% (wt/vol) BSA (PBS for 1 h, followed by overnight incubation at 4 °C with MUC4 antibody (8G7 from Dr Batra) at a 1:100 dilution in filtered 3% (wt/vol) BSA. Cells were washed three times for 5 min each in PBS, and then incubated with goat anti-mouse AlexaFluor 647 at a dilution of 1:400 in filtered 3% (wt/vol) BSA for 1 h at room temperature, followed by two 5 min washes in PBS. Cells were then washed in PBS + DAPI for 5 min. Coverslips were mounted using ProLong Gold anti-fade agent and cells were imaged on a Zeiss LSM 710 confocal microscope (Supplementary data, Fig. S1).

**Statistical analysis**

Statistical analyses were performed using a two-tailed student’s t-test (GraphPad InStat program; GraphPad Software, Inc., San Diego, CA, USA).

**Results**

**Mucin mRNA profiles in normal endometrium across the menstrual cycle**

Levels of MUC1 and MUC16 mRNAs have been previously profiled in human endometrial tissues (Hey et al., 1994; Gipson et al., 1997, 2008; Hebbar et al., 2005). These studies did not account for variations in uterine epithelial cell populations during the menstrual cycle. We therefore examined mucin mRNA expression levels by qRT–PCR in the different stages of the cycling endometrium relative to the epithelial marker, cytokeratin-18, KRT18 (Fig. 1). The mucins, MUC1, MUC4 and MUC16, were expressed in all stages of the cycle with very low expression of MUC4 detected in all cases. There was no significant change in MUC1 and MUC16 mRNA expression across the cycle when compared with KRT18. Since this was in apparent conflict with previous reports, we also compared MUC1 and MUC16 expression relative to ACTB used by others as a load control in similar studies. In agreement with previous reports (Hey et al., 1994; Gipson et al., 2008), we observed a rise in MUC1 and MUC16 mRNA expression relative to ACTB during the secretory phase; however, no significant changes were observed in the expression of these mucin mRNAs relative to KRT18 (Fig. 2A and B, respectively). Thus, the apparent rise in MUC1 and MUC16 reported previously parallels the increased epithelial content of the endometrium during the secretory phase.

**Mucin mRNA profiles in normal endometrium and endometrium of women with endometriosis**

Mucin glycoproteins have been assessed as potential biomarkers or diagnostic tools in endometriosis because of their utility as markers of

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**Table I** Oligonucleotide primer sequences used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>5′ AGAGAAATCTCTGAGGCGGAC</td>
<td>5′ TGACATCTTGCCCTGAGTG</td>
<td>114 bp</td>
<td>Dharmaraj et al. (2010)</td>
</tr>
<tr>
<td>MUC4</td>
<td>5′ GAGCCCACTGCTGACTGC</td>
<td>5′ AATGGTGCGGATGAATTGTTT</td>
<td>102 bp</td>
<td>Argueso et al. (2002)</td>
</tr>
<tr>
<td>MUC4-CT</td>
<td>5′ CATGGAAGCCAGTGCCGAC</td>
<td>5′ GTGTCACAGTGCTGCCCC</td>
<td>94 bp</td>
<td>*This Paper</td>
</tr>
<tr>
<td>MUC16</td>
<td>5′ GCCCTCTACCTAAGCGTCAATGAA</td>
<td>5′ GGATCGCATGACCTGGTG</td>
<td>114 bp</td>
<td>Argueso et al. (2003)</td>
</tr>
<tr>
<td>ACTB</td>
<td>5′ GATGAGATGGCATGGCTTT</td>
<td>5′ ACCCCTACGCCGGATTT</td>
<td>100 bp</td>
<td>Dharmaraj et al. (2010)</td>
</tr>
<tr>
<td>KRT18</td>
<td>5′ GACACCAAATATACACAGACTG</td>
<td>5′ GGCTGTTAGGCTTTTACTCC</td>
<td>108 bp</td>
<td>*This Paper</td>
</tr>
</tbody>
</table>

*Refer to materials and methods for primer validation.
Figure 1  Mucin mRNA profile in normal endometrium. Box plots were used to express levels of transmembrane mucin mRNA levels in the cycling human endometrium. Mucin mRNA expression was determined relative to that of epithelial marker, cytokeratin-18 (KRT18) by qRT–PCR as described in Materials and Methods. Mucins were expressed at similar levels at all stages of the cycle with very low expression of MUC4. The box plot shades the second and third quartiles of the data. The black line in the box is drawn at the median value. The whiskers above and below show the maximum and minimum values, respectively.

Figure 2  Mucin mRNA levels relative to those of KRT18 (A) and ACTB (B). MUC1, MUC16, KRT18 and ACTB mRNA levels were determined by qRT–PCR as described in Materials and Methods. Box plots were used to express these data as described in the legend to Fig. 1. No significant differences in mucin expression were observed across the cycle when compared with KRT18 mRNA levels.
malignant diseases (May et al., 2010). We sought to profile MUC1, MUC4 and MUC16 mRNA expression in normal endometrium and in endometriosis of women with endometriosis again relative to the epithelial marker, KRT18 mRNA. MUC1 and MUC16 mRNA expression did not vary significantly between these conditions (Fig. 3). MUC4 mRNA was much more difficult to detect than MUC1 and MUC16 in normal endometrium and in endometriosis from endometriosis patients (Fig. 3). Primers to the cytoplasmic tail (CT) region of the MUC4 gene were therefore designed, in addition to the primers to the tandem repeat (TR) region, to detect MUC4 mRNA expression to account for any variability in MUC4 mRNA expression (Table I). We confirmed minimal levels of MUC4 mRNA expression in both normal endometrium and that of women with endometriosis (Fig. 4). Collectively, these data indicated little variation in MUC1, MUC4 and MUC16 mRNA expression in the eutopic endometrium of women with endometriosis when compared with normal endometrium.

**Mucin mRNA profiles in stage-matched endometriotic tissue**

We then sought to compare mucin expression in stage-matched eutopic and ectopic samples from endometriosis patients relative to KRT18 mRNA (Fig. 5). qRT–PCR analyses revealed that MUC1 (Fig. 5A), MUC4 (Fig. 5B) and MUC16 (Fig. 5C) mRNA expression did not vary between eutopic and ectopic stage-matched endometriotic tissues. MUC4 mRNA expression was not significantly higher in ectopic endometriotic tissue (Fig. 5B) and was still very low relative to that of MUC1 and MUC16. Collectively, these results were in accordance with the above experiment and showed little variation in mucin expression in stage-matched eutopic and ectopic endometriotic tissue.

**Transmembrane mucin protein detection by immunohistochemistry**

To determine the localization of each mucin, immunohistochemistry was performed on frozen sections from normal and mid-secretory endometriosis grade II endometrium (Fig. 5A–L). Robust MUC1 and MUC16 localization was confirmed on the apical surface of epithelia (Fig. 5A–D). Localization of MUC16 protein expression to epithelia was confirmed using the pan-cytokeratin antibody (Fig. 5E–L). We performed immunohistochemistry for MUC4 only in endometrium from endometriosis patients since MUC4 mRNA expression was barely detectable in normal cycling endometrium. Confirming the results from MUC4 qRT–PCR analyses, a lack of reactivity with the MUC4-specific antibody was observed in sections of mid-secretory endometriotic tissue (Fig. 5E–H). Pan-cytokeratin staining was performed to identify epithelial cells in each case (Fig. 5G and 5J). MUC4 immunohistochemistry on frozen sections from proliferative stage endometrium from endometriosis patients was also performed (Supplementary data, Fig. S1). Similar to the results obtained in mid-secretory endometriosis grade tissues, MUC4 protein expression was undetectable (Supplementary data, Fig. S1B) while MUC1 and MUC16 were expressed (Supplementary data, Fig. S1A). Recognition of MUC4 by the 8G7 antibody was confirmed by positive immunostaining for MUC4 in the pancreatic adenocarcinoma cell line, HPAF (Supplementary data, Fig. S1C and D). Collectively, these data confirm that MUC1 and MUC16 expression are restricted to endometrial epithelial cells and that MUC4 protein is not present at appreciable levels in endometriosis.

**Discussion**

Mucin expression and distribution changes in several gynecological pathologies, including endometrial cancer and endometriosis (He et al., 2008). 1734 Dharmaraj et al.
Endometriosis is a prevalent endocrine-driven disease occurring in almost 45% of all women receiving laparoscopic examination and in \( \sim 10\% \) of all women presenting with clinical symptoms (Lessey, 2000; Winkel, 2003; de Ziegler et al., 2010). Symptoms range from mild to severe pelvic pain and infertility (Winkel 2003; de Ziegler et al., 2010) and are cyclical following the rise and fall of ovarian steroid hormones through the menstrual cycle that act on endometriotic tissue as well as the endometrium (Winkel, 2003). The etiology of endometriosis remains unclear, but a common theory is that retrograde menstruation leads to the deposition of endometrial tissue on the ovaries and within the peritoneal cavity. Retrograde menstruation is common in menstruating women, but the reasons that some women get endometriosis and others do not remain an area of active investigation.

The current study focused on quantitative analyses of membrane-tethered mucin (MUC1, MUC4 and MUC16) expression in normal endometrium and eutopic and ectopic endometrium of endometriosis patients. This is the first comprehensive study of these three key membrane-associated mucins in these contexts. Only three reports have examined MUC4 expression by immunohistochemistry in human endometrium (Gipson et al., 1997; Koscinski et al., 2006; Alameda et al., 2007) with no agreement on the expression pattern of MUC4. Gipson et al. (1997) reported a lack of MUC4 expression in normal endometrium while Koscinski et al. (2006) report isolated patches of MUC4 expressed in luminal and glandular epithelium using the same 8G7 antibody utilized in the present studies in infertile and women undergoing IVF. A lack of information on MUC4-specific antibody used by Alameda et al. (2007) hinders interpretation of their results which indicate endometrial MUC4 protein expression in a subset of women. There also is limited information on MUC16 expression in the cycling endometrium, although qRT–PCR has been used to assess levels of MUC16 mRNA levels during the cycle (Gipson et al., 2008). Previous reports have indicated that MUC1 and MUC16 vary in the cycling endometrium with maximal expression in the secretory phase (Hey et al., 1994; Gipson et al., 2008). These studies relied on the use of ACTB or 28S RNA as RNA load controls, i.e. an RNA species found in all endometrial cells. When MUC1 and MUC16 mRNA levels were compared relative to ACTB mRNA, our results were in agreement with previous reports (Hey et al., 1994; Gipson et al., 2008).

We used KRT18 mRNA as a control for potential changes in the epithelial populations in endometrial samples (Moll, 1983). Our data indicate that endometrial MUC1 and MUC16 mRNA expression does not change significantly relative to KRT18 and rather seems to vary in parallel with the changes in the epithelial populations in the endometrium. While it is possible that KRT18 is hormonally regulated, this has not been described. A number of agents including progesterone and proinflammatory cytokines modulate transmembrane mucin expression in uterine cell types and endometrium of various animal models (Hoffman et al., 1998; Brayman et al., 2006, 2007; Dharmaraj et al., 2010). Nonetheless, the current studies indicate that changes in mucin expression are not observed during the normal menstrual cycle nor in endometriotic tissues. Several reports also indicate that serum MUC1 levels are not changed in endometriosis (reviewed in May et al. 2010). One report describes a reduction of MUC1 in endometriosis (Margaret et al. 2010). Endometriosis has been suggested as a precursor for ovarian cancer (Worley et al., 2010) and the serum MUC16 levels test (detected as CA-125) is a widely used test for ovarian cancer. Elevated serum MUC16/CA-125 has also been used as a marker for endometriosis, particularly for Stage III–IV disease (Mol et al., 1998; Vodolazkaia et al., 2012). Our data indicate that MUC16 mRNA expression does not vary in endometriotic tissue through the cycle relative to the levels observed in normal endometrium. Nonetheless, our samples were primarily obtained from patients presenting with stage I–II endometriosis and thus we can only support the notion that MUC16/CA 125 is not a useful marker for early-stage disease.

Previous reports have examined MUC4 expression in the human endometrium (Gipson et al., 1997; Koscinski et al., 2006; Alameda et al. 2010).

**Figure 5** (A) MUC1, (B) MUC4 and (C) MUC16 mRNA expression in stage-matched eutopic and ectopic endometria of endometriosis patients. Six menstrual stage-matched samples, either from secretory or proliferative stage of the menstrual cycle and stage II or III (one sample) endometriosis were analyzed by qRT–PCR for the different mucins as described in Materials and Methods. Box plots were used to express these data as described in the legend to Fig. 1. Changes in levels of MUC4 expression was observed in endometriotic tissue when compared with eutopic endometriotic tissue.
et al., 2007). Using an established qRT–PCR protocol that detects a region of MUC4 mRNA encoding a portion of the ectodomain, we found that MUC4 mRNA expression is at very low levels under all conditions examined. Consistent with this, MUC4 protein was undetectable by immunohistochemistry in proliferative and mid-secretory stage endometriosis. Thus, we conclude that full-length MUC4 is not expressed at appreciable levels in normal endometrium and endometriosis. Although we found a trend toward higher MUC4 mRNA levels in ectopic versus eutopic endometrium of stage-matched endometriosis patients, this change was not significant. It would be interesting to determine whether a significant accumulation of MUC4 expression occurs in advanced stages of endometriosis.

Progesterone resistance is now considered a key player in the etiology of endometriosis (Brosens et al., 2012). Nuclear receptors and nuclear receptor coregulator dysregulation are associated with the progression of endometriosis either due to an increase in estrogen or due to signaling by inflammatory molecules (Han and O’Malley, 2014). Several of these factors, such as PR-A and PR-B, PPARγ and TNFα regulate the expression of transmembrane mucins in various systems (Brayman et al., 2006; Dharmaraj et al., 2010; Wang et al., 2010; Morgado and Carson, unpublished observations). Thus, an understanding of mucins in endometriosis is important. There is great need for endometriosis biomarkers to aid clinical diagnosis and also to monitor the disease. Changes in mucin glycoforms have been suggested to characterize endometrial disease (Sivridis et al., 2002; Seeber et al., 2008). Although we have found no alterations in mucin expression in endometriosis, a comprehensive profile of transmembrane mucin expression, including changes in the oligosaccharide moieties carried by the mucin core proteins, in normal and endometriotic tissues may yet allow for development of meaningful biomarkers. In addition, changes in the oligosaccharide structures carried by mucins may have important biological consequences such as binding of selectins (Carson et al., 2006) or suppression of the immune system (Daniels et al., 2002; Hauselmann and Borsig, 2014). Future studies should focus on how glycoforms of MUC1 and MUC16 change in endometrial disease states in ways that might promote disease progression.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements
The authors appreciate the excellent secretarial and graphics assistance of Ms Sharron Kingston. We also greatly appreciate the routine critical input of Drs Pamela Constantinou-Papadopoulos and Cindy Farach-Carson as well as Mr Brian Engel during the course of these studies.

Authors’ roles
N.D., P.J.C. and M.M. performed all the qRT–PCR and immunostaining experiments, participated in the experimental design and co-wrote the manuscript. S.M.H., S.L.Y. and B.A.L. secured the patient samples and participated in the experimental design, critical discussions and manuscript editing. D.D.C. participated in the experimental design and manuscript preparation.

Figure 6 MUC1, MUC4 and MUC16 expression in normal endometrium and in endometriosis. Frozen sections were stained with antibodies to the indicated mucins as described in Materials and Methods. Pan-cytokeratin staining was used to detect epithelia in each case (F, H, J and L). DAPI staining was used to detect nuclei as described in Materials and Methods. Robust staining for both MUC1 (B and D) and MUC16 (C, D, K and L) were observed in all cytokeratin positive (epithelial) cells. No MUC4 staining was detected (G and H). DAPI: blue; pan-cytokeratin or MUC1: green; MUC4 or MUC16: red. Magnification for all fields is as indicated in (L), 50 μm.
Funding
This study was supported by: NIH R01HD29963 to D.D.C.; NIH U54HD007495 to S.M.H.; and NIH R01HD067721 to S.L.Y. and B.A.L.

Conflict of interest
None declared.

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IL-1β induces p62/SQSTM1 and represses androgen receptor expression in prostate cancer cells

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Abstract

Chronic inflammation is associated with advanced prostate cancer (PCa), although the mechanisms governing inflammation-mediated PCa progression are not fully understood. PCa progresses to an androgen independent phenotype that is incurable. We previously showed that androgen independent, androgen receptor negative (AR−) PCa cell lines have high p62/SQSTM1 levels required for cell survival. We also showed that factors in the HS-5 bone marrow stromal cell (BMSC) conditioned medium can upregulate p62 in AR+ PCa cell lines, leading us to investigate AR expression under those growth conditions. In this paper, mRNA, protein, and subcellular analyses reveal that HS-5 BMSC conditioned medium represses AR mRNA, protein, and nuclear accumulation in the C4-2 PCa cell line. Using published gene expression data, we identify the inflammatory cytokine, IL-1β, as a candidate BMSC paracrine factor to regulate AR expression and find that IL-1β is sufficient to both repress AR and upregulate p62 in multiple PCa cell lines. Immunostaining demonstrates that, while the C4-2 population shows a primarily homogeneous response to factors in HS-5 BMSC conditioned medium, IL-1β elicits a strikingly heterogeneous response; suggesting that there are other regulatory factors in the conditioned medium. Finally, while we observe concomitant AR loss and p62 upregulation in IL-1β-treated C4-2 cells, silencing of AR or p62 suggests that IL-1β regulates their protein accumulation through independent pathways. Taken together, these in vitro results suggest that IL-1β can drive PCa progression in an inflammatory microenvironment through AR repression and p62 induction to promote the development and survival of androgen independent PCa.

Keywords

Interleukin-1β; p62/Sequestome-1; androgen receptor; prostate cancer; bone marrow stromal cells; inflammation

Inflammation is the seventh hallmark of cancer, where pro-inflammatory cytokines activate signaling cascades that promote tumor cell survival, proliferation, angiogenesis, and...
metastasis [Hanahan and Weinberg, 2011]. In primary and metastatic tumors, bone marrow-derived immune cells can infiltrate the tumor and secrete pro-inflammatory cytokines into the tumor microenvironment [Multhoff et al., 2012]. Interleukin-1 beta (IL-1\(\beta\)) is one such pro-inflammatory cytokine that can support tumor progression through paracrine activation of pro-survival pathways in tumor cells [Multhoff et al., 2012].

IL-1\(\beta\) is produced as a 35 kDa pro-IL\(\beta\) protein that is cleaved by the inflammasome complex into the 17 kDa biologically active IL-1\(\beta\) protein [Martinon et al., 2002]. Mature IL-1\(\beta\) is then secreted and binds to its heterodimeric IL-1RI/IL-1RaCP receptor on target cells to initiate IL-1\(\beta\) signaling [Dinarello, 2009]. Monocytes are the primary source of IL-1\(\beta\) [Dinarello, 2009]; but IL-1\(\beta\) can also be produced by other cell types such as fibroblasts [Erez et al., 2009] and epithelial cells [Kogan-Sakin et al., 2009] as a paracrine or autocrine signal.

As part of the inflammation response, IL-1\(\beta\) signaling initiates Nuclear Factor Kappa B (NF\(\kappa\)B) activation [Lawrence, 2009]. NF\(\kappa\)B is a transcription factor that induces expression of pro-inflammatory cytokines, anti-apoptotic proteins, angiogenic proteins, adhesion molecules, extracellular matrix remodeling enzymes, and metastatic factors [Nguyen et al., 2013]. Thus, in the context of chronic inflammation and cancer, IL-1\(\beta\) secreted by bone marrow-derived immune cells can activate NF\(\kappa\)B in tumor cells to drive tumor cell proliferation, survival, and metastasis.

One mechanism of IL-1\(\beta\) activation of NF\(\kappa\)B requires the multifunctional adaptor protein, p62 (also known as Sequestome-1) [Nakamura et al., 2010; Sanz et al., 2000]. As an adaptor protein, p62 has several distinct protein-protein interaction domains, two of which are the ubiquitin binding domain and the TRAF6 binding domain [Moscat and Diaz-Meco, 2012]. Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6) is an E3 ubiquitin ligase that mediates NF\(\kappa\)B activation[Cao et al., 1996; Deng et al., 2000]; and in response to stimuli such as IL-1\(\beta\) and Nerve Growth Factor, p62 binds to and facilitates the polyubiquitination of TRAF6, leading to I\(\kappa\)B Kinase activation and downstream NF\(\kappa\)B nuclear translocation and transactivation [Nakamura et al., 2010; Sanz et al., 2000; Wooten et al., 2005].

Chronic inflammation causes the cytotoxic accumulation of reactive oxygen and nitrogen species, resulting in tissue necrosis and DNA damage that can initiate tumorigenesis [Multhoff et al., 2012]. Once transformed, tumor cells can attenuate intracellular damage from cytotoxic reactive oxygen and nitrogen species through various antioxidant pathways, including activation of the Nuclear Factor Erythroid 2-Related Factor 2 (NRF2) transcription factor [Mitsuishi et al., 2012].

Under homeostatic conditions, NRF2 is sequestered in the cytoplasm by the adaptor protein, Kelch-Like ECH-Associated Protein 1 (KEAP1), marking NRF2 for proteasomal degradation [Mitsuishi et al., 2012]. When ROS is elevated, p62 binds KEAP1 and disrupts the KEAP1-NRF2 interaction, allowing NRF2 to translocate to the nucleus and transactive antioxidant response genes [Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010].

Interestingly, p62 is a gene target for NF\(\kappa\)B [Ling et al., 2012] and NRF2 [Jain et al., 2010]. Thus, p62 participates in a positive feedback loop for signaling pathways regulated by both...
transcription factors. Because NFκB and NRF2 regulated pathways can be antagonistic [Bellezza et al., 2010], p62 is expected to be tightly regulated and function in a dynamic, context-specific manner.

We previously reported that bone marrow stromal cell paracrine factors upregulate p62 in bone metastatic prostate cancer (PCa) cell lines [Chang et al., 2014]. Based on the pleiotropic effects of bone marrow stromal cell paracrine factors on PCa cell lines, including induction of apoptosis, autophagy, or neuroendocrine differentiation [Delk and Farach-Carson, 2012; Zhang et al., 2011], p62 likely has pleiotropic roles in maintaining cellular homeostasis in our model. To begin to gain insight into the role(s) of p62 in PCa cells exposed to bone marrow stromal cell paracrine factors, we first sought candidate paracrine factors that might mediate induction of p62 and chose to explore IL-1β as a candidate cytokine.

IL-1β levels are elevated in tumors [Liu et al., 2013] and serum [Saylor et al., 2012] of prostate cancer (PCa) patients with advance disease. In agreement, IL-1β was shown to induce PCA neuroendocrine differentiation (NED) in vitro [Albrecht et al., 2004; Chiao et al., 1999; Diaz et al., 1998] and promote the skeletal colonization and growth of metastatic PCa cell lines in mice [Liu et al., 2013]. PCa NED is associated with disease progression, poor prognosis, and treatment resistance [Sun et al., 2009]. PCa NED cells produce and secrete proteins that promote tumor cell proliferation, survival, and tumor angiogenesis and do not express the therapeutic target, the androgen receptor (AR) [Sun et al., 2009]. Likewise, PCa bone metastases are aggressive and incurable [Msaouel et al., 2008] and there is evidence that IL-1β accumulation negatively correlates with AR activity and positively correlates with NED in PCa patient bone metastases [Liu et al., 2013].

In this paper, we report that IL-1β can induce p62 mRNA and repress AR mRNA in PCa cell lines and we believe these in vitro results reflect mechanisms by which IL-1β can drive PCa progression and treatment resistance in an inflammatory tumor microenvironment. We propose a model wherein IL-1β, secreted by immune cells in the inflammatory tumor microenvironment or secreted by bone marrow stromal cells in the metastatic niche, can promote the transformation of PCa cells into treatment resistant PCa cells that survive the harsh inflammatory or bone metastatic environments through processes mediated by cell survival proteins like p62.

MATERIALS AND METHODS

Cell Culture

PCa cell lines (LNCaP, C4-2, MDA PCa 2a) and bone marrow stromal cell lines (HS-5, HS-27a) were grown in a 37°C, 5.0% (v/v) CO₂ growth chamber and maintained as described in Chang et al., 2014. Briefly, LNCaP and C4-2 cell lines were cultured in T-medium (Gibco/Invitrogen) supplemented with 5% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals), MDA PCa 2a cell line was cultured in BRFF-HPC1 medium (AthenaES; 0403) supplemented with 20% (v/v) FBS, and HS-5 and HS-27a cell lines were cultured in low glucose DMEM medium (Gibco/Invitrogen) supplemented with 10% FBS.
**Conditioned Medium Treatment**

Bone marrow stromal cell conditioned media was obtained as described in Chang et al., 2014. Briefly, conditioned T-medium was collected from bone marrow stromal cells after 3 days incubation.

**Cytokine and siRNA Treatments**

*Cytokines:* Recombinant human interleukin-1 beta (IL-1β) (R&D Systems; 201-LB/CF), recombinant human interleukin-6 (IL-6) (R&D Systems; 206-IL/CF). IL-1β was diluted in 0.1% (w/v) filtered bovine serum albumin (BSA) (Sigma-Aldrich; A7906). IL-6 was diluted in 1X sterile phosphate buffered saline (PBS) (VWR; 71002-822). 0.1% BSA served as the vehicle control. *siRNA treatments:* Cells were transfected with a pool of four unique AR siRNA duplexes (Thermo Scientific; M-003400-02-0005) or a pool of three unique p62/ SQSTM1 siRNA duplexes (Origene; SR305865) using siTran 1.0 transfection reagent (Origene; TT300001). The Trilencer-27 Universal Scrambled Negative Control siRNA Duplex was used as a negative control (Origene; SR30004). Immunostaining was used to confirm protein loss.

**Western Blot Analysis and Antibodies**

Western blot analysis was performed as described in Chang et al., 2014. Briefly, protein was isolated from cells using NP40 lysis buffer, loaded onto sodium dodecyl sulfate polyacrylamide gel, and transferred from the gel to nitrocellulose membrane. Total protein was visualized using Ponceau S (Sigma; P7170). Protein blot bands were visualized using SuperSignal West Femto chemiluminescent substrate (Fisher Scientific; PI34095) and imaged using the Fujifilm LAS-4000 imager (Fuji). Ponceau S stain or β-actin was used as the loading control. *Primary antibodies:* Androgen Receptor (AR N-20) (Santa Cruz; sc-816), p62/SQSTM1 (Abnova; H00008878-M01), β-actin (Novus Biologicals; NB600-505). *Secondary antibodies:* sheep anti-mouse (Jackson ImmunoResearch Laboratories; 515-035-062), goat anti-rabbit (Sigma-Aldrich; A6154).

**RNA Extraction and Quantitative Polymerase Chain Reaction (QPCR)**

RNA was extracted as described in Chang et al., 2014. Reverse transcription and QPCR were done in a one-step reaction using the Verso 1-step RT-qPCR SYBR Green kit according to the manufacturer’s instructions (Thermo Scientific; AB-4104/A). Primers specific for Androgen Receptor or p62/SQSTM1 were used and data was normalized to the β-actin transcript levels. Relative mRNA levels were calculated using the 2−ΔΔCT method. *Androgen Receptor primers:* Forward: AAG ACG CTT CTA CCA GCT CAC CAA; Reverse: TCC CAG AAA GGA TCT TGG GCA CTT. *p62 primers:* Forward: AAA TGG GTC CAC CAG GAA ACT GGA; Reverse: TCA ACT TCA ATG CCC AGA GGG CTA. *β-actin primers:* Forward: GAT GAG ATT GGC ATG GCT TT; Reverse: CAC CTT CAC CGG TCC AGT TT.

**Immunofluorescence**

Immunofluorescence was carried out as described in Chang et al., 2014. Briefly, cells were fixed and permeabilized with 100% methanol, blocked with 5% BSA in 1X PBS, and
incubated with antibodies at 4°C overnight. Cells were mounted using ProLong Gold Antifade with DAPI (Life Technologies; P36935). **Primary antibodies:** Androgen Receptor (AR N-20) (Santa Cruz; sc-816), p62/SQSTM1 (Abnova; H00008878-M01). **Fluorescently labeled secondary antibodies:** Alexafluor 488, goat anti-mouse (Invitrogen; A11001), Alexafluor 568, goat anti-rabbit (Invitrogen; A11061).

**Microscopy**

Images were taken and processed using the Eclipse TE300 inverted microscope (Nikon) and NIS Elements software (Nikon) or Zeiss axiscope (Carl Zeiss GmbH) and AxioVision software (Carl Zeiss GmbH). Scale bar = 50 μm.

**Cell Response Percentage**

To determine the percentage of cells in a cell population that down regulated nuclear AR accumulation or enhanced p62 cytoplasmic accumulation, images of immunostained cells were acquired on the Eclipse TE300 inverted microscope (Nikon) and immunostained control-treated cells were used to establish baseline fluorescence intensity for nuclear AR and p62 diffuse cytoplasmic accumulation using NIS Elements software (Nikon). Cells with nuclear AR fluorescence intensity lower than baseline or p62 diffuse cytoplasmic intensity higher than baseline were counted as responsive to treatment. Cells grown in control condition medium, vehicle control, or IL-1β accumulated little to no p62 speckles and were therefore assayed for cytoplasmic p62 intensity. HS-5 BMSC conditioned medium-treated cells were assayed for p62 speckle accumulation. Total cells counts were obtained from five or more microscopy fields for each treatment. Total cell counts for each treatment were as follows: control growth medium, n > 2300; HS-5 conditioned medium, n > 850; vehicle control, n > 2300; IL-1β, n > 3800.

**Statistics**

Statistical significance was determined using unpaired student t test.

**RESULTS**

**HS-5 bone marrow stromal cell paracrine factors repress AR mRNA, protein, and nuclear accumulation in C4-2 PCa cells**

We previously reported that HS-5 BMSC paracrine factor(s) upregulate p62 mRNA and protein in AR+ PCa cell lines [Chang et al., 2014]. On the other hand, AR− PCa cell lines have high basal p62 mRNA [Chang et al., 2014]. This led us to speculate that the loss of AR and the upregulation of p62 accumulation are linked. In accordance, we discovered that HS-5 BMSC conditioned medium down regulated AR mRNA, protein, and nuclear accumulation in AR+ C4-2 cells (Fig. 1A–C). However, relative to control growth medium, HS-27a BMSC conditioned medium did not repress AR mRNA, protein, or nuclear accumulation in C4-2 cells (Fig. 1A–C). Thus, as with the induction of p62 [Chang et al., 2014], the repression of C4-2 AR mRNA, protein, and nuclear accumulation is also specific to the HS-5 BMSC paracrine factor milieu.
Interleukin 1-beta (IL-1β) is sufficient to repress AR mRNA and induce p62 mRNA in C4-2 PCa cells

The regulation of AR and p62 described above occurs for C4-2 cells grown in HS-5 BMSC conditioned medium, but not for C4-2 cells grown in HS-27a BMSC conditioned medium (Fig. 1; [Chang et al., 2014]). Therefore, we sought candidate paracrine factor(s) that might mediate HS-5 BMSC regulation of AR and p62 in C4-2 cells. Comparative gene expression analysis of HS-5 BMSCs versus HS-27a BMSCs led us to test the effect of interleukin-1 beta (IL-1β) on AR and p62 accumulation in C4-2 cells. IL-1β mRNA levels are approximately 83 times higher in HS-5 BMSCs than HS-27a BMSCs [Graf et al., 2002] and HS-5 BMSCs secrete IL-1β protein [Roecklein and Torok-Storb, 1995]. Furthermore, IL-1β has been shown to block AR activity in a PCa cell line [Culig et al., 1998]. Indeed, we found that 25 ng/ml IL-1β was sufficient to represses AR mRNA levels and nuclear accumulation in C4-2 cells (Fig. 2A & B). In addition, IL-1β upregulated p62 mRNA levels and protein accumulation in C4-2 cells (Fig. 2A & B).

p62 sequesters ubiquitinated protein aggregates into the autophagosome for degradation, therefore, p62 accumulation could indicate aberrant autophagy-mediated degradation of p62 [Bjorkoy et al., 2005]. Treatment with the autolysosomal inhibitor, chloroquine, increased p62 accumulation in IL-1β-treated cells, demonstrating that autophagy-mediated degradation of p62 was preserved in IL-1β-treated cells (Fig. 2C). Thus, as with HS-5 BMSC conditioned medium, IL-1β can repress AR and upregulate p62.

The C4-2 PCa cell population shows a heterogeneous response to IL-1β regulation of AR and p62

While, both HS-5 BMSC conditioned medium and IL-1β can repress AR mRNA and induce p62 mRNA in C4-2 cells (Fig. 1 & 2, [Chang et al., 2014]), IL-1β is likely not the only factor in the HS-5 BMSC milieu regulating these proteins. For example, HS-5 BMSC conditioned medium reproducibly enhanced p62 speckle accumulation in C4-2 cells (Fig. 3, data not shown), while treatment with 25 ng/ml IL-1β for a similar period of time primarily enhanced diffuse p62 accumulation (Fig. 2B & 3, data not shown). p62 speckles or aggregates are the active organizing centers for various p62-mediated signaling pathways [Moscat and Diaz-Meco, 2009]. This suggests that while both factors in HS-5 BMSC conditioned media and IL-1β can induce p62 mRNA, their posttranslational regulation of p62 may differ.

In preliminary experiments, various recombinant human IL-1β concentrations and treatment time points could induce p62 mRNA or protein accumulation in multiple cell lines, including the LNCaP, C4-2, and MDA PCa 2A prostate cancer cell lines and the T47D breast cancer cell line (Supplemental Data; data not shown). However, the level of p62 induction observed in pooled cell populations using QPCR or western blot analysis was either subtle or inconsistently detectable (data not shown). Therefore, we employed a more sensitive and informative approach by using immunostaining to analyze the effects of IL-1β on individual cells. Focusing on the C4-2 cell line, we co-immunostained for AR and p62 and discovered that, while the C4-2 cell population showed a largely uniform response to factors in HS-5 BMSC conditioned medium, only a portion of the cells responded to...
treatment with recombinant human IL-1β (Fig. 3 & 4). For example, we found that 93% of the C4-2 cell population grown in HS-5 BMSC conditioned medium for two days showed reduced or no nuclear AR staining, 100% showed p62 speckle accumulation, and 93% of the population showed concomitant loss of nuclear AR and enhanced p62 speckle accumulation (Fig. 4). On the other hand, 66% of the C4-2 cell population grown in 25 ng/ml IL-1β for two days showed AR loss, 18% showed p62 induction, and 9% showed concomitant AR loss and p62 induction (Fig. 4). Thus, the response of the C4-2 cell population to HS-5 BMSC conditioned medium regulation of AR and p62 is primarily homogenous, while the response to IL-1β alone is strikingly heterogeneous.

The heterogeneous response of the C4-2 PCa cell population to IL-1β could not be attributed to treatment with insufficient recombinant IL-1β protein concentration; for, while our western blot method could detect the 17 kDa IL-1β protein in 20 μl of 25 ng/ml of recombinant IL-1β protein, we were not able to detect IL-1β protein in a comparable volume of the HS-5 BMSC conditioned medium (Supplemental Data). Thus, factors other than, or in addition to, IL-1β in the HS-5 BMSC conditioned medium regulate AR and p62 mRNA and protein levels in C4-2 PCa cells.

**IL-1β represses AR and upregulates p62 through independent pathways**

Given that factors in HS-5 conditioned medium and IL-1β can both regulate AR and p62 in C4-2 cells (Fig. 1–4) and given that AR- PCa cell lines have high basal p62 levels [Chang et al., 2014], we hypothesized that AR and p62 regulation are interdependent. To test our hypothesis, we used siRNA to down-regulate AR mRNA and protein in AR+ C4-2 PCa cells and co-immunostained cells for AR and p62 to determine the effect on p62 accumulation. Under control growth conditions, loss of AR did not upregulate p62 accumulation (Fig. 5). Likewise, loss of AR protein did not enhance IL-1β upregulation of p62 in C4-2 PCa cells (Fig. 5). Conversely, siRNA-mediated loss of p62 protein in C4-2 PCa cells did not prevent the down regulation of AR induced by IL-1β (Fig. 5). Furthermore, analysis of the heterogeneous response of C4-2 cells to IL-1β, revealed that of the 66% of cells that showed reduced nuclear AR accumulation, 57% lost nuclear AR without a concomitant increase in p62; and of the 18% of cells that increased p62 accumulation, 9% upregulated p62 without the loss of nuclear AR (Fig. 4). Taken together, these data suggest that the IL-1β regulation of p62 induction and AR repression are independent pathways.

**DISCUSSION**

**IL-1β can contribute to androgen-independent PCa by repressing AR expression and upregulating p62**

Chronic inflammation is implicated in PCa progression [Gueron et al., 2012]. One course of PCa progression is the development of androgen independent PCa, which is often metastatic and is incurable [Grossmann et al., 2001]. Androgen independent PCa cells have reduced or no dependence on androgen for survival due to AR over-accumulation or AR gain-of-function mutations [Grossmann et al., 2001]. Androgen independences can also result from loss of AR expression and the upregulation of compensatory cell survival mechanism (e.g. Bcl-2 overexpression) [Grossmann et al., 2001; Sun et al., 2009]. As such, chronic
inflammation likely promotes PCa progression to androgen-independent disease through inflammatory cytokine signaling. As demonstrated in this report, the inflammatory cytokine, IL-1β, can repress AR expression in PCa cell lines (Fig. 2, Supplemental Data). Thus, we propose that IL-1β secreted by bone marrow-derived immune cells infiltrating the primary PCa tumor or secreted by bone marrow stromal cells in the PCa bone metastatic niche, can repress AR expression, thereby contributing to androgen independence.

Because the loss of AR can be cytotoxic for PCa cells, AR− PCa subtypes (e.g. small cell PCa, neuroendocrine PCa) have high levels of pro-survival proteins [Sun et al., 2009]. Indeed, we have shown that the AR− PCa cell lines, DU145 and PC3, have high basal p62 levels that is required for their cell survival [Chang et al., 2014]. In this report, we have shown that IL-1β upregulates p62 expression in PCa cell lines (Fig. 2, Supplemental Data). Therefore, it is intriguing to speculate that chronic exposure to IL-1β in an inflammatory tumor microenvironment contributes to androgen independence, not only by repressing PCa AR expression, but also by upregulating p62 to help maintain cellular homeostasis.

According to the GeneCard database, p62 forms various complexes with at least 30 different proteins. Therefore, p62 could be involved in a myriad of processes that protect PCa cells in an inflammatory environment. For example, it is plausible that IL-1β-induced p62 expression increases the pool of p62 available to integrate cues from the NFκB inflammatory response, NRF2 antioxidant response, and autophagy, in order to attenuate cytotoxic ROS accumulation and clear ROS-damaged protein aggregates. It will be important to identify the role(s) of p62 in PCa cell response to IL-1β to elucidate potential mechanism of PCa disease progression.

One aspect of PCa progression is development of treatment resistance; and androgen independence is characteristic of treatment-resistant, metastatic disease. For example, the AR-targeting therapies, androgen deprivation therapy (ADT) and anti-androgens are initially effective at attenuating PCa progression. However, many patients subsequently develop androgen independent PCa cell growth and relapse within a few years of treatment [Beltran et al., 2011]. Cytotoxic chemotherapy is employed as a first line of defense against androgen independent PCa, but has historically shown limited efficacy [Beltran et al., 2011]. Our data suggests that IL-1β could contribute to such therapeutic resistance by repressing expression of the therapeutic target, AR, thereby rendering ADT or anti-androgens ineffective. Furthermore, by upregulating pro-survival p62, IL-1β could protect PCa cells from cytotoxic chemotherapy. Despite extensive clinical studies of various drugs, including chemotherapeutic agents, radiopharmaceuticals, and bisphosphonates, androgen independent PCa, particularly bone metastatic PCa, remains incurable [Beltran et al., 2011; Mukherji et al., 2014]. Therefore, based on our data, we contend that IL-1β-targeted therapy should be explored as an adjuvant to both mitigate and treat androgen independent PCa. Ongoing clinical trials using an IL-1 receptor antagonist in combination with chemotherapy for various advanced cancers (ClinicalTrials.gov) will provide useful insight into this treatment strategy.
Factors other than IL-1β likely mediate HS-5 BMSC regulation of AR and p62 in PCa cells

Among its pleiotropic effects on PCa cells, HS-5 BMSC conditioned media induces neuroendocrine differentiation [Zhang et al., 2011], induces cytoprotective autophagy [Delk and Farach-Carson, 2012], represses AR expression (Fig. 1), and upregulates p62 expression and speckle accumulation (Fig. 3, [Chang et al., 2014]). Taken together, the HS-5 BMSC conditioned medium paracrine factors promote the development and maintenance of androgen independent PCa by altering molecular programs (e.g. loss of AR expression) and capitalizing on cytoprotective mechanisms (e.g. autophagy induction); and identifying these paracrine factors will be important for developing therapeutic interventions.

Using the results of differential gene expression analysis [Graf et al., 2002], we focused on IL-1β and, previously, on IL-6, as candidate paracrine factors because their genes are highly expressed in HS-5 BMSCs, they are secreted by HS-5 BMSCs [Roecklein and Torok-Storb, 1995], and both IL-1β and IL-6 can induce PCa neuroendocrine differentiation in vitro [Diaz et al., 1998; Sun et al., 2009]. We previously reported that IL-6 induces autophagy [Delk and Farach-Carson, 2012] and in this report, we demonstrate that IL-1β both represses AR and induces p62 expression (Fig. 2, Supplemental Data) in PCa cells. Unlike IL-6, which is readily detectable by western blot in HS-5 BMSC conditioned medium [Delk and Farach-Carson, 2012], under the same conditions, we do not detect IL-1β in the HS-5 BMSC conditioned by western blot, but can detect recombinant human IL-1β (Supplemental Data).

In addition, the recombinant human IL-1β concentrations we used in our experiments elicit only a heterogeneous response for AR and p62 regulation in the PCa cell population, while HS-5 BMSC conditioned media elicits a nearly homogeneous response (Fig. 3 & 4). Thus factors other than, or in addition to, IL-1β mediate HS-5 BMSC repression of AR and upregulation of p62 expression in PCa cells.

Finally, in addition to upregulating p62 expression, factors in HS-5 BMSC conditioned medium promote p62 speckle accumulation in PCa cells (Fig. 3). However, treatment with IL-1β for the same period of time does not induce p62 speckles (Fig. 3). Thus, paracrine factors other than IL-1β are likely signaling the formation of active p62 complexes in PCa cells exposed to HS-5 BMSC conditioned medium.

The heterogeneous response of the C4-2 PCa cell line to IL-1β is a useful model for tumor heterogeneity

While virtually the entire C4-2 cell population shows concomitant AR loss and p62 upregulation when grown in HS-5 BMSC conditioned medium, it is unclear why only a small percentage of the C4-2 cell population shows the same response when grown in a sufficient concentration of IL-1β for the same time period (Fig. 3 & 4). We cannot attribute the heterogeneous response of the C4-2 cells to degraded, inactive recombinant human IL-1β, because full length IL-1β is remains detectable in the media by western blot throughout the duration of the experiment (data not shown). In addition, the cells are grown in a monolayer in vitro, allowing equal exposure to the recombinant human IL-1β. The strikingly heterogeneous response suggests that there is a subpopulation of IL-1β sensitive cells in the otherwise isogenic cell line that may ultimately have a survival advantage by upregulating proteins like p62 or by developing into androgen independent cells through the
loss of AR expression. Importantly, we can take advantage of the heterogeneous response to IL-1β and use systems biology approaches to identify relevant molecular networks that can predict those cells in a heterogeneous tumor that will develop into aggressive disease as a result of chronic inflammation and predict cell populations that would respond to cytokine-targeted therapy.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

Grant sponsor: Grant numbers: NIH/NCI K01 CA160602; NIH/NCI F32 CA128296; NIH/NCI P01 CA098912.

We would like to thank all of the members of the labs of Drs. Mary C. Farach-Carson, Daniel Carson, and Nora Navone and all of the members of the Prostate Cancer P01 group for scientific discussion and/or technical support. This work was supported by NIH Funding support from NIH/NCI K01 CA160602, NIH/NCI F32 CA128296, and NIH/NCI P01 CA098912.

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J Cell Biochem. Author manuscript; available in PMC 2015 December 01.


Nguyen DP, Li J, Yadav SS, Tewari AK. Recent insights into NF-kappaB signalling pathways and the link between inflammation and prostate cancer. BJU Int. 2013


Figure 1. HS-5 BMSC conditioned medium represses AR in C4-2 PCa cells
C4-2 cells were grown in control growth medium or conditioned medium from HS-5 or HS-27a BMSCs for two days. C4-2 cells were analyzed for AR mRNA, protein, or subcellular localization. A) QPCR, B) western blot, and C) immunostaining for AR revealed that, in comparison to control or HS-27 BMSC conditioned media, HS-5 BMSC conditioned medium repressed AR mRNA, protein, and nuclear accumulation in C4-2 cells. For QPRC, relative mRNA levels were normalized to control growth medium; error bars represent standard deviation of three biological replicates; p-value (*) = 0.008. For western blot, Ponceau stain serves as a loading control. For immunostaining, dapi is a nuclear stain and scale bar = 50 μm.
Figure 2. IL-1β represses AR and induces p62 in C4-2 PCa cells

C4-2 cells were grown in vehicle control or 25 ng/ml recombinant human IL-1β protein for two days and analyzed for AR or p62 accumulation. A) QPCR demonstrated that IL-1β was sufficient to repress AR mRNA accumulation (p-value = 0.01) and upregulate p62 mRNA levels (p-value = 0.08) in C4-2 cells. Relative mRNA levels were normalized to control growth medium and error bars represent standard deviation of three biological replicates. B) Co-immunostaining for AR and p62 protein revealed that IL-1β repressed nuclear accumulation and induced p62 subcellular protein accumulation in C4-2 cells. Scale bar = 50 μm. C) C4-2 cells were grown for three days in vehicle control or 25 ng/ml recombinant human IL-1β in the absence or presence of 40 μM chloroquine (CQ), and analyzed for p62 protein by western blot. CQ blocks autophagy-mediated degradation of p62 in the vehicle control and in the presence of IL-1β, indicating that IL-1β induction of p62 is not due to abrogated p62 degradation.
Figure 3. HS-5 BMSC conditioned medium and IL-1β differently regulate p62 subcellular accumulation
C4-2 cells were grown in HS-5 BMSC conditioned medium or 25 ng/ml recombinant human IL-1β protein for two days and co-immunostained for AR and p62. HS-5 BMSC conditioned medium repressed AR nuclear accumulation and induced p62 speckle accumulation (inset). IL-1β repressed AR nuclear accumulation and induced diffuse p62 accumulation (representative cells indicated by arrows). Scale bar = 50 μm.
Figure 4. The C4-2 PCa cell population shows a heterogeneous response to IL-1β regulation of AR and p62

C4-2 cells were grown in HS-5 BMSC conditioned medium or 25 ng/ml recombinant human IL-1β protein for two days and co-immunostained for AR (red) and p62 (green). Image: Shown is a merged image of a representative responsive cell showing concomitant nuclear AR loss and diffuse cytoplasmic p62 upregulation (arrow). Graph: The percentage of cells showing the loss of nuclear AR, the upregulation of speckled/diffuse p62, or both was graphed. Ninety-three percent of C4-2 cells grown in HS-5 BMSC conditioned medium showed concomitant loss of nuclear AR and induction of p62 speckles, while 9% of the C4-2 cells grown in IL-1β showed concomitant AR loss and diffuse p62 induction, indicating that the response of the C4-2 cell population to IL-1β is heterogeneous.
Figure 5. IL-1β regulates AR and p62 through independent pathways
C4-2 cells were grown vehicle control or 25 ng/ml recombinant human IL-1β protein for two days in the presence of 40 μM control siRNA, AR siRNA, or p62 siRNA and then co-immunostained for AR and p62. Loss of AR protein did not induce p62 accumulation in vehicle control-treated or IL-1β-treated cells. Loss of p62 protein did not down regulate AR in vehicle control-treated cells or inhibit IL-1β-mediated AR repression. Arrows indicate representative cells.