EXPLORATION OF YPEL3 RESPONSE TO HORMONES AND ABILITY TO INDUCE SENESCENCE.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

JOSEPH EDWARD ROTSINGER
B.S., The University of Toledo, 2009

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2012
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION
BY Joseph Rotsinger ENTITLED EXPLORATION OF YPEL3 RESPONSE TO HORMONES AND
ABILITY TO INDUCE SENESCENCE BE ACCEPTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract


p53 activation through different cellular senescence pathways can trigger cell cycle arrest via regulation of p53 target genes. One such target gene is YPEL3 which is expressed upon binding of tumor suppressor protein p53 at its p53 binding sites (Kelley, 2010). The ability of p53 to induce YPEL3 gene expression led to the discovery that YPEL3 is one of several p53 target genes which induce cellular senescence (Kelley, 2010). Additionally YPEL3 can be regulated independently of p53 by estrogen signaling through estrogen receptor α (Tuttle, 2011). The loss of estrogen receptor α or removal of estrogen induces YPEL3 gene expression and leads to cellular senescence, indicating that estrogen bound to estrogen receptor α represses YPEL3 gene expression (Tuttle, 2011). Although YPEL3 induction results in cellular senescence the mechanism by which YPEL3 elicits cellular senescence is not well understood. It is also unknown if other steroid hormones, such as testosterone play a role in regulating YPEL3 gene expression.

To further understand hormone regulation of YPEL3 the first part of this thesis tested if testosterone regulates YPEL3 gene expression in MCF7 breast cancer cells and LnCAP prostate cancer cells. Like MCF7 breast cancer cells, LnCAPs cultured in the absence of
steroid hormones induced YPEL3 expression indicating that YPEL3 gene expression in LnCAPs is repressed by steroid hormones. This induction of YPEL3 expression was blocked by the addition of testosterone to LnCAP cells. In contrast the addition of testosterone to steroid deprived MCF7 cells resulted in YPEL3 induction. Based on the results in LnCAP prostate cancer cells and MCF7 breast cancer cells it appears that testosterone's effect on YPEL3 gene expression is tissue specific.

In part two of this thesis MCF7 and IMR90 cells were employed to determine if over expression of YPEL3 leads to increased reactive oxygen species (ROS) levels. First an optimized method for detecting reactive oxygen species levels in breast cancer cells using DCFDA was developed. Utilizing this method, MCF7 human breast cancer cells harboring a Tet-On system expressing YPEL3 induced with tetracycline did not show increased levels of reactive oxygen species over LacZ expressing MCF7 cells. Additionally Infecting MCF7 cells with lentivirus expressing YPEL3 and probing with DCFDA showed no increase of ROS levels. Alternatively IMR90 primary diploid human fibroblasts containing a normal repertoire of genes and fully functional pathways were infected with lentivirus expressing YPEL3 and also did not show an increase in ROS levels. These results suggest that YPEL3 activates senescence in a ROS independent manner.

The third part of this thesis was to identify YPEL3 interacting proteins. Epitope tagged YPEL3 proteins obtained from MCF7 tetracycline responsive cells expressing YPEL3 were captured from cell extracts by co-immunoprecipitation, followed by elution and denaturing. Denatured proteins were separated by SDS-Page gel electrophoresis.
and potential protein bands excised for composition analysis by LC/MS/MS. LC/MS/MS analysis identified potential proteins that interact with YPEL3.

The cumulative findings of this thesis were designed to aid in the understanding of YPEL3 regulation by testosterone and to assist in locating potential downstream targets of YPEL3 that may lead to senescence.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>2</td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>5</td>
</tr>
<tr>
<td>Induction of ROS during cellular senescence</td>
<td>6</td>
</tr>
<tr>
<td>Yippee Like 3 (YPEL3)</td>
<td>9</td>
</tr>
<tr>
<td>YPEL3 is inactivated human cancers</td>
<td>13</td>
</tr>
<tr>
<td>Purpose</td>
<td>14</td>
</tr>
<tr>
<td>II. Materials and Methods</td>
<td>16</td>
</tr>
<tr>
<td>Cells and Reagents</td>
<td>16</td>
</tr>
<tr>
<td>Virus Infections</td>
<td>16</td>
</tr>
<tr>
<td>Generating and Detecting Intracellular ROS</td>
<td>18</td>
</tr>
<tr>
<td>RNA Isolation</td>
<td>19</td>
</tr>
<tr>
<td>Reverse Transcription</td>
<td>19</td>
</tr>
<tr>
<td>Taqman Based PCR</td>
<td>20</td>
</tr>
</tbody>
</table>
Protein Extraction

Co-Immunoprecipitation

SDS-PAGE Electrophoresis

Western Blot

Colloidal Blue and Silver Staining

III. Hormone Regulation of YPEL3

The effects of testosterone on YPEL3 expression in LnCAPs

The effects of Testosterone on YPEL3 Expression in MCF7s

IV. Reactive oxygen species do not regulate YPEL3 induced cellular senescence

V. Co-Immunoprecipitation of YPEL3-V5 reveals potential bound proteins

VI. Discussion

YPEL3 gene expression is repressed in prostate cancer by testosterone

YPEL3 gene expression is repressed in breast cancer cells following the addition of testosterone, but is not affected by the addition of progesterone.

Increased levels of YPEL3 gene expression does not trigger increased ROS levels

Potential Proteins that Interact With YPEL3 Proteins.

Conclusion
List of Figures

Figure 1: YPEL3 Expression Is Induced In LnCAP Cells Grown In CSS. 27

Figure 2: YPEL3 is repressed in LnCAP cells in the presence of Testosterone. 29-30

Figure 3: YPEL3 Repression is inversely related to PSA expression in LnCAP Cells exposed to 10 nM Testosterone for 48 hours. 32

Figure 4: Testosterone Induction and β-estradiol Repression of YPEL3 in MCF7 Cells. 35

Figure 5: In MCF7 Cells YPEL3 is Induced in the Presence of Increasing Doses of Testosterone. 37

Figure 6: Hydrogen Peroxide produces cellular reactive oxygen species levels with detectable DCFDA. 41

Figure 7: MCF7 cells scraped in DPBS provides a better mechanism to isolate MCF7 cells for vehicle for analysis by flow cytometry. 43

Figure 8: Allowing DCFDA treated MCF7 cells to recover in complete media after DCFDA exposure does not lead to improved ROS positive detection. 46-47
Figure 9: Tetracycline induced YPEL3 expression in MCF7 cells does not lead to higher ROS production when compared to LacZ induced MCF7 cells.

Figure 10: MCF7 cells infected with lentivirus expressing YPEL3 does not lead to increased ROS production.

Figure 11: IMR90 Cells Infected With YPEL3 Expresing Lentivirus Does Not Lead To Increased ROS Production.

Figure 12: Extraction of YPEL3-V5 by co-immunoprecipitation.

Figure 13: Comparison of three Silver Stain Methods.

Figure 14: Secondary elution revealing 2 bands in the YPEL3 only lane.

Band 1 was between 140kDa-100kDA and band 2 between 70kDa-50kDA.

Figure 15: Band 3 located between the 50kDa and 70kDa molecular weight markers.

Figure 16: The fourth extracted band from immunoprecipitated material appeared between the 25kDa and 35kDa molecular weight bands.
List of Tables

Table 1: Lentiviral Plasmid and Antibiotic Doses for MCF7 and IMR90 cell lines. 17

Table 2: Liquid chromatography tandem mass spectrometry analysis 74-80
(LC/MS/MS)
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In Loving Memory of Joseph H. Smith
I. Introduction

Compiled data from 1970 to 2002 showed heart disease as the leading cause of death in the United States, with cancer ranking second but predicted to overtake heart disease as the leading cause of death (Ahmedin, 2005). This is not due to an increase in the number of cancer related deaths; rather it is due to a 52.1% reduction in the number of deaths from heart disease, but only a decline of 2.7% in cancer related deaths (Ahmedin, 2005). In 2012 cancer is still projected to be the second leading cause of death in the United States (Siegel, 2012). It is estimated that 1,638,910 new invasive cancer cases will be diagnosed with 577,190 cancer related deaths being projected (Siegel, 2012). In males and females prostate cancer and breast cancer both have the second highest number of diagnoses respectively, behind only skin cancer (Siegel, 2012).

Breast cancer is projected to account for 229,060 cases with the mortality rate projection being 39,920 in both sexes, with males accounting for 1% of new cases as well as deaths (Siegel, 2012). Prostate cancer is projected to account for 241,740 new cases and 28,170 deaths (Siegel, 2012). With nearly 70,000 projected deaths from breast or prostate cancer it becomes even more important to understand the molecular basis of the disease and to improve our ability to prevent and detect these cancers earlier.
Breast Cancer

Critical to early detection of breast cancer is self or clinical breast exams to identify masses that may be forming. Growth progression of a cancerous mass is dependent on the level of the growth stimulating hormone estrogen, and the presence or absence of its receptors. Follicle stimulating hormone is the major stimulator of estrogen production, which occurs mainly in the placenta, corpus luteum and developing follicles in the ovaries. Also, to a lesser extent it is produced in granulosa cells of the ovaries, adrenal glands, fat cells and the breasts. After synthesis estrogen diffuses into cells where it binds one of two types of nuclear hormone receptors in the cytoplasm and is subsequently translocated into the nucleus to impart growth stimulating effects by interacting with estrogen response elements of genes. Nuclear hormone receptors are ligand activated transcription factors that bind to specific DNA sequences known as hormone response elements and are able to repress or activate transcription of genes harboring hormone response elements. There are two estrogen binding nuclear hormone receptors, estrogen receptor α (ER-α) and estrogen receptor β (ER-β), which share a high level of sequence homology overall (Dutertre, 2000). However, they have very little sequence homology at one of the two activation of function domains (AF-1) which is critical to ER-α’s ability to recruit co-regulatory protein complexes for gene expression (Hall, 1999). ER-β contains a repressor at AF-1 that antagonizes ER-α function through dimerization of ER-α and ER-β (Hall, 1999 and Dutertre, 2000). ER-β also causes
competitive repression of ER-α by blocking the ability of ER-α to bind hormone response elements of target genes (Leung, 2007). Another unique characteristic is their different gene expression patterns (Couse, 1997). For example, estrogen receptor α is highly expressed in breast tissue, whereas in prostate tissue estrogen receptor β is the main receptor expressed (Couse, 1997). The most extensively studied estrogen receptor is ER-α, which imparts pro-growth effects by inducing expression of genes that positively regulate cell cycle progression and cell division while repressing genes that are growth suppressive (Preston-Martin, 1990). ER-β is the other nuclear estrogen receptor, however its function is not fully known. ER-β has gone unnoticed until it was recently discovered that it may play a role in preventing metastasis of cancerous cells and may also have antiproliferative functions (Roy, 2011). This correlates with findings that suggest ER-β positive breast cancers have a better prognosis than ER-β negative breast cancers (Leung, 2007). ER-β also has the ability to increase cell sensitivity to selective estrogen receptor modulators (SERMs) by mediating their antiestrogenic properties. SERMs elicit an antiestrogenic effect when bound to estrogen receptors by reducing the ability of estrogen to signal cell proliferation. When ER-β binds SERMs it can homodimerize with ER-α and modulate ER-α’s ability to induce proliferation (Leung, 2007). The levels of ER-β expressed can also be used as a marker to determine how well cancer may respond to SERM treatment (Leung, 2007). All of this taken together shows the importance of estrogen levels which may correlate to the rate of estrogen dependent tumor progression, therefore the expression of estrogen’s potential target genes may serve as good prognostic markers.
Estrogen is known to induce pS2 gene expression which produces a pS2 protein (Horiguchi, 1996). In breast tumors the level of pS2 protein has become a potential indicator for predicting treatability, relapse potential and survival (Foekens, 1990). pS2 may also be an indicator of early stage breast cancer, since it may not be expressed in late stage breast cancer that have become independent of growth stimulation by estrogen (Foekens, 1990). Although high levels of pS2 protein may indicate the presence of proliferating breast cancer, it also indicates the presence of ER-α (Foekens, 1990). The presence of ER-α decreases the chance of relapse and increase survival due to a fully functional estrogen receptor that is treatable (Foekens, 1990). ER-α positive breast cancer may benefit from the ability to block growth progression by inhibiting the estrogen receptor with selective estrogen receptor modulators (Park, 2002).

In ER+ breast cancer cells, not only are the levels of estrogen important, but also the levels of testosterone are important due to aromatase activity in breast cancer tissue that can convert androgen substrates into estrogen (Smith, 2003). Aromatase therapy is used clinically to prevent the conversion of testosterone to estrogen, in estrogen receptor positive breast cancers (Smith, 2003). Inhibiting aromatase activity in breast cancer is beneficial in two ways, it results in decreased estrogen levels due to the loss of testosterone conversion to estrogen, and testosterone may actually have a protective effect on male and female breast cancer by inducing cell cycle arrest (Zhou, 2003, Dimitrakakis, 2003, and Lobaccaro, 1993). This protective cell cycle arrest by testosterone could potentially come from the ability of testosterone bound androgen receptors triggering increased p21 expression via an androgen response element.
located 200 bp upstream of the p21 gene and proximal to the promoter region (Lu, 1999). However, testosterone regulation of other genes could play a significant role in arresting a cell when treated with testosterone.

**Prostate Cancer**

Screening for prostate cancer often begins when men receive digital rectal exams as part of a yearly physical to locate any irregularities in the back of the prostate. This region of the prostate is where 85% of prostate cancers occur (Chodak, 1989). If irregularities are found one of the next steps is screening for increased levels of a specific serine protease, known as Prostate Specific Antigen (PSA), due to the correlation of increased PSA levels with testosterone levels and positive prostate cancer diagnosis (Thompsons, 2004). Increased levels of testosterone bound androgen receptors will bind the known target gene PSA’s androgen response element located in its promoter and signal increased gene expression (Thompsons, 2004). The potential for positive diagnosis of high grade cancer with a Gleason score above seven (scale 0-10, with 10 having worst prognosis) increased in men with PSA levels above 4.0 ng/mL of blood serum, and significantly increased when PSA levels increased above 10.0 ng/mL of blood serum (Thompsons, 2004). A concern with this test is that obese men tend to have decreased PSA levels, which may lead to an increase in false negative results (Fowke, 2006). Additionally there are other stresses such as long distance ambulation, ejaculation and aggressive cycling which may lead to increased PSA levels at the time of testing (Leibovitch, 2005). False diagnosis can potentially lead to unnecessary medical
procedures, such as biopsy, or chemotherapy. Prostate cancer may also be associated to low free serum testosterone levels and therefore PSA is not an accurate indicator of potential prostate cancer (Morgenthaler, 1996). It was recently discovered that there may not be an overall benefit to PSA screening. When comparing the survival rates of prostate cancer patients who participated in PSA monitoring to those who did not have PSA screenings there were no survival benefits (Andriole, 2012). A thirteen year study recently concluded that the death rates per 10,000 patients were nearly that same (3.7 Vs. 3.4) when comparing a group undergoing scheduled PSA screening and digital rectal exams to another group which did not undergo scheduled testing. It is possible that PSA screening can false positively diagnose patients with high testosterone levels who do not have prostate cancer but have PSA levels in the positive range, or patients can be negatively diagnosed with androgen independent prostate cancer (Andriole, 2012).

**Induction of Reactive Oxygen Species During Cellular Senescence.**

In the mid 1960’s it was reported that in cell culture primary fibroblasts undergo growth arrest after a finite number of cellular divisions resulting from the erosion of telomeres (Hayflick, 1965). This finite number of divisions became known as Hayflick’s limit (Hayflick, 1965). After Hayflick’s limit is reached cells enter an arrested state and division ceases (Hayflick, 1965). This growth arrest was coined intrinsic replicative senescence. As telomeres deteriorate the exposed chromosomal ends are sensed as DNA damage and a DNA damage response occurs. The DNA damage response can be activated by intrinsic factors such as telomere shortening, as well as extrinsic factors,
such as culture conditions, UV or chemical damage. DNA damage activates ATM and ATR, which lead to activation of p53 via phosphorylation by CHK1/CHK2 (Bennett, 2001). Activation of p53 increases gene expression of p21 which leads to a transient G1 cell cycle arrest and an increase in ROS levels (Passos, 2010). This increase in ROS maintains growth arrest by causing additional DNA damage until the cell is permanently arrested (Passos, 2010). If ROS levels are decreased during transient cell cycle arrest, a cell can reenter the cell cycle and begin replication (Lu, 2009). Not all cellular senescence mechanisms are dependent on DNA damage, but rather a third senescence pathway exists.

Oncogene induced senescence is caused by mitogenic stimuli which can activate RAS and lead to increased levels of ROS in both immortalized cells and human diploid fibroblasts (Moiseeva, 2009). The mechanism by which RAS causes increased ROS production is not fully understood, however via signaling through the RAS/RAF/MEK/ERK signaling pathway, activation of ERK can disrupt the mitochondrial membrane, signal cell cycle arrest through p21 and activate NADPH oxidase which produces superoxide, a form of ROS (Cagnol, 2010 and Serrano, 1997). When p21 gene expression increases it can also increase ROS production which inhibits phosphatases that could inactivate RAS, RAF, MEK or ERK and allows continuous signaling to increase p53 levels through ERK (Passos, 2010 and Serrano, 1997). Cells also trigger increased p53 protein levels via RAF inactivation of AKT which causes dephosphorylation of Mdm2 and blocks Mdm2 mediated degradation of p53 (Cagnol, 2009). Oncogenic signaling through such transcription factors as DMP1 can also activate ARF which blocks MDM2
and stabilizes p53, leading to an increase of p53 (Zindy, 1998). When the level of reactive oxygen species overwhelms the natural antioxidant defenses an oxidative stress response may occur causing mutations and damage to DNA, which induces a DNA damage response (Campisi, 2007).

Common to replicative senescence, the DNA damage response pathway and oncogene induced senescence is the activation of p53. p53 is one of the most important tumor suppressors, and has been given the moniker “Guardian of the Genome” for its ability to actively suppress cellular growth (Lane, 1992). p53 primarily functions as a transcription factor that is kept inactive due to rapid degradation by Mdm2, however various cellular stresses can block Mdm2 mediated degradation leading to p53 accumulation (Louria-Hayon, 2003). This accumulation causes p53 to activate cell cycle inhibitors which induces apoptosis, cellular senescence, or a transient growth arrest that can be reversed (Lowe, 2004 and Vousden, 2002). Common to all tumor formation is inactivation of p53 (Itahana, 2001). When p53 is inactivated it cannot activate its target genes and the ability to maintain controlled cellular growth is lost, which is a hallmark of tumor progression (Itahana, 2001).

Determining the activity of p53 target genes can help explain the functions of p53, however there are hundreds of p53 target genes and complex activation patterns that are cell type and stress specific (Harms, 2004). Some of these gene products are redundant indicating that not just one p53 target is the basis for p53 dependent senescence (Harms, 2004). Some of the targets of p53 that have been linked to a
senescent response include p21, Promyelocytic leukemia PML, Plasminogen Activator Inhibitor 1, and DEC1 (Harms, 2004). Although all of these target proteins elicit cellular senescence, their mechanisms are different, PML acts to stabilize p53 by inhibiting degradation by Mdm2, Plasminogen Activator Inhibitor 1 can trigger replicative senescence and DEC1 mediates p53 dependent G1 cell cycle arrest (Qian, 2010). This signifies the importance of p53 activating many targets to induce growth arrest (Harms, 2004). PML, p21, PAI-1 and DEC1 lead to cellular senescence after activation by p53; however in the absence of these targets p53 can still elicit a senescence response, which indicates that there are other p53 targets that activate senescence and the importance of discovering novel p53 targets that cause cellular senescence (Harms, 2004).

Recently the YPEL3 gene was reported to be directly activated by p53 and capable of eliciting growth arrest in tumor cells (Kelley, 2010). Based on several assays it was determined that YPEL3 induction triggers cellular senescence (Kelley, 2010). However, YPEL3 is not only regulated by p53. It was recently discovered that in breast cancer cells YPEL3 is repressed by estrogen signaling through estrogen receptor α (Tuttle, 2011). Blocking estrogen signaling triggered p53 independent cellular senescence (Tuttle, 2011). The mechanism by which YPEL3 leads to cellular senescence is not readily known. One goal of this thesis is to explore whether YPEL3 expression triggers ROS.

Yippee Like 3 (YPEL3)

Yippee was discovered in Drosophila via a yeast interaction trap that screened for proteins with potential interactions to hemolin of Cecropia Moth, also known by its
Binomial nomenclature as *Hyalophora cecropia* (Roxstrom, 2001). Hemolin is a constitutively active member of the IG superfamily that is expressed at increased levels during development and bacterial infection (Roxstrom, 2001). Increased hemolin levels serve an immunological function by binding to bacteria and lipopolysaccharides which enhance phagocytosis and activate protein kinase C (Roxstrom, 2001). Further research to determine the role of Yippee binding to hemolin has not been conducted (Roxstrom, 2001). Sequencing of subcloned fragments revealed that the Yippee gene has four exons which encode a 121 amino acid protein harboring a conserved putative zinc binding ring finger motif comprised of four cysteine residues (Roxstrom, 2001). An EMBL database search located a human protein sequence, with 76% sequence homology to the Drosophila Yippee protein, which was later found to harbor the same conserved zinc finger motifs found on the Drosophila Yippee protein (Roxstrom, 2001 and Honoso, 2004). This human sequence was later determined to be a paralog of the human YPEL family and subsequently named YPEL5 (Honoso, 2004).

While characterization of the Yippee gene ceased, comprehensive sequence analysis of a 350 kb region of chromosome 22 revealed a Di Georges synteny region in mice which contained a novel mouse gene associated with 22q deletion syndrome (Farlie, 2001). Deletion at the 22q chromosomal region in humans led to the development of craniofacial abnormalities, specifically Di George syndrome, as well as cardiac abnormalities and thymic hypoplasia (Scrambler, 2000 and Farlie, 2001). The novel mouse gene, named YPEL1, was subject to a BLAST sequence analysis search and a highly homologous human sequence was found (Farlie, 2001). This sequence was
determined to be a human homolog of the mouse YPEL1 gene, which had previously been identified as a homolog of the Yippee gene in Drosophilia (Farlie, 2001 and Honoso, 2004).

In 2003, it was announced that sequencing of the human genome had been completed which allowed for sequence analysis of unknown genes or sequences against the whole human genome. A blast search using the human YPEL1 sequence against the entire human genome identified four additional human paralogs (Honoso, 2004). The five human paralogs discovered were subsequently named YPEL1-5 after the Yippee like gene found in Drosophila and were found to have high sequence homology from slime mold to humans (Honoso, 2004). Interestingly, using RT-PCR analysis it was discovered that YPEL1, YPEL2 and YPEL4 display a restrictive pattern of expression in adult and fetal tissue while YPEL3 and YPEL5 are found to be constitutively active in all tissues tested (Honoso, 2004).

A potential function for YPEL3 was discovered when Murine Small Unstable Apoptotic Protein (SUAP), which has detrimental effects on actively dividing cells, was determined to be the mouse homolog of human YPEL3 (Kelley, 2010). Prior to making this connection it was discovered that mouse YPEL3 gene has 100% sequence homology to human YPEL3 indicating a potential orthologous gene (Hosono, 2004). SUAP (mouse YPEL3) in murine myeloid precursor cells suppresses IL-3 dependent proliferation and its overexpression induced apoptosis following IL-3 withdraw (Baker, 2003). The
detrimental effect SUAP (mouse YPEL3) has on proliferating cells suggests human YPEL3 may play a role in blocking cell cycle progression.

Interest over the YPEL3 paralog increased due to microarray analysis linking YPEL3 as a potential target of p53 (Heminger, 2009). Increasing the level of p53 by knockdown of the p53 negative regulators HdmX and Hdm2 induced YPEL3 gene expression when compared to cells with fully functional HdmX and Hdm2 (Heminger, 2009). The YPEL3 gene was found to be induced in a p53 dependent manner in the presence of DNA damaging agents (Kelley, 2010). The activation of a cotransfected luciferase reporter vector harboring three putative p53 half sites with a wild type p53 indicated that p53 can directly bind to the YPEL3 gene promoter when damage is induced (Kelley, 2010). A chromatin immunoprecipitation assay further proved that p53 binds the YPEL3 gene promoter in vivo (Kelley, 2010). Taken together, it was determined that YPEL3 acts downstream of p53 (Kelley, 2010).

Since YPEL3 gene expression is regulated by p53, and p53 is known to induce growth arrest, potential functions of YPEL3 were examined. Increasing YPEL3 gene expression in a p53 independent manner indicated growth suppression which was shown by decreased levels of colonies in a colony formation assay and growth arrest indicated by an increase in cells in the G1 or S phase (Kelley, 2010 and Berberich, 2011). Growth arrest in cells overexpressing YPEL3 was determined to be cellular senescence; which was confirmed by an increase in β-Galactosidase levels, a hallmark of cellular senescence (Kelley, 2010). This indicates that human YPEL3 can cause cellular senescence independent of p53 (Kelley, 2010). Although it was previously predicted
that YPEL3 may lead to apoptosis, as does mouse YPEL3, no increase in apoptotic cells were seen when YPEL3 gene expression was increased (Kelley, 2010). However, it was noted that both mouse and human YPEL3 are rapidly degraded by ubiquitin mediated proteasomes (Baker, 2003 and Kelly Miller Personal Communication).

**YPEL3 Is Inactivated In Human Cancers.**

Due to growth suppressive effects of YPEL3 it was predicted that YPEL3 is downregulated in human cancers since cancer exhibits unregulated growth progression. As expected, in colon, lung and ovarian tumors YPEL3 was found to be downregulated, hence suppressing its growth repressive mechanism (Kelley, 2010 and Tuttle, 2011).

Further analysis of various cell lines uncovered that YPEL3 downregulation can occur epigenetically through hypermethylation of a 950bp CpG island found near the promoter and histone acetylation, as well as through a p53 independent pathway involving estrogen signaling via estrogen receptor α (Kelley, 2010 and Tuttle, 2011). When estrogen is bound to estrogen receptor α it elicits a suppressive effect on YPEL3 gene expression (Tuttle, 2011). Estrogen receptor α is imperative to YPEL3 gene suppression because following ER-α knockdown YPEL3 gene expression increases, even in the presence of estrogen (Tuttle, 2011). However, upon the removal of estrogen in estrogen receptor positive cells an increase in YPEL3 gene expression is also seen indicating the importance of estrogen as well as its receptor in gene suppression. The growth suppressive effects of an estrogen antagonist such as selective estrogen receptor modulator Tamoxifen is able to induce YPEL3 gene expression (Tuttle, 2011).
The culmination of these findings, coupled with the growth promoting activities of testosterone led us to explore YPEL3 gene expression regulation by testosterone in breast and prostate cancer cells. It has also led to studies that examine the downstream effects of YPEL3 that elicit senescence, given that the senescence pathway that is activated upon activation of YPEL3 is not well understood. One potential mechanism that was examined in this thesis was whether YPEL3 triggered an increase in intracellular ROS.

**Purpose**

The purpose of this study is to further examine the effects of hormone regulation on YPEL3 gene expression, determine if increased YPEL3 gene expression can cause ROS production, and find potential proteins that interact with YPEL3. It has been previously shown that increased YPEL3 levels induce cellular senescence; however this pathway is not yet fully understood. In the first objective of this thesis, which is derived from previous work revealing the ability of estrogen to suppress YPEL3 in breast cancer, the effects of testosterone on LnCAP cells and MCF7 cells were examined. We have previously seen that overexpression of YPEL3 induces cellular senescence and wanted to determine if this may have any correlation to Reactive Oxygen Species production. For the second part of this thesis the hypothesis that YPEL3 expression leads to increased ROS production was tested in IMR90 and MCF7 cells. The third part of this thesis will present preliminary findings of potential proteins that interact with the YPEL3 protein.
The results from this thesis may provide insight into new potential therapeutic targets and get us closer to identifying the pathway by which YPEL3 induces cellular senescence.
II. Materials and Methods

Cells and Reagents

LnCAP cells derived from a prostate adenocarcinoma were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 10 μg/mL gentamycin. MCF7 breast carcinoma cells and IMR90 primary human diploid fibroblasts (less than 20 passages) were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 10 μg/mL gentamycin (complete media). All cell lines were purchased from American Type Culture Collection.

Reagents used to treat cells include doxycycline or tetracycline (Clontech), β-estradiol (Sigma), and testosterone (Sigma).

Virus Infections

Lentivirus that had previously been produced were thawed and used to infect MCF7 cells, as well as IMR90 cells (Table 1). Cells were plated at a minimum density of 50K to a maximum density of 200K in complete media. After 24 hours the complete media was removed and a mixture containing viral supernatant (1 mL), complete media (1 mL) and Polybrene (6 μg/mL) was added. The infected cells were incubated for 24 hours before being refed with complete media. After an additional 24 hours antibiotic selection was
<table>
<thead>
<tr>
<th>Lentiviral Plasmid</th>
<th>Antibiotic Resistance</th>
<th>MCF7</th>
<th>IMR90</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLenti4-YPEL3-V5</td>
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<td>Blasticidin</td>
<td>6 µg/mL</td>
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<td>pLenti6-GFP</td>
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</tbody>
</table>

Table 1: Lentiviral Plasmid and Antibiotic Doses for MCF7 and IMR90 cell lines.

Lowest dose of selecting agent needed to eradicate cells not expressing a resistance gene. Blasticidin and Zeocin were obtained from Invitrogen. (Experiments conducted by Kate Heminger)
added and selection continued until no viable cells were left in a parallel mock infected plate (Table 1). The lowest dose of Zeocin or Blasticidin used for antibiotic selection had previously been determined by kill curve experiments in MCF7 and IMR90 cells (Table 1). Antibiotic selection exposure ranged from 7 to 12 days, after which cells were refed with complete media for 24 to 72 hours.

**Generating and Detecting Intracellular Reactive Oxygen Species**

IMR90 and MCF7 cells were plated at a density of 200k cells per well in a six well or 6 cm culture plate. To elicit reactive oxygen species (ROS) activity, after 24 hours cells were treated with 10 μM of Hydrogen Peroxide (Cumberland Swan Inc) in DMEM + FBS for 2 hours. Media was removed and adherent cells were rinsed with Dulbecco’s Phosphate Buffered Saline (DPBS). DPBS was removed and 1mL of DPBS was added to each well along with 8 μM of DCFDA (D399 H₂DCFDA and C6827 CM-H₂DCFDA Invitrogen) reconstituted in Dimethyl Sulfoxide (DMSO). After preliminary studies a switch from H₂DCFDA to CM-H₂DCFDA was made due to availability, but this was beneficial due to better retention in live cells. The dye (H₂DCFDA or CM-H₂DCFDA) was added to six well or 6 cm culture plates. Cells were incubated at 37°C for 30 minutes wrapped in aluminum foil to avoid light induced oxidation. Adherent cells in 6 well or 6 cm culture plates were rinsed with DPBS, scraped and resuspended in DPBS in 5 mL snap cap tubes for flow cytometry.

Cells undergoing flow cytometry analysis were scraped in DPBS into 5 mL snap cap tubes and wrapped in aluminum foil to deter light induced oxidation. Flow cytometry
was performed on a BD Accuri C6 Personal Flow Cytometer with a run limit of 30,000 events and using the preset medium fluidics setting of 35 µL per minute and a core size setting of 16 µm. Results were analyzed using CF-20 CFlow Plus Analysis Software and FCS Express version 3 or 4 (De Novo Software). Histogram plots were analyzed in log scale on the X-axis by setting it to FL1-A and linear scale on the Y-axis by setting to cell count.

RNA Isolation

Cells were rinsed 1 time with Dulbecco's Phosphate Buffered Saline (DPBS) and then lysed with TRK lysis buffer supplemented with 2% β-mercaptoethanol. The lysate was transferred to an Eppendorf tube and frozen at -80°C until needed. After thawing, 1 volume of 70% ethanol was added to each sample followed by vortexing. RNA was isolated following the e.Z.N.A. Total RNA kit (Omega Bio-Tek) protocol. RNA was eluted in 40µL of DEPC water that had been heated to 70°C and incubated on the spin column pad for 5 minutes. RNA quantification was performed on a NanoDrop DN-1000 spectrophotometer with RNA purity being determined by the absorbance ratio at 260 nm and 280 nm. A ratio of 1.8-2.1 signified good quality RNA.

Reverse Transcription

CDNA was created by mixing 500 ng of RNA, 4 µL of qScript cDNA supermix (Quanta Biosciences Inc.) and enough sterile distilled water to bring the reaction to a total of 20 µL. The reaction was incubated for 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at
85°C followed by indefinite holding at 4°C (ABI 2700). The cDNA was then diluted with 200 µL of sterile distilled water prior to quantitative PCR.

Taqman Base PCR

The ABI 7900HT sequence detection system (Applied Biosystems) was used to perform quantitative real time PCR in quadruplicate on a 96 well microtiter plate. The 20 µL Taqman based PCR reaction was prepared by combining 9 µL of the diluted cDNA mix with a 11 µL master mix containing 10 µL of 2X Taqman Universal PCR master mix (Applied Biosystems) and 1 µL of the appropriate 20x Taqman Assay-on-Demand Gene Expression product (Applied Biosystems). The reactions were then subjected to 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Target gene expression was normalized to GAPDH expression and analysis of gene expression was performed using SDS 2.2.2 software (ABI) setting a confidence interval of 95%. Outlier Ct values were assessed based on the ΔCtSD values. Outliers were automatically and manually removed to improve the RQ minimum and maximum range.

Protein Extraction

Cells were rinsed with 5 mL of DPBS and harvested by scraping into 5 mL of fresh DPBS. Cellular suspensions of five 15 cm plates were combined into a 50 mL test tube and pelleted by centrifugation at 4000 RPM for 2 minutes. The supernatant was carefully drawn off the pellet. Pellets were stored at -80°C until needed. Based on the cell pellet size, five volumes of lysis buffer (50 mM Tris pH 8.0, 5mM EDTA, 150nM NaCl,
0.5% Nonident p-40, 1mM phenylmethanesulfonylfluoride (Sigma), 1mM Dithiothreitol (Sigma) and Protease Inhibitor Coctail (Sigma)) were added and the frozen pellet was allowed to thaw on ice. The pellet was gently resuspended and transferred to a new 50 mL screw cap tube for centrifugation in a 4°C environment at 11,000 RPM (15,000G) for 15 minutes. The soluble protein fraction was transferred from the insoluble material to a clean 15mL test tube. A Bradford Protein Assay was used to determine the protein concentration of the cell extract. Bovine Serum Albumin acetylated (BSA) (Promega) was used to generate a standard curve at by adding 2 μg, 5 μg or 10 μg to 800 μL of sterile distilled water (SDW) and 200μL of Bradford Protein Assay Dye Reagent (Bio-Rad). Unknown protein concentrations were determined by adding 5 μL of protein that had been diluted tenfold to 800 μL of SDW and 200 μL of Bradford Protein Assay Dye Reagent. The samples and standards were transferred to plastic cuvettes and absorbance was measured at 595nM on either a Genesys 5 Spectrophotometer or Genesys 6 Spectrophotometer (Thermospectronic). The protein samples concentrations were determined by comparing their absorbencies to the standard BSA absorbencies.

**Co-Immunoprecipitation**

A total of 100 mg of protein obtained by the protein extraction method was moved to a new test tube diluted to 10 mL with lysis buffer. To each reaction either 40 μg (80 μL) of Agarose Immobilized Rabbit Anti V5 beads (Bethyl), or 80 μL Anti V5 Agarose Gel produced in mouse (Sigma) was added and allowed to incubate overnight at 4°C. Each sample was centrifuged at 4000 rpm for 8 minutes at 4°C. Lysis buffer was removed by
pipetting and resuspended in 6 mL of fresh lysis buffer followed by incubation for 5 minutes at 4°C on a rotating platform. This washing process was repeated 6 times and a fraction (F#) of each wash was saved. Following the washes, the beads were transferred to a 1.5 mL Eppendorf test tube by adding 1 mL of lysis buffer to the beads and resuspending. The beads were centrifuged in a 4°C microfuge at 10,000 rpm for 5 minutes and excess lysis buffer was removed from the bead pellet. At this point the bound proteins were either eluted by adding 50 μL of 1X SDS Page Running Buffer to the beads and heating for 5 minutes at 37°C, or 0.8 mg (200 μL) of competing V5 peptide (Sigma) added to the beads, inverted at 4°C for 5 minutes, 4°C cold centrifuged for 5 minutes at 10,000 rpm and repeated to obtain 2 eluted fractions (EF#). When samples were eluted with V5 peptide after the second elution the beads were resuspended in 50 μL of 2X SDS Dye and heated to 70°C for 5 minutes. The beads were centrifuged in a 4°C microfuge at 10,000 rpm for 5 minutes and the bead fraction was removed from the bead pellet. The 2 eluted fractions (EF) from each cell set were combined and concentrated by adding 3.6 mL of lysis buffer to the elutions and loading into an Amicon Ultra Centrifugal Filter 3k molecular weight ultracell (Millipore) which was centrifuged at 3600 g for 70 minutes at 4°C. Alternatively, the EF was concentrated by loading each fraction into a Microcon Ultracel YM-10 centrifugal filter (Sigma) which was centrifuged at 10,000 RPM for 10 minutes at 4°C. For both concentration methods the flow through was removed and additional lysis buffer was added to desalt the proteins. The proteins were concentrated in a volume of 20 μL of Lysis buffer. To the retentate, 20 μL of lysis buffer was added and vigorous pipetting was used to loosen proteins from the
membrane. The retentate was combined with an equal amount of 2X SDS loading Dye and stored at -80°C until further use.

**SDS-PAGE Electrophoresis**

Protein extracts were combined with an equal volume of 2X SDS loading dye (1X = 60 mM Tris, pH7.6, 2% SDS, 10% glycerol) and loaded into either a large 12% SDS-Page gel with a 4% stacking gel run in a V16 electrophoresis apparatus (Gibco Bethesda Research Laboratories) with 1X SDS running buffer (25 mM Tris pH 8.3, 250 mM glycine, 0.1% SDS) at 150V for approximately 30 minutes then 250V for approximately 1.5 hours, or a precast 8-16% Precise Protein Tris Hepes Gradient Gel (Thermo Scientific) run in BupH Tris Hepes SDS running buffer (Thermo Scientific), reconstituted in deionized water, for approximately 45 minutes at 150V using an Owl P8D (Owl Separation Systems Inc).

**Western Blot**

Denatured proteins were transferred from the SDS-PAGE gel to polyvinylidene difluoride (PVDF) membrane (Millipore), that had been presoaked in 100% methanol for 15 seconds and then transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 5 minutes, via a Bio-Rad Mini Protean 2 transfer system for 1 hour at .5 Amps in transfer buffer. After transfer the PVDF membrane was dried and then placed in PBS blocking buffer (1X PBS, 5% non-fat dry milk (NFDM), 0.1% Tween-20) for 24 hours on a moving platform at 4°C. Primary antibody (monoclonal V5) (Sigma) was added at a concentration from 1:1000-1:5000 in diluted PBS blocking buffer (1:10) for a minimum of 5 hours (Room Temperature) and a maximum of 24 hours (4°C). The PVDF
membrane was washed 5 times for 15 minutes each in diluted PBS blocking buffer. The secondary antibody with conjugated horseradish peroxidase enzyme (Goat Anti Mouse) (Promega) was added at a concentration of 1:2500 in diluted PBS blocking buffer and allowed to incubate for 1 hour at room temperature. The PVDF membrane was again washed 5 times for 15 minutes each in diluted PBS blocking buffer and exposed to Super Signal West Pico Chemiluminescence (Thermo Scientific) for 2-3 minutes. Chemiluminescent images of the blot were taken in a FUJI FILM LAS 4000 image reader and the images were visualized using Fujifilm Multi Guage software. To reprobe the PVDF membrane, it was first stripped with two 30 minute treatments in Western Strip Buffer (25 mM glycine, 1% SDS, pH 2.0). The membrane was rinsed extensively in DPBS and then reblocked with DPBS blocking buffer before repeating the above protocol.

**Colloidal Blue and Silver Staining**

SDS-Page protein gels were fixed in 40% methanol and 5% acetic acid overnight on a shaker. Gels were then stained following the NuPage Bis Tris protocol contained in the Novex Colloidal Blue Stain kit (Invitrogen). To silver stain a gel it was first fixed in 40% ethanol and then one of the following three methods were employed. The first method was to follow the protocol from the Bio-Rad Silver stain kit (Bio-Rad). The second method exposed the gel 2 times to 10% glutaraldehyde for 3 minutes each. The gel was then placed in 200 nM Dithiothreitol for 10 minutes, followed by .1% silver nitrate for 15 minutes. The gel was rinsed 2 times with sterile distilled water (SDW) for 1 minute each
to remove excess silver nitrate. Formalin (0.4%) plus 2% sodium carbonate was used to develop the gel with an exposure time between 4 to 8 minutes. Development of the gel was stopped by placing it in 1% acetic acid. The third method fixed the gel in 20mg/mL sodium thiosulfate for 2 minutes. The gel was then rinsed in SDW twice for one minute each. After rinsing, the gel was exposed to .1% silver nitrate for 30 minutes. The excess silver nitrate was then removed by washing twice with SDW for 1 minute each. The gel was developed with .04% formalin plus 2% sodium carbonate with an exposure time between 4 to 8 minutes. Acetic acid (1%) was used to stop development of the gel indefinitely.
III. Hormone Regulation of YPEL3

The effects of testosterone on YPEL3 Expression in LnCAPs

It has previously been shown in estrogen receptor positive MCF7 breast cancer cells that growth in the presence of estrogen leads to a repression of YPEL3 gene expression (Tuttle 2011). In addition to estrogen receptors, MCF7 cells also harbor androgen receptors (Ortmann, 2002). However, the effects of testosterone on YPEL3 gene expression are unknown in MCF7 cells. Also unknown are the effects that steroid hormones play on YPEL3 gene expression in non breast cancer cell lines. Steroid hormones, which include estrogen, progesterone and testosterone, diffuse into cells where they bind their respective cytoplasmic receptor and are translocated into the nucleus to impart growth stimulation or inhibition. LnCAP cells, which are derived from human prostate adenocarcinoma, are a hormonally responsive cell line containing both androgen and estrogen receptors (Horoszewicz, 1983). The presence of estrogen or testosterone has been shown to have growth stimulating effects on LnCAPs (Castagnetta, 1995 and Kampa, 2002)

To determine whether hormones regulate YPEL3 gene expression in prostate cancer cells, LnCAP cells were grown in complete media or charcoal stripped serum (CSS) for up
Figure 1: YPEL3 Expression Is Induced In LnCAP Cells Grown In CSS.

LnCAP cells were plated in complete media for 24 hours before being switched to CSS, or refed with complete media. RNA from cells grown in complete media was isolated 24 hours after the first feeding (CM). Cells grown in charcoal stripped serum were either fed every 24 hours (CSS *), or the media remained unchanged (CSS). RNA was isolated after 24, 48 and 72 hours. YPEL3 mRNA levels were analyzed using RT-PCR normalized to GAPDH mRNA levels. Error bars represent the 95% confidence intervals resulting from triplicate assays for both YPEL3 and GAPDH expression.
to a total of 72 hours (Figure 1). Growing cells in CSS, which is devoid of all hormones as well as other lipophilic compounds including certain growth factors and cytokines, gives the ability to analyze the impact of hormone removal on YPEL3 gene expression. LnCAP cells were grown in complete media or CSS media for 24, 48 or 72 hours prior to total RNA isolation and RT-PCR analysis for YPEL3 mRNA levels. YPEL3 gene expression increased 2 fold in the LnCAP cells cultured in CSS after 24 hours and continued to increase up to 4.7 fold at 72 hours when fed every 24 hours. Regardless of feeding schedules if the LnCAP cells were fed fresh CSS media every 24 hours or not there is an induction of YPEL3 gene expression caused by culturing LnCAP cells in CSS media, when compared to complete media.

Having demonstrated that growing LnCAP cells in CSS media induces YPEL3 expression the next series of experiments tested the effect of administering testosterone on YPEL3 gene expression. Testosterone is an important regulator of cell cycle progression and proliferation in LnCAP cells, however it is unknown if testosterone plays a role in regulating YPEL3 gene expression. By performing a dose response the effects of various testosterone doses on YPEL3 gene expression was determined. LnCAP cells were initially grown in complete media for 24 hours then switched to CSS and varying doses of testosterone (0-100 nM) were administered to the CSS media (Figure 2). RNA was isolated 24 hours post testosterone treatments and mRNA levels of PSA and YPEL3 were assessed by RT-PCR. PSA was used as a positive control since its induction by testosterone in LnCAP cells has been previously reported (Kampa, 2002). As expected the addition of testosterone resulted in a dose-dependent induction of
Figure 2: YPEL3 is repressed in LnCAP cells in the presence of Testosterone.

LnCAP cells were grown in complete media for 24 hours before being switched to charcoal stripped serum. After 24 hours testosterone was added at doses of 2, 5, 10, 25, and 100 nM to the CSS media. RNA was isolated 24 hours after testosterone exposure. (B) YPEL3 and (A) PSA mRNA levels were analyzed using RT-PCR normalized to GAPDH mRNA levels. Error bars represent the 95% confidence intervals resulting from triplicate assays for PSA, YPEL3 and GAPDH expression.
PSA gene expression (Figure 2A). In contrast, YPEL3 showed a consistent 80% reduction in gene expression across the range of testosterone treatments (Figure 2B). These results suggest that YPEL3 expression is repressed by testosterone in LnCAP cells.

Prostate cells normally do not possess aromatase activity which converts androgens to estrogen, however upon conversion to a malignant state; LnCAP prostate cells begin to express aromatase (Ellem, 2004). By monitoring the expression of pS2, which is a target gene of estrogen, we could indirectly assess whether testosterone was being converted to estrogen in LnCAP cells. LnCAPs were grown in CSS to induce YPEL3 and a testosterone time course using 10 nM was set up (Figure 3). A dose of 10 nM was chosen because not only was it shown in the dose response from Figure 2 to give the largest repression of YPEL3 gene expression and the second highest PSA induction, but also because aromatase activity in LnCAP cells has been shown to not exceed a conversion rate of .54 nM of testosterone to estrogen over 72 hours (Castagnetta, 1997). By saturating the aromatase enzymes it can be determined if testosterone has an impact on YPEL3 gene expression, or if its conversion to estrogen potentially regulates YPEL3 gene expression. Higher doses of testosterone may give a marginally higher PSA induction that is not statistically different from 10 nM, but higher doses do not repress YPEL3 gene expression as well. Isolating total RNA at hourly time points of 2, 4, 8, 24, and 48 hours followed by quantification of mRNA levels for YPEL3 and PSA by RT-PCR analysis, was used to generate a time course curve to determine PSA and YPEL3 gene expression levels. Analysis of PSA gene expression by time course curve revealed that MCF7 cells grown in the presence of 10 nM testosterone induced PSA gene
Figure 3: YPEL3 Repression is inversely related to PSA expression in LnCAP Cells exposed to 10 nM Testosterone for 48 hours. LnCAP cells were plated in complete media for 24 hours before being switched to CSS. After 24 hours 10 nM testosterone was added to the CSS media. RNA was isolated at 2, 4, 8, 24 and 48 hour timepoints. YPEL3, PSA and PS2 mRNA levels were analyzed using RT-PCR normalized to GAPDH mRNA levels. Error bars represent the 95% confidence intervals resulting from triplicate assays for PSA, YPEL3, PS2 and GAPDH expression.

*Outlying data at 8 hours was removed for YPEL3.
expression increases up to 24 hours before PSA gene expression began to return to baseline levels. Alternatively, testosterone (10 nM) caused maximal repression of YPEL3 gene expression at 24 hours and continued maximal repression at 48 hours. At 24 hours there was a 12 fold increase in PSA, which was the highest, and also a 38% reduction in YPEL3, which was the largest reduction. However, at 48 hours there was a 4 fold induction of PSA, which may be attributed to testosterone degradation (t½ = 2-4 Hr) as well as the turnover and synthesis rate of androgen receptors (Eckert, 1984). Levels of pS2 mRNA were also quantified by RT-PCR, revealing no change in pS2 gene expression in LnCAPs over 48 hours, suggesting that testosterone is not being converted to estrogen under these conditions.

Testosterone replacement therapy can have beneficial effects when administered in the event of Hypogonadism, or low testosterone levels. However, if an androgen receptor positive prostate cancer is present testosterone may lead to a more aggressive cancer.

After a prostatectomy, which removes the prostate, testosterone replacement therapy is used to maintain lost testosterone production; however testosterone may also induce growth progression of metastatic prostate cancer cells that have evaded prostatectomy and chemotherapeutic treatment. The addition of testosterone to LnCAP prostate cells has a repressive effect on YPEL3 gene expression which may lead to cell cycle progression. This strengthens the idea that testosterone replacement therapy
is not the best choice when androgen responsive prostate cancer is suspected, due to its ability to repress expression of YPEL3.

The effects of Testosterone on YPEL3 Expression in MCF7 Cells

The progression of estrogen receptor positive breast cancer has been linked to estrogen induced cell proliferation (Katzenellenbogen, 1987). Testosterone on the other hand has been shown to inhibit growth in androgen receptor positive breast cancer cells (Ortmann, 2002). Cells that harbor both the androgen and estrogen receptor like MCF-7 cells may proliferate in the presence of estrogen but proliferation is inhibited in the presence of testosterone (Ortmann, 2002). The level of inhibition by testosterone is slightly less in cells with aromatase activity due to its ability to convert testosterone to estrogen (Ortmann, 2002). However, unlike LnCAP cells aromatase expression is repressed at the transcriptional level in MCF7 breast cancer cells (Castagnetta, 1997 and Zhou, 1993).

With the knowledge that YPEL3 is repressed in MCF7 cells in the presence of estrogen we set forth to determine how testosterone impacted YPEL3 expression in MCF7 cells when given alone or in combination with estrogen. MCF7 cells were utilized since they contain estrogen and androgen receptors (Horwitz, 1975). MCF7 cells grown in CSS were exposed to 1nM estrogen, 5nM testosterone, or a combination of both (Figure 4). Estrogen was chosen at a dose of 1 nM due to previous research that shows no further repression of YPEL3 in MCF7 breast cancer cells at higher doses (Tuttle, 2011). A dose of 5 nM testosterone was chosen because it was found to be near, or slightly more than
Figure 4: Testosterone Induction and β-estradiol Repression of YPEL3 in MCF7 Cells.

MCF7 cells were plated in complete media for 24 hours before being switched to CSS. The media was changed to CSS with the addition of either 1 nM Estrogen, 5 nM testosterone, or a combination of Estrogen (1 nM) and testosterone (5 nM). RNA was isolated after 24 hours. YPEL3 mRNA levels were analyzed using RT-PCR normalized to GAPDH mRNA levels. Error bars represent the 95% confidence intervals resulting from triplicate assays for both YPEL3 and GAPDH expression.
the threshold of high to normal testosterone production (Schroder, 1998). As expected, MCF7 cells treated for 24 hours with 1 nM of estrogen showed a 25% repression of YPEL3 gene expression (Figure 4). MCF7 cells exposed to 5nM of testosterone resulted in a 5.9 fold increase in YPEL3 mRNA levels, when compared to CSS control, which is opposite of what was observed in LnCAP cells. Treatment with both estrogen (1 nM) and testosterone (5 nM) showed a slight increase in YPEL3 gene expression when compared to CSS control, suggesting that the two steroids have opposing effects on YPEL3 mRNA expression.

To expand on the results obtained in Figure 4 comparing testosterone and estrogen combination in MCF7 cells, a dose response curve with 1 nM estrogen and various doses of testosterone was performed. By growing MCF7 cells in CSS media and adding various doses of testosterone in addition to 1nM estrogen a dose response can be generated. MCF7 cells were grown in CSS media for 24 hours before the addition of testosterone (0.5 -10 nM) combined with 1 nM estrogen (Figure 5). Total mRNA was isolated after 24 hours, and as expected the CSS sample showed a near 2 fold induction of YPEL3 gene expression. The addition of 0.5 nM testosterone and 1 nM estrogen represses YPEL3 gene expression back to levels that are statistically the same as MCF7 cells grown in complete media. When testosterone and estrogen are administered both at 1 nM there is an increase in YPEL3 gene expression relative to MCF7 cells grown in complete media, but there is no statistical difference when compared to MCF7 cells grown in CSS. However, YPEL3 gene expression increases in a dose dependant manner as testosterone levels are increased above 1 nM. A combination of 5 nM testosterone and 1 nM
Figure 5: In MCF7 Cells YPEL3 is Induced in the Presence of Increasing Doses of Testosterone. MCF7 cells were plated in complete media for 24 hours before being switched to CSS. After 24 hours of growth in CSS media MCF7 cells were exposed to 1 nM estrogen combined with either 0.5 nM, 1 nM, 5 nM, or 10 nM of testosterone. RNA was isolated after 24 hours. YPEL3 levels were analyzed using RT-PCR normalized to GAPDH mRNA levels. Error bars represent the 95% confidence intervals resulting from triplicate assays for both YPEL3 and GAPDH expression.
estrogen yielded a 3 fold increase in YPEL3 gene expression when compared to complete media, and the first statistically significant increase when compared to MCF7 cells grown in CSS media. A further increase in YPEL3 gene expression to 4 fold was seen when 10 nM testosterone was combined with 1nM estrogen. This indicates there may be a counteracting effect between testosterone and estrogen.
IV. Reactive Oxygen Species is not produced during YPEL3 induction.

Cellular senescence is the irreversible loss of cellular division which can be mediated by an increase in the level of reactive oxygen species (ROS) (Passos, 2010). A cell normally produces ROS as a byproduct of cellular respiration; however it has mechanisms to convert potentially damaging reactive oxygen species to more stable forms leading to minimal DNA damage (Nohl, 2004). A cell may regulate its ROS levels by many different mechanisms, some are known, and others are novel mechanisms being discovered. Recently our laboratory discovered that YPEL3 could trigger cellular senescence when over expressed, however the mechanism leading to this senescence is currently unknown (Kelley, 2010).

By adding hydrogen peroxide and cell permeable fluorescent indicator DCFDA, a cell population positive for increased ROS can be segregated from a negative population. When DCFDA enters a cell its acetate groups are cleaved by intracellular esterases to DCF and oxidation by hydrogen peroxide causes DCF to emit fluorescent wavelengths after excitation. Fluorescence is due to an increase in the number of pi orbital electrons that can be excited, conversion of DCFDA to a planar molecule and disruption of conjugate electrons. DCF has an excitation absorbance at 490 nm, therefore when exposed to an Accuri c6 flow cytometer’s 488 nm laser an excitation
emission occurs at 527 nm which is detected by the FL1 optical filter that has a maximum emission detection peak at 533 nm and a high or low detection range spanning +/-15 nm from 533 nm. An increase in positive ROS producing cells will be indicated by a shift to the right in the fluorescent peak on a histogram caused by excitation of DCF electrons emitting a longer wavelength of energy than cells producing a lower amount of ROS or not emitting a fluorescent signal. A shift to the right indicates a higher level of fluorescence in a cell. To establish the peak fluorescence of DCFDA treated cells I compared DCFDA treated MCF7 or IMR90 cells with a second population of MCF7 or IMR90 cells treated with DCFDA and hydrogen peroxide. The shift in population of hydrogen peroxide treated cells when compared to dye only cells allowed me to determine the fluorescence threshold for positive and negative ROS producing cells (Figure 6C and 6D, 8% untreated Vs. 19% H2O2).

To assess the best method to treat cells with DCFDA to optimize detection of ROS positive cells two different approaches of DCFDA treatment were tested. In the first experiment, MCF7 cells were preloaded with DCFDA and then exposed to hydrogen peroxide for 2 hours (Figure 6A and 6B). The second experiment involved treating MCF7 cells with Hydrogen peroxide for 2 hours, then loading the cells with DCFDA for 30 minutes (Figure 6C and 6D). Following these treatments both sets of cells were rinsed with DPBS and then scraped into DPBS for flow cytometry analysis. From the flow cytometry results it appears that cells loaded with DCFDA for 30 minutes after hydrogen peroxide exposure produced a slightly higher number of ROS positive cells than when
Preloaded

Figure 6: Hydrogen Peroxide produces cellular reactive oxygen species levels detectable with DCFDA. MCF7 cells were plated in complete media 24 hours prior to treatment. (A and B) Preloaded with DCFDA (10 μM), followed by treatment of (B) with H₂O₂ (10 μM) for 2 hours. (C) Treated with H₂O₂ (10 μM) for 2 hours, followed by 30 minute DCFDA (10 μM) exposure to (C and D). Cells were scraped in 1 mL DPBS and resuspended into a single cell population before flow cytometry analysis on an Accuri C6 was performed. (A, B, C and D) Histograms that represent negative and positive ROS producing populations. X-axis represent fluorescence (log scale) and Y-axis represents cell count (linear scale).
preloaded with DCFDA for 30 minutes and subsequently exposed to hydrogen peroxide treatment. Pre or post treatment of DCFDA had no effect on the percentage of ROS positive cells in the untreated samples (8%; Figures 6A and 6C). Although both methods of DCFDA treatment detected an increase in ROS positive cells, all subsequent experiments were performed using the DCFDA post-treatment approach.

Through hydrodynamic focusing the flow cytometer passes individual particles or cells through the interrogation point containing a laser that determines cell size through light scattering or excites fluorophores that emit a fluorescent signal. If cell damage or cell clumping occurs while harvesting the cells it could lead to inaccurate readings caused by debris or doublets. Although debris and doublets can be removed through gating this may result in an inaccurate representation of the total population. To determine optimized cell harvesting conditions two methods were tested. In the first approach cells were scraped in DPBS and the cells were vortexed to create a single cell population. Alternatively cells were trypsinized using 1 mL of trypsin. Trypsinized cells were transferred to complete media to inactivate the trypsin, pelleted by centrifugation and resuspended in DPBS. Comparison of the dot plots (Figure 7A) shows that the trypsinized cells have a population with a high level of forward and side scatter that is absent in the dot plot of the cells scraped in DPBS. The trypsinized cells have three distinct peaks in their histogram compared to one in the DBPS scraped cells (Figure 7B). To determine if cell doublets are forming a histogram comparing cell count and side scatter pulse width was utilized. As individual cells pass through the fluidics system the
Figure 7: MCF7 cells scraped in DPBS provides a better mechanism to isolate MCF7 cells for vehicle for analysis by flow cytometry.

MCF7 cells were plated at 200k/per well in complete media 24 hours prior to treatment. MCF7 cells were treated with DCFDA in DPBS for 30 minutes and scraped in DPBS (Row 1). Trypsinized cells were suspended in DMEM + 10% fbs before pelleting and resuspending in DPBS (Row 2). Analysis was performed on an Accuri C6 flow cytometer after samples were vortexed. (A) Dot plot analyzing forward scatter and side scatter. (B) Histogram representation of FL1-A and cell count. (C) Histogram representation of side scatter pulse width and cell count. (D) Histogram representation of gated single cell population (0-100 w) from (C).
laser will determine their size using three different pulse parameters which includes height, area and width. Cells moving through the flow cytometer encounter the laser one at a time. When a doublet is encountered it is passed through the fluidics system with the doublets orientation parallel to the fluidics system and perpendicular to the laser. The doublets orientation will cause formation of a second peak that will appear on the side scatter width and cell count histogram (Figure 7C). The first peak to the left corresponds to single cells and the second peak, which has a higher fluorescence and different light scatter will occur to the right on the histogram. The scraped cells do not have a second peak in the side scatter width histogram and the majority of the cells are present as single cell events (Figure 7C). Trypsinized cells form a second peak indicating doublets, with more than two thirds of the cell population forming doublets (Figure 7C). By gating out the doublets and reanalyzing (Figure 7D), histogram analysis shows that there is a higher level of ROS production when cells are trypsinized, indicating trypsinization may induce ROS production. This revealed that cells isolated by scraping into DPBS gave interpretable results, whereas the trypsinized cells gave a false ROS positive and exhibited clumping or the formation of doublets. By gating out the doublets the trypsinized cells also gave a less accurate interpretation of the data because less than a third of the population was represented, whereas the scraped cells single cell population represents over 90% of the cells. Moving forward the method of cell isolation was rinsing cells with DPBS after DCFDA treatments and scraping in 1 mL of DPBS into 5 mL snap cap tubes, followed by vortexing to create a single cell population before flow cytometry analysis.
According to Invitrogen, as well as recent literature, reseeding cells in full serum media for a short recovery period after DCFDA exposure gives cellular esterases time to cleave acetate groups on the DCFDA molecules and for oxidation of DCF by ROS to cause fluorescence (Eruslanov, 2010). Employing serum free media, in addition to full serum media, allowed us to determine if increases in fluorescence are due to growth factors stimulating metabolism, or esterases present in serum. To assess ROS levels after a period of recovery MCF7 cells were treated with hydrogen peroxide (2 hours) followed by DCFDA exposure (30 min) and were allowed to recover in full serum media (Figure 8A) or serum free media (Figure 8B) for 0, 30 minutes, 1 hour and 2 hours before scraping into 1 mL DPBS for flow cytometry analysis.

Cells grown in full serum media (Figure 8A) were switched to DPBS while adding DCFDA because according to Invitrogen the high level of primary and secondary amines in full serum media can hydrolyze DCFDA to its active form which changes its charge and impedes its entry into the cell. MCF7 cells grown in serum free media (SFM) remained in SFM upon addition of hydrogen peroxide or DCFDA. Counter to the literature, the level of ROS positive DCFDA fluorescence declines in MCF7 cells as the cells recover in full serum media (Figures 8A and 8C). From the flow cytometry results it appears that cells grown in full serum media and exposed to DCFDA for 30 minutes after hydrogen peroxide exposure and no recovery period produced a larger number of ROS positive cells than when grown in SFM and exposed to the same treatment (Figures 8A and 8B). MCF7 cells recovering in full serum media or SFM after treatment with hydrogen peroxide and DCFDA exhibit the same trend of ROS positive cells with the highest
A) Recovery in full serum media.

B) Recovery in serum free media.
Figure 8: Allowing DCFDA treated MCF7 cells to recover in complete media after DCFDA exposure does not lead to improved ROS positive detection. MCF7 cells were cultured in full serum media over night. (A) MCF7 cells were treated with H2O2 (10 μM), followed by 30 minute exposure to DCFDA (10 μM) in DPBS. The negative control (DCFDA loaded in PBS) was only exposed to DCFDA. Following treatment cells were washed with DPBS and fed with full serum media. Cells were isolated at time points of 0, 30 minutes, 1 hour and 2 hours. (B) MCF7 cells were treated with H2O2 (10 μM), followed by 30 minute exposure to DCFDA (10 μM) in serum free media. The negative control (MCF7 DCFDA loaded in SFM) was only exposed to DCFDA. Following treatment cells were fed with serum free media. Cells were isolated at time points of 0, 30 minutes, 1 hour and 2 hours by scraping in 1 mL DPBS and resuspended into a single cell population before flow cytometry on an Accuri C6 was performed. (C) Graphical representation of the percentage of ROS positive populations in each treatment condition.
increase in ROS positive DCFDA fluorescence occurring with no recovery time, when compared to cells receiving DCFDA only. A gradual decline was observed at each isolation point during the recovery period (Figure 8C). This indicates that MCF7 cells grown in full serum media provide higher levels of ROS positive DCFDA fluorescence when compared to MCF7 cells grown in serum free media and exposed to the same treatments. Flow cytometry results indicate that recovery after DCFDA exposure is unnecessary and actually lowers the amount of ROS positive DCFDA fluorescence. To treat cells in the future I determined optimal DCFDA loading conditions would be 30 minutes in DPBS, followed immediately by scraping to isolate the cells and analysis by flow cytometry.

Having established optimized conditions for monitoring ROS positive cells using DCFDA, I can set out to examine whether inducing YPEL3 triggered increased ROS within human cells. Initially I tested YPEL3 overexpression by employing MCF7 cells which harbored either a tetracycline inducible expression vector that expressed either YPEL3, or the LacZ gene. These cells were grown either in the presence (induced) or absence (repressed) of 1 µg/mL tetracycline (Figure 9). When tetracycline is added to cells harboring tetracycline inducible expression vectors gene expression is induced. The dose of 1 µg/mL tetracycline was chosen because it had previously been shown to trigger a physiologically relevant 8 fold induction of YPEL3 gene expression (Kelley, 2010). Before performing DCFDA/flow cytometry experiments cells were exposed to tetracycline and RNA was isolated for quantitative PCR. MCF7 cells were used to normalize expression. MCF7 cells expressing LacZ were employed to test the integrity of
MCF7 cells harboring inducible YPEL3 or LacZ -/+ Tetracycline

A)

Expression

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<th>LacZ tet-</th>
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B)

- MCF7 + DCFDA
- YPEL3 Tet-
- YPEL3 1ug/ml Tet
- LacZ tet-
- LacZ 1ug/ml Tet

Counts and percentages for each condition.
Figure 9: Tetracycline induced YPEL3 expression in MCF7 cells does not lead to higher ROS production when compared to LacZ induced MCF7 cells. A) QPCR results for YPEL3 expression in MCF7 cells and MCF7 cells with inducible YPEL3 or LacZ grown in the presence or absence of 1µg/mL tetracycline for 24 hours after which total RNA was isolated. Y axis represents the relative expression comparing YPEL3 mRNA levels normalized to GAPDH mRNA levels. Error bars represent 95% confidence intervals from triplicate PCR reactions analyzing YPEL3 and GAPDH cDNA’s respectively.

B) Flow cytometry results for MCF7 cells containing a tetracycline inducible element expressing either YPEL3 or LacZ grown in the presence or absence of 1 µg/mL tetracycline. MCF7 cells treated with 10 µM hydrogen peroxide for 2 hours were used as a positive control for identifying the ROS positive cell population following DCFDA and flow cytometry. MCF7 cells treated only with DCFDA dye were used as a ROS negative cell population. Cells were rinsed with DPBS, scraped into DPBS and vortexed before analysis by an Accuri C6 flow cytometer.

C) Bar Graph representing the percent of positive ROS producing cells in each cell population (X-axis) analyzed in Figure 9B.
the Tet-On system. The qPCR results show a 4 fold induction of YPEL3 in the YPEL3 tet inducible cells when grown in the absence of tetracycline and compared to MCF7 cells (Figure 9A). This indicates that the tetracycline inducible YPEL3 promoter is not completely repressed in the Tet-On system which may be caused by the Tet repressor protein weakly binding the Tet operon in the absence of tetracycline, or the Tet repressor protein not fully inhibiting transcription factors from binding the promoter expressing YPEL3. In the presence of 1 µg/mL tetracycline the YPEL3 inducible cells show an 8 fold induction over untreated MCF7 cells and almost twice as much expression as YPEL3 inducible cells grown in the absence of tetracycline (Figure 9A). As expected, the lacZ tetracycline inducible cells did not show a change in YPEL3 expression regardless of the presence or absence of tetracycline when compared to MCF7 cells (Figure 9A). The tetracycline inducible YPEL3 cells, which have higher YPEL3 gene expression than control cells, do not show increased ROS levels when compared to MCF7 cells expressing LacZ (Figure 9B and 9C). This indicates that the overexpression of YPEL3 in MCF7 cells does not lead to an increase in ROS. It should be noted however that the YPEL3 and LacZ infected cells show an increase in ROS positive cells when compared to MCF7 cells (Figure 9B and 9C).

Flow cytometry results reveal an increase in ROS production in YPEL3 and LacZ infected cells independent of tetracycline treatment when compared to MCF7 cells (Figure 9C). The fact that the percentage of ROS positive cells do not increase suggests elevation of ROS in cells is the result of the viral infection, the TetR protein, or selection
with Zeocin and Blasticidin. This approach does not provide a good system to assess ROS levels due to high levels of ROS detection in all of the infected cells.

MCF7 cells harboring a tet inducible expression vector expressing YPEL3 were previously shown to have no effect on ROS levels, however leaky expression, or the phenotype of MCF7 TetR cells may increase ROS levels. Therefore an alternative method of expressing YPEL3 in MCF7 cells was utilized, as well as exposing the cells to an extended period of YPEL3 overexpression to determine if ROS production is a delayed response to YPEL3 overexpression. To determine if MCF7 cells infected with YPEL3 can increase ROS levels after infection, immortalized MCF7 cells were infected with lentivirus expressing YPEL3, or H-RAS as a positive control (Figure 10). Cells were selected with Zeocin (750 µg/mL) or blasticidin (6 µg/mL) for 12 days, and then reseeded for an additional two days before RNA isolation and RT-QPCR were performed to monitor YPEL3 levels. RT-QPCR results show that MCF7 cells infected with lentivirus producing YPEL3 showed a 56-fold increase in YPEL3 when compared to uninfected MCF7 cells. Interestingly YPEL3 expressing MCF7 cells also demonstrated an 11-fold increase in p21 expression (Figure 10A). H-RAS infected MCF7 cells possessed a more modest 4-fold increase in p21 gene expression and a 4-fold increase in YPEL3 expression, compared to MCF7 cells. RAS expression was undetectable after many attempts to quantify mRNA levels, however overexpression of RAS has previously been shown to increase mRNA expression of p21 through p53 (Agarwal, 2001). Under this assumption it would appear that RAS was expressed, however the level at which it was expressed is unknown.
A) 

![Gene Expression Bar Graph]

Relative Gene Expression

Lentivirus Infection

B) 

![Cytometry Graphs]

MCF7

H2O2

YPEL3

RAS
Figure 10: MCF7 cells infected with lentivirus expressing YPEL3 show increased gene expression of YPEL3. (A) MCF7 cells were plated in complete media for 24 hours before being infected with lentivirus expressing YPEL3 or RAS. RNA was isolated after 24 hours. YPEL3 and p21 mRNA levels were analyzed using RT-PCR normalized to GAPDH mRNA. Error bars represent the 95% confidence intervals resulting from triplicate assays for both YPEL3 and GAPDH expression. (B) MCF7 cells were exposed to hydrogen peroxide plus DCFDA, or just DCFDA, infected cells were exposed to DCFDA only, and then scraped in DPBS. These samples were vortexed and flow cytometry was performed.
To assess ROS production by flow cytometry, MCF7 cells infected with lentivirus expressing YPEL3, or H-RAS were selected for 12 days with Zeocin (750 µg/mL) or blasticidin (6 µg/mL) and then reseeded for an additional 2 days. Infected MCF7 cells were exposed to DCFDA (30 min) followed by scraping to isolate the cells in DPBS (1 mL) and then vortexed to ensure a single population for flow cytometry analysis. Flow cytometry results show infection of MCF7 cells with H-RAS and cells treated with hydrogen peroxide show an increase in ROS production when compared to MCF7 cells (16% and 14% Vs. 5%) (Figure 10B). MCF7 cells overexpressing YPEL3 did not show an increase in positive ROS producing cells when compared to MCF7 cells (5% Vs. 5%) (Figure 10B).

Having established that increased level of YPEL3 did not appear to cause an increase of ROS in MCF7 breast cancer cells, IMR90 cells were utilized to determine if YPEL3 senescence in primary fibroblasts is dependent on ROS. IMR90 primary human diploid fibroblast cells were utilized due to their non transformed nature, intact pathways and ability to undergo replicative senescence, as well as oncogene induced senescence. IMR90 cell were also utilized because an increase in ROS dependent on YPEL3 may be related to the genetic context of the cell.

To further investigate any connections between YPEL3 and ROS, IMR90 cells were infected with Lentivirus expressing YPEL3, or expressing H-RAS as a positive control (Figure 11). RNA was isolated 2 days post infection and RT-QPCR performed to monitor the levels of YPEL3 expression. RT-QPCR results show that IMR90 cells infected with
lentivirus producing YPEL3 showed a 1000 fold increase in YPEL3 when compared to uninfected IMR90 cells. Interestingly YPEL3 expressing IMR90 cells also demonstrated an 8 fold increase in p21 expression, but no significant alteration in endogenous RAS gene expression (Figure 11A). H-RAS infected IMR90 cells possessed a more modest 5-fold increase in RAS gene expression compared to IMR90 cells, as well as a 10-fold induction of p21 and a 5-fold induction of YPEL3 (Figure 11A).

Using DCFDA and flow cytometry I next examined how YPEL3 and H-RAS infection of IMR90 cells impacted ROS activity. Interestingly, the addition of hydrogen peroxide resulted in only a modest increase in ROS positive cells (Figure 11B, 19% Vs. 11%). While YPEL3 overexpressing IMR90 cells showed no significant increase in ROS positive cells (Figure 11B, 10% Vs. 11%) The inability of H-RAS to trigger increased ROS activity (Figure 11B, 12% Vs. 11%) makes it difficult to conclude whether or not YPEL3 overexpression is capable of eliciting ROS activity in IMR90 cells. Since the H-RAS infected cells were not selected and showed a more modest level of mRNA induction (Figure 11A) it is possible that the failure to detect increased ROS activity as previously reported (Moiseeva, 2009) was the result of a low transduction rate or no selection applied. If that assumption is accepted then these results suggest YPEL3 overexpression does not lead to increased ROS activity in IMR90 cells. These results indicate that YPEL3 does not cause an increase in ROS, and may not trigger senescence in a ROS dependant manner in non cancerous IMR90 cells.
A) 

Lentivirus Infection of IMR90 Cells

![Bar chart showing log expression levels of p21, Ras, and YPEL3 in IMR90, IMR90 Y3, and IMR90 RAS cells.]

B) 

![Histograms showing the FL1-A count for H2O2 and YPEL3 treated IMR90 cells, and for H2O2 and RAS treated IMR90 cells.]

![Histograms showing the FL1-A count for YPEL3 and RAS treated IMR90 cells, along with the percentage of FL1-A fluorescence.]
Figure 11: IMR90 Cells Infected With YPEL3 Expressing Lentivirus Does Not Lead To Increased ROS Production. (A) IMR90 cells were infected with Lentivirus expressing YPEL3 or RAS. RNA was isolated at 24 hours. YPEL3, RAS and p21 mRNA levels were analyzed using RT-PCR normalized to GAPDH mRNA levels. Error bars represent 95% confidence intervals from triplicate PCR reactions analyzing YPEL3, p21, RAS and GAPDH cDNA’s respectively. (B) Flow Cytometry for IMR90 cells infected with YPEL3 or RAS, as well as a negative control exposed to DCFDA (30 Min) and a positive control exposed to DCFDA (30 Min) plus the addition of hydrogen peroxide (2 Hr). Cells were isolated by scraping into 1mL DBPS and immediately analyzed by flow cytometry.
V. Co-Immunoprecipitation of YPEL3-V5 Reveals Potential Bound Proteins.

Previous studies have linked increased expression of YPEL3 to cellular senescence (Kelley, 2010 and Tuttle, 2011). However, the mechanism of senescence activation by YPEL3 is unknown. In an attempt to discover putative binding proteins that interact with YPEL3 proteins, a Tet-On expression system was utilized followed by co-immunoprecipitation of YPEL3 and any bound proteins. The Tet-On system allows expression of a gene of interest in the presence of tetracycline, or its derivative doxycycline and contains a strong constitutively active cytomegalovirus promoter which expresses a tetracycline repressor gene that produces a tetracycline repressor protein (TetR). The TetR protein binds to a tetracycline operon that overlaps with a second promoter ligated to an expression gene, which in this case expresses YPEL3. The second promoter is normally repressed by TetR, however in the presence of tetracycline the TetR protein is released from the tet operon and transcription of YPEL3 along with a V5 epitope tag occurs. The V5 epitope tag allows us to co-immunoprecipitate YPEL3 by binding it to anti V5 agarose beads and precipitating it from whole cell extracts. Activation of the second promoter in the presence of tetracycline will cause amplification of YPEL3-V5 protein levels, which can be extracted upon lysis of the cells. In order to perform a co-immunoprecipitation the conditions under which cells are lysed
must be optimized to conserve protein interactions and decrease the chance of protein
degradation. By utilizing anti V5 agarose beads YPEL3-V5 can be extracted along with
any YPEL3 linked proteins. The protein sequences of the co-immunoprecipitated
proteins were determined by liquid chromatography tandem mass spectrometry
analysis (LC/MS/MS) at Oregon Health and Science University. To analyze proteins by
LC/MS/MS, proteins are first enzymatically digested into peptides at Oregon Health
Science University and introduced into a mass spectrometer via a liquid chromatography
system. Once inside the mass spectrometer the peptides are fragmented to produce
MS/MS spectra. The patterns of fragmentation are matched against theoretical spectra
from a protein database and the sequence of each individual peptide is derived. Scoring
algorithms and statistical tools are then used to determine their identification and
quantity.

To determine if we were able to extract YPEL3 by co-immunoprecipitation, MCF7
cells that had previously been infected on two separate occasions with a Tet-On system
followed by infection with lentivirus expressing YPEL3 fused to a V5 epitope tag were
grown in complete media containing 10 μg/mL of tetracycline for 24 hours to induce
YPEL3 gene expression. The two separate infections were identified as Y3O, to indicate
cells created by Kelly Miller, and Y3N to indicate cells created by Dr. Steven Berberich.
Alternatively MCF7 cells were infected with a Tet-On system lacking YPEL3 or a V5 tag
(MCF7 TetR) were also subject to 10 μg/mL of tetracycline for 24 hours. Post
tetracycline exposure, MCF7 cells were scraped into 5 mL of DBPS and pelleted. After
freezing the cells (-80°C minimum of 24 hours) they were thawed and lysed in 5 volumes
of lysis buffer components and centrifuged to separate soluble and insoluble material. A Bradford assay was performed on the extracts to determine the protein concentration. Protein extract (50 mg) was diluted in lysis buffer (10 mL) with the addition of anti V5 agarose beads (40µg) and inverted overnight at 4°C before being subjected to 3 washes to remove unbound proteins. Covalently bound proteins were eluted from the V5 agarose beads in 6X SDS loading dye and proteins were separated on an SDS-PAGE gradient gel by electrophoresis. After Western transfer, the blot was probed for YPEL3 with a primary monoclonal V5 antibody (1:5000 for 4 hours) and a secondary goat anti mouse antibody conjugated to horseradish peroxidase (1:2500). Chemiluminescence analysis of the probed blot showed the presence of YPEL3 in the Y3O and Y3N lanes between the 15-25 kDa molecular weight markers, indicating YPEL3-V5 was eluted in the co-immunoprecipitation (Figure 12). As expected, a band was not present in the extracts not expressing YPEL3-V5 (MCF7 TetR), as indicated by the absence of a band in lane 5 between 15-25 kDa. The higher molecular weight bands indicate V5 antibody binding to human proteins lacking V5 antibody. This could have been caused by insufficient blocking or too high of a primary or secondary antibody concentration. Moving forward growth of the YPEL3 inducible cells known as YPEL3 New or Y3N were discontinued in favor of the cells known as YPEL3 Old, or Y3O, due to a higher level YPEL3 expression determined by qPCR after Y3O and Y3N were treated with 10µg/mL tetracycline (data not shown). In addition the protocol was optimized by exposing the beads to an additional 3 washes in lysis buffer to remove unbound
Figure 12: Extraction of YPEL3-V5 by co-immunoprecipitation.

MCF7 cells harboring a tetracycline expression vector expressing YPEL3-V5 (Y3O or Y3N), or not expressing YPEL3 (TetR) were grown in DMEM minus tetracycline until reaching 80% confluency. Tetracycline was added to the media (10 μg/mL of media) for 24 hours and the cells were scraped in 5 mL DPBS for pelleting. Cells were frozen then lysed (50 mM Tris pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonident p-40, 1 mM phenylmethanesulfonylfluoride (Sigma), 1 mM Dithiothreitol (Sigma) and Protease Inhibitor Cocktail (Sigma)) and centrifuged to remove insoluble material. Protein extracts (40 mg) were co-immunoprecipitated and eluted for separation on an SDS page gel by electrophoresis. Proteins were transferred by Western analysis and probed with primary V5 antibody (1:5000 for 4 hours) and secondary goat anti mouse antibody conjugated to horseradish peroxidase (1:2500). Molecular weights were determined by comparing to the molecular weight marker (M). Lane 2 is blank (B). Arrow is pointing to YPEL3-V5 tagged proteins.
proteins. A colloidal blue stain of each fraction taken after a wash indicated the absence of unbound proteins after 6 washes (data not shown). All co-immunoprecipitation beads from this point were washed 6 times in lysis buffer.

The ability to extract YPEL3-V5 is crucial for the discovery of potential YPEL3 bound proteins. After extraction and separation any bands occurring in the Y3O lane that does not occur in the TetR lane could be potential binding proteins of YPEL3 that would be excised and analyzed by mass spectrometry. To visualize these potential bands a highly sensitive method of protein detection known as silver staining was implemented. To determine the best silver stain method that is mass spectrometry compatible, three silver staining methods were compared, each using an identical amount of protein. Protein for this analysis was obtained either from the whole cell extracts of MCF7 cells (MCF7 TetR and Y3O) after treatment with tetracycline, or protein was eluted off of anti V5 agarose beads after co-immunoprecipitation of whole cell extracts (Figure 1). The Bio-Rad method developed distinct bands with little background noise and would be the preferred method of choice; however this silver stain method is incompatible with mass spectrometry due to the use of a strong oxidizer (Figure 13A). Method 2 had a high level of background noise and either revealed very faint yellow bands, or was not sensitive enough to pick up bands that appeared in the other 2 methods (Figure 13B). The lanes appeared either clear or slightly yellow in method 2. Method 3 was nearly as free of background noise as the Bio Rad kit method and showed an equivalent number of visible bands (Figure 13C). This method is also compatible with mass spectrometry and yields a high level of protein recovery for analysis because it does not cause cross linking.
Figure 13: Comparison of three Silver Stain Methods.

SDS-Page protein gels were fixed in 40% methanol and 5% acetic acid overnight on a shaker.  (A) Method 1 Bio-Rad Silver stain kit (Bio-Rad). (B) Method 2 10% glutaraldehyde, 200 nM dithiothreitol, .1% silver nitrate.  (C) Method 3 sodium thiosulfate, .1% silver nitrate.  (A, B, C) Develop with .04% formalin plus 2% sodium carbonate.  Stop development in 1% acetic acid. The molecular weight marker is indicated by (M). Whole cell extracts were indicated as Tet-On (T) and Tet-On expressing YPEL3 (Y3). The samples labeled (Tf and Y3f) were insoluble protein fractions not bound to V5 agarose beads.

* The gel in method 1 is on the same scale as method 2 and 3 however it appears smaller due it tearing during removal from the electrophoresis apparatus. The majority of the gel that was lost was below the SDS dye front.
of proteins and utilizes a weak oxidizer so silver ions can be easily removed before mass spectrometry. All subsequent experiments use silver staining method 3.

To isolate potential proteins interacting with the YPEL3 protein, whole cell extracts (TetR and Y3) of tetracycline treated cells (10 µg/mL) were co-immunoprecipitated and bound protein was subsequently eluted by a competitively binding V5 peptide (30 min). After the first elution, the anti V5 agarose beads were exposed to 1X SDS loading dye to elute any additional proteins still bound to the beads (BF). The eluted fraction (EF) and BF fraction were separated on an SDS page gel and analyzed by silver staining (Figure 14). The wells loaded with EF appear to be absent of any detectable protein, indicating elution by competing V5 peptide may not be the best method of elution or the elution should have been for a longer period of time. The bead fraction (BF) had two bands appear in the Y3 lane that were absent in the Tet lane. Excision of the bands, followed by Liquid chromatography-tandem mass spectrometry analysis revealed a larger band near 140 kDa (Band 1) contained Mov10 (Putative Helicase), HNRL1 (Heterogeneous nuclear ribonucleoprotein U like protein), Lima1 (Lim domain and actin binding protein) and RBM14 (RNA binding protein 14) (Table 2). Interestingly, these proteins were not found in any of the other analyzed bands. In addition to Band 1, another band near 60 kDa was extracted and analyzed by mass spectrometry (Figure 14). The second band (Band 2) contained two proteins, CARM-1 (Histone Arginine Methyl Transferase) and FA98A (isoform belonging to FAAM98A protein family), not found in any of the other analyzed bands (Table 2).
Figure 14: Secondary elution revealing 2 bands in the YPEL3 only lane. Band 1 was between 140kDa-100kDa and band 2 between 70kDa-50kDa. MCF7 TetR and Y3O cell extracts (40 mg) were co-immunoprecipitated with anti V5 agarose beads and YPEL3-V5 was competitively eluted from the beads after being washed 6 times to remove non specifically bound proteins (EF). The beads were subsequently exposed to SDS loading dye to elute any remaining proteins (Beads). The eluted proteins were separated on an SDS Page gel by electrophoresis and stained with silver stain. Two bands (indicated by arrows) were excised and incubated twice for 30 minutes in 1.0 mL of solution made of acetonitrile and 100 mM ammonium bicarbonate mixed in a 1:1 ratio. After the second washing the mixture was fully removed and the extracted bands were dried in a speed vac.
Under the assumption that the eluted fraction had a low level of protein due to the inability of the competing peptide to efficiently compete off YPEL3-V5 from the beads in 30 minutes, the eluted fractions were concentrated from 160 µL to 20 µL. This also removed any proteins below 3000 Da in size due to the concentrator’s molecular weight cutoff. Silver stain analysis revealed a band above 50 kDa in the Y3 lane (Band 3) however no additional bands were discovered (Figure 15, EFc). Mass spectrometry analysis of this band determined that the majority of the protein was caused by contamination (Table 2). The bead fraction was separated by electrophoresis on an SDS page gel and stained by silver stain (Figure 15 Beads), which showed a similar pattern to the bead extraction in figure 14.

To assess if an alternative method of eluting proteins from anti V5 agarose beads will give us a higher protein yield, the extracts of treated (10 µg/mL tetracycline) MCF7 cells (TetR and Y30) were co-immunoprecipitated by anti V5 agarose beads, however protein was eluted by heating (75°C) the samples for 5 minutes in 1X SDS running buffer (Figure 16). Silver staining was utilized to locate variations in bands between the two samples. After developing the gel, thick bands appeared in each lane above the 50 kDa molecular weight marker and two additional bands appeared at 35 kDa and 25 kDa in each lane. The only variation that occurred was a darker band in the YPEL3 expressing cells near 25 kDa. This band was also visible in MCF7 (TetR) cells, but much more faint. The difference in band intensity does not appear to be loading related because the other bands in each lane exhibit the same level of intensity. Extraction of the band and
Figure 15: Band 3 located between the 50kDa and 70kDa molecular weight markers.

MCF7 TetR and Y3O cell extracts (40 mg) were co-immunoprecipitated with anti V5 agarose beads and YPEL3-V5 was competitively eluted from the beads after being washed 6 times to remove non specifically bound proteins (EFC). The EF fractions were concentrated from 160µL to 20 µL and SDS loading dye was added. The beads were subsequently exposed to SDS loading dye to elute any remaining proteins (Beads). The eluted proteins were separated on an SDS Page gel by electrophoresis and stained with silver stain. One band (indicated by arrow) was excised and shook twice for 30 minutes in 1.0 mL of solution made of acetonitrile and 100 mM ammonium bicarbonate mixed in a 1:1 ratio. After the second washing the mixture was fully removed and the extracted bands were dried in a speed vac.
Figure 16: The fourth extracted band from immunoprecipitated material appeared between the 25kDa and 35kDa molecular weight bands. MCF7 TetR and Y3O cell extracts (68.4 mg) were co-immunoprecipitated with anti V5 agarose beads and YPEL3-V5 was eluted in 1X SDS running buffer after the beads were washed 6 times (EF). The EF fractions were concentrated from 200µL to 20 µL and SDS loading dye was added. The beads were subsequently exposed to SDS loading dye to elute any remaining proteins (Beads). The eluted proteins were separated on an SDS Page gel by electrophoresis and stained with silver stain. One band (indicated by arrow) was excised and shook twice for 30 minutes in 1.0 mL of solution made of acetonitrile and 100 mM ammonium bicarbonate mixed in a 1:1 ratio. After the second washing, the mixture was fully removed, and the extracted bands were dried in a speed vac.
subsequent mass spectrometry analysis did not determine the presence of any potential YPEL3 binding proteins (Table 2).
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## Table 2: Liquid chromatography-tandem mass spectrometry analysis

Analysis of 4 isolated bands reveals potential proteins that may bind to YPEL3.

Emphasis on certain proteins was based on their number of hits in each sample, as well as their molecular weight corresponding to the excised bands. Proteins listed in normal font are not specific to a sample, do not correlate with the molecular weight of the excised band, have a very low hit number, or may not impact senescence. The band numbers above the protein hit number columns correlate to the excised bands.

Proteins highlighted blue correlate to a high level of protein specificity for a certain band. Analysis was performed at Oregon Health and Science University. Abbreviations:

- **OS** = Organism
- **GN** = Gene Name
- **PE** = Evidence at the Protein Level
- **SV** = Sequence Version

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VI. Discussion

YPEL3 gene expression is repressed in prostate cancer cells by testosterone.

Estrogen has been implicated in estrogen receptor positive breast cancer as a pro-growth steroid hormone leading to cell proliferation. A connection was established between estrogen and YPEL3 when the removal of estrogen, from estrogen receptor positive breast cancer cells, caused an induction of YPEL3 gene expression (Tuttle, 2011). This discovery linked estrogen to the senescence inducing YPEL3 gene and demonstrated a potential mechanism by which selective estrogen receptor modulators can block breast cancer proliferation.

To build off of this previous work we expanded the research to include LnCAP prostate cancer cells, as well as utilizing testosterone in an attempt to determine if this steroid hormone also regulates YPEL3 expression. It was discovered that testosterone signals proliferation in androgen receptor positive prostate cancer cells, which indicates testosterone may have the same effect on YPEL3 expression in prostate cancer cells, as estrogen does on ER+ breast cancer cells.

My first objective was to show the removal of hormones induce YPEL3 gene expression, indicating that its repression is dependent on steroid hormones. Like estrogen receptor positive breast cancer cells, growing LnCAP cells in CSS media devoid
of steroid hormones and quantifying YPEL3 mRNA levels we were able to determine that steroid hormones inhibit YPEL3 gene expression in LnCAP cells. Growing LnCAP cells in CSS media with the addition of testosterone treatments repressed YPEL3 gene expression at doses as low as 2 nM when compared to LnCAP cells grown in CSS media only. As expected the known testosterone target gene PSA was induced in the presence of testosterone when compared to LnCAP cells grown in CSS media only. Cells that were harvested 24 hours after treatment did not appear to have any significant differences in density or morphology at the time of harvest indicating a need for additional experiments. Further experiments would need to be conducted to determine if the removal of testosterone leads to YPEL3 dependent cellular senescence. The length of time that 10 nM of testosterone can impact YPEL3 expression was assessed and it was determined that at a time point after 24 hours and between 48 hours PSA expression begins to decline, however YPEL3 is still maximally repressed at 48 hours. This is due to testosterone having a half life between 2 and 4 hours which may indicate that at a lower dose, testosterone can repress YPEL3, but induce PSA as well. Quantification of the estrogen target gene pS2’s mRNA expression showed no change in expression, indicating that testosterone is not being converted to estrogen in LnCAPs even though LnCAPs are reported to express aromatase (Ellem, 2004). Androgen ablation causes growth arrest in androgen receptor positive prostate cancer cells (Agus, 1999). Implications for these findings are a potential mechanism by which selective androgen receptor modulators antagonize androgen receptors which block repression of YPEL3 and induce growth arrest. However, additional experiments would need to be
completed to confirm this link. Knockdown of the androgen receptor in testosterone treated LnCAP cells grown in CSS media followed by YPEL3 gene expression analysis would provide us with the knowledge that testosterone is repressing YPEL3 expression in either an androgen receptor dependent, or independent manner. If YPEL3 gene repression is dependent on the presence of testosterone and the androgen receptor, it would be beneficial to determine if senescence is dependent on YPEL3 expression in LnCAP prostate cancer cells. This could be accomplished by measuring β-galactosidase activity in LnCAP cells grown in full serum media or CSS (+ or – testosterone), and knocking down the androgen receptor in another set grown in CSS (+ or – testosterone). If the β-galactosidase test is positive for senescence in the cells grown in CSS only this indicates senescence is dependent on YPEL3 in the absence of testosterone or the androgen receptor. Therapeutic effects could then be tested by administering selective androgen receptor modulators to the LnCAP cells. Another potential target that may regulate YPEL3 in LnCAPs is ER-β, which is the major estrogen receptor expressed in prostate cells, due to its ability to suppress growth progression through increased levels of p21 (Pravettoni, 2006).

**YPEL3 gene expression is repressed in breast cancer cells following the addition of testosterone.**

Since testosterone has been shown to have a growth inhibiting effect on breast cancer cells, we tested the ability of testosterone to regulate YPEL3 expression as a potential mechanism of growth inhibition. We were able to show that in MCF7 breast
cancer cells testosterone treatment increases YPEL3 gene expression levels. Interestingly, we were able to show that the increase of YPEL3 gene expression is dose dependent and also depends on the dose of estrogen present, indicating these hormones may antagonize each other’s ability to regulate YPEL3 gene expression. These findings suggest a mechanism and also an argument for administering a cocktail of testosterone agonists or testosterone derivatives combined with selective estrogen receptor modulators, as a therapy for endocrine responsive breast cancers. To determine if senescence is testosterone and androgen receptor dependent, this can be accomplished by measuring β-galactosidase activity in MCF7 cells grown in complete media or CSS (+ or – testosterone) and comparing this to MCF7 cells containing a knocking down of the androgen receptor grown, also in CSS (+ or – testosterone).

Further analysis of YPEL3 gene expression in the presence of steroid hormones could move toward analyzing the effects of progesterone. When bound to estrogen, estrogen receptor α can increase gene expression of the progesterone receptor (Kastner, 1990). This increase in progesterone receptor expression plus the addition of progesterone can either, evoke cell cycle progression, or cause cellular differentiation (Alkhalaf, 2003). Transient pulses of progesterone regulate these two counteracting functions in breast cancer cells by evoking cell cycle progression for the first cycle and arresting cells at the G1 /S checkpoint of the second cycle to allow differentiation (Owen, 1998). Differentiation continues until progesterone is degraded and the process repeats (Owen, 1998). Progesterone accomplishes this by initially upregulating p21 gene expression through the transcription factor sp1 (Owen, 1998). This biphasic effect of
progesterone is antagonized by the ability of the ER-α which suppresses p21 activity (Brekman, 2011). This pathway appears to have the potential to respond to endocrine therapy and it has an important link to estrogen receptors, indicating its use as a potential biomarker.

**Increased levels of YPEL3 gene expression does not trigger increased ROS levels**

Reactive oxygen species play a major signaling role in the event of cellular stresses. Replicative senescence, DNA damage response and oncogene mediated senescence all lead to the activation of p53 which is capable of activating p21 and causing growth arrest. Increased expression of p21 increases the cellular levels of ROS in a feedback mechanism to stabilize p53 activation and cause the cell to enter permanent senescence (Passos, 2010). Another target gene of p53 that mediates cellular senescence is YPEL3 (Kelley, 2010).

Conditions were first optimized to detect hydrogen peroxide (H2O2) in MCF7 cells which allows us to determine the threshold that separates cells exhibiting DCFDA fluorescence (positive) from those that do not (negative). This assessment of conditions includes treatments of MCF7 cells with hydrogen peroxide (2 Hr), followed by DCFDA exposure (30 Min), rather than preloading DCFDA at the time of peroxide treatment. Lower levels of fluorescence in the preloaded MCF7 cells could potentially be caused by oxidation of DCFDA outside of the cells which changes its charge and causes it to become impermeable to the cell membrane.
Trypsinizing cells for flow cytometry analysis causes an increased level of ROS production and clumping when compared to scraping in DPBS. This may be caused by an extended length of time that the cells were exposed to trypsin, however scraping in DPBS was used in future isolations. Allowing the MCF7 cells a period of exposure to full serum media after hydrogen peroxide and DCFDA exposure should in theory cause higher levels of DCFDA detections due to reestablishing growth factors in the media that signal metabolism. Counter to this rational, MCF7 cells recovering in full serum media or serum free media actually showed the same pattern of decline in DCFDA fluorescence detection when compared. Declining fluorescence could be attributed to the ability of catalase to convert hydrogen peroxide to oxygen and water, which would leave less to oxidize DCFDA to DCF, or it may be caused by the ability of fluorescent DCF to leak out of live cells and be washed away in DPBS at the time of harvest (Chen, 2003).

Overexpression of YPEL3 in MCF7 cells controlled by a Tet-On system showed by RT-qPCR an increase in YPEL3 gene expression in the absence of tetracycline and an even higher increase when tetracycline was present when compared to MCF7 cells. This increase in YPEL3 expression was not seen in Tet-On cells expressing LacZ. Treatment with DCFDA showed an increase in ROS levels when compared to MCF7 cells in the absence of tetracycline, however this level remained the same when tetracycline was added. This would suggest that the lower level of YPEL3 expression in the cells growth without tetracycline treatment is sufficient to maximally activate ROS production. However the LacZ expressing Tet-On MCF7 cells expressed the same levels of ROS production indicating that a component of the Tet-On system may lead to ROS
production. This increase in ROS could also be caused by the cells reaching confluency and senescing, or cell culture conditions. However, this is unlikely due to the MCF7 cells not having high fluorescence indicating ROS because all cells were subject to the same conditions.

Since the Tet-On system left us with potentially ambiguous results regarding ROS production, alternatively, MCF7 cells were infected with lentivius expressing YPEL3, selected, and hydrogen peroxide levels were measured 2 days post selection. Although a 56-fold induction of YPEL3 gene expression was shown, we were unable to detect an increase in the levels of hydrogen peroxide, relative to MCF7 cells, by flow cytometry analysis, as well as by fluorescent microscopy of DCFDA treated cells counterstained with Hoechst stain. We were able to see an increase in ROS levels by flow cytometry and fluorescent imaging when MCF7 cells express H-RAS. However this could be caused by p21 since RAS levels were undetermined by RT-QPCR analysis.

To assess the potential of YPEL3 everexpression to produce ROS in another cell line, IMR90 cells were utilized. IMR90 primary human diploid fibroblast cells were utilized due to their non transformed nature and ability to undergo replicative senescence, as well as oncogene induced senescence. Assessing IMR90 cells expressing YPEL3 for ROS production, we see that there is not an increase in ROS levels when compared to untreated MCF7 cells. Surprisingly we did not see an increase in ROS levels when IMR90 cells were infected with H-RAS, however, this could be related to using cells that were not selected after infection. This would indicate a low level of infection, which would
correlate to the low levels of RAS induction seen by RT-QPCR. Surprisingly YPEL3 and H-RAS infected cells exhibited 8 and 10 fold increases of p21 respectively. H-RAS expression also caused a 5 fold increase in YPEL3 expression.

**Potential Proteins that Interact With YPEL3 Proteins.**

Since YPEL3 may function as a tumor suppressor activating senescence, it is beneficial to discover putative proteins which interact with YPEL3 proteins. By inducing expression of YPEL3-V5 in MCF7 cells and lysing the cells to make whole cell extracts, we were able to extract YPEL3 and any potential YPEL3 bound proteins by co-immunoprecipitation with anti V5 agarose beads. Analysis of co-immunoprecipitated proteins by western blot analysis with V5 antibody showed YPEL3 bands between 15-25 kDa in the expected lanes indicating we were able to extract the 18 kDa YPEL3-V5 protein from the whole cell extracts (Figure 12).

After optimization of silver staining methods we were able to extract 4 bands not found in MCF7 cells. The bands were analyzed by Liquid chromatography-mass spectrometry, compared against a database of protein sequences and the number of times the sequence appears in the extracted band determines its relevance. After elimination of known contaminating bands and reverse sequences from the results we ended up with 79 potential proteins; however some of these may still may be caused by contamination during handling of the samples. Of the four extracted bands analyzed by mass spectrometry, 3 of the 4 provided useable results, with band 3 composed of mostly contamination. Two of the bands had relatively high hit numbers for proteins
that correspond to the molecular weight where the protein bands were excised from the gels. Gel 1 had bands extracted near 140 kDa as well as 70 kDa. Potential proteins that had high hit levels and may correspond to the 140 kDa band includes MOV10, HNRL1, and RBM14. A potential protein corresponding to the 70 kDa band was CARM1. The inability to detect more proteins in bands 1 and 2, and the inability to detect proteins in bands 3 and 4 could be due to rapid degradation of YPEL3, or due to low affinity or transient transactions that are not conserved outside of a cell's physiological state.

Expression of co-activator associated arginine methyl transferase (CARM-1) had previously been shown in estrogen receptor positive MCF7 cells to slow the rate cell growth in an estrogen dependent manner by associating with estrogen receptor α and upregulating expression of the negative cell cycle regulators p21 and p27 (Al-Dhaheri, 2011). CARM-1’s ability to increase p21 and p27 causes growth arrest by blocking cell cycle entry into S phase (Al-Dhaheri, 2011). Interestingly it was also found to relieve estrogen dependent repression of approximately 56% of estrogen repressed genes (Al-Dhaheri, 2011). This implicates CARM-1 as having a potential role in inhibiting estrogen receptor α’s ability to repress YPEL3 which would trigger growth arrest.

MOV10 is a putative RNA helicase which has been implicated as a novel telomere associated protein that is essential for telomerase activity in transformed cells (Wang, 2010). Telomerase activity will extend the length of telomeres and inhibit replicative senescence in cancer cells. RNA helicases can also modulate the structure of critical
RNA molecules and make them available for processing which leads to protein expression (Wang, 2010). If YPEL3 has the ability to bind to MOV10 it could potentially inhibit its ability to recruit telomerase to the telomeres which would lead to telomere degradation and replicative senescence (Wang, 2010).

HNRL1 and RNA binding protein 14 have functions that have not been fully elucidated and very little is known about them. HNRL1 is a splice variant of N-Methyl-D-aspartate receptor. N-Methyl-D-aspartate receptor belongs to a subcategory of post-synaptic ionotropic glutamate receptors which are important for sodium and calcium ion entry into the neurons. Overstimulation of these receptors has been associated with chronic neurodegenerative conditions due to elevations of calcium concentrations in neurons (Camacho, 2002).

RNA binding protein 14 has been shown to interact with RISC loading complex subunit TARBP2 (Melo, 2009). Inactivation of TARBP2 causes destabilization of the DICER1 protein which leads to tumor formation and defects in the expression of mature miRNA, while activation of TARBP2 inhibits tumors (Melo, 2009).

Conclusion

The goals of this thesis were to expand on the impact that hormones have on YPEL3 gene expression, to determine if YPEL3 has an impact on reactive oxygen species generation and to identify potential proteins that bind to YPEL3 proteins. It was demonstrated that testosterone regulation of YPEL3 is tissue type dependent. Testosterone has an inhibitory effect on YPEL3 gene expression in androgen receptor
positive prostate cancer cells and triggers expression of the YPEL3 gene in androgen receptor positive breast cancer cells. Also demonstrated were the counteracting effects on YPEL3 gene expression by combined testosterone and estrogen treatments. Progesterone did not have an effect on YPEL3 gene expression.

Increasing YPEL3 levels did not demonstrate an increase in reactive oxygen species levels in breast cancer cells, or primary diploid human fibroblasts, indicating YPEL3 must mediate cellular senescence in a ROS independent manner. Co-immunoprecipitation and LC/MS/MS analysis of proteins found 79 potential binding proteins, however this was narrowed down to four potential proteins due to their high frequency levels, their molecular weight corresponding to the size of the excised band and having a potential senescence function. The potential proteins discovered were MOV10, HNRL1, RBM14 and CARM1. Taken together, these findings aim to provide mechanisms of YPEL3 gene expression that may be targeted in endocrine therapy, as well as identifying potential YPEL3 binding proteins that may help us understand the function of YPEL3 better and facilitate the discovery of how it causes cellular senescence.
**VII. Literature Cited**


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