Investigation of Human recombinant Müllerian Inhibiting Substance:
Expression Vector Analysis and Analytical Method Development

A Major Qualifying Project Submitted to the Faculty of

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Abstract

Although chemotherapy is available to patients with ovarian cancer, these patients will eventually become drug resistant, which factors into the disease’s high mortality rate. Müllerian Inhibiting Substance (MIS) has been shown as a potentially effective treatment for ovarian cancer. Under normal biological conditions, MIS causes the regression of the Müllerian Duct during fetal male development, which in females becomes the reproductive organs. MIS works through the Bone Morphogenetic Protein (BMP) pathway, which utilizes the transcriptional regulatory element designated the Bone Morphogenetic Response Element (BRE), resulting in apoptosis of ovarian cancer stem cells partially responsible for reoccurring cancer. Recombinant Human MIS (rhMIS), however, has never been produced in high enough quantities for translation to clinical trials. This MQP focused on the development of new analytical and developmental approaches for purification and detection of rhMIS. rhMIS is found in nature as a proprotein that is cleaved in one location to from a heterodimer. The rhMIS N-terminus is known to stabilize the rhMIS C-terminus. MIS Type II receptor positive OVCAR-8 cells were transfected with a BRE-luciferase report plasmid to identify the effect of active rhMIS. OVCAR-8 cells proved to be unacceptable hosts to create a stable BRE-Luc assay. Contrary to published reports, the relative affinity of rhMIS N-terminus appears not high enough to be used as an affinity ligand for rhMIS C-terminus.
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1. Introduction

1.1. Overview of Cancer

Cancer is a disease that affects people in the United States and around the globe. The United States National Library of Medicine defines cancer as a disease where abnormal cells uncontrollably grow and divide, possessing the ability to invade nearby tissues\(^1\). Currently in the United States, cancer is the second leading cause of death in adults, following heart disease\(^2\). In 2015, it is estimated that approximately 1.6 million new cases of cancer will be diagnosed and approximately 500,000 deaths will be associated with cancer\(^2\). From the 1.6 million cases, 98,000 will be diagnoses of gynecological cancers in women\(^2\). Gynecological cancers are cancers in the uterine cervix, uterine corpus, ovary, vulva, and vagina\(^2\). Among the 98,000 diagnosed gynecological cancers, it is estimated that approximately 21,000 will be of ovarian cancer\(^2\). Approximately 14,000 women will die of ovarian cancer, making it the deadliest of gynecological cancers\(^2\). Research has suggested that many gynecological cancers originate from Müllerian derived tissue\(^2\). During fetal development, the development of male/female reproductive structures is dependent upon the Müllerian Inhibiting Substance (MIS). This hormone signals regression of the müllerian duct through apoptosis, thus allowing the development of the male reproductive organs. One of the primary reasons why treatment for ovarian cancer is largely unsuccessful is because chemotherapy targets cancer cells rather than cancer stem cells. MIS has been researched and seen as a potential therapeutic to ovarian cancer because it induces apoptosis of gynecological stem cells. Although MIS shows great potential as a therapeutic agent, it has never been produced in a large enough scale to move into clinical
trials. This MQP developed initial analytical and purification techniques in an attempt to purify MIS protein in a large scale so that it could move from bench to bedside.

2. Background

2.1. Biological Development of Müllerian Inhibiting Substance (MIS)

The development of primary sex characteristics in embryonic development is partly influenced by the Müllerian Inhibiting Substance (MIS). During early fetal development, embryos have the potential to develop either male or female reproductive organs. Detailed effects of MIS in embryonic development is shown in Figure 1. The absence or presence of MIS dictates the regression of the Müllerian duct. In male embryos, MIS is produced by the sertoli cells of the testis and binds to the MIS Type II receptors (MISRII) located on the primordial Müllerian duct, inducing regression of the duct. In males, serum MIS levels remain high until puberty and then drop to lower levels. The absence of MIS allows for the development of the female reproductive organs. The Müllerian duct forms the upper vagina, cervix, uterus, ovaries and fallopian tubes. In females, MIS is absent in fetal development and becomes slightly detectable during puberty and then undetectable during menopause.

2.2. What are the Cancers Affected by MIS?

While the focus of this project is on ovarian cancer, there are several gonadal cancers that are known to be affected by MIS including: prostate cancer, breast cancer, endometrium cancer, and cervical cancer.

2.2.1. Ovarian Cancer

The ovary consists of three primary cell types, epithelial, germ, and stromal cells. Epithelial tumors arise from the cells that cover the outermost surface of the ovary and most
ovarian carcinomas are epithelial. Germ cell carcinomas arise from the cells responsible for the production of the ova and Stromal tumors form from the structural tissue cells responsible for keeping the ovary intact.

Most cases of ovarian cancer are sporadic and approximately 5-10% are genetic. BRCA1 and BRCA2 are two major genes linked to ovarian cancer. In healthy females, these genes produce important tumor suppressor proteins which repair cells containing damaged DNA. Mutated forms of these genes are present in approximately 10-15% of all ovarian cancer patients and result in a protein product that cannot properly correct damaged DNA, resulting in cancerous cells to grow and proliferate. In humans, the BRCA1 gene is comprised of 24 exons and encodes a protein consisting of 1863 amino acids. Some known tumor suppressor proteins encoded by BRCA1 include p21, ER, and Gadd45.

Ovarian cancer proves to be extremely lethal partly because it remains undetected until it has metastasized beyond the ovaries. Despite the wide range of technological advancements in medicine, the lack of early detection for ovarian cancer continues to be a major problem. Diagnostic techniques have consisted of a serum measurement of the glycoprotein cancer antigen CA-125 as well as transvaginal sonography. This test, although useful, lacks specificity and ultrasound screening has low predictive ability. To this day, there has been no developed technique for rapid early detection of ovarian cancer.

Although the mortality rate is high in ovarian cancer, treatment options are available for patients. They generally consist of a combination of surgical removal of the tumor, platinum–containing medications, alkylating agents and (or) taxol. Despite the therapies available to reduce and eliminate cancer cells, most ovarian cancers will become resistant to treatment,
making ovarian cancer very difficult to permanently eliminate. Because of the high resistance nature of ovarian cancer, new therapeutics have been looked into, one being the use of MIS.

In females, MIS has been shown to have a presence post-embryonic development. In ovarian cancer cells, MIS induces the Bone Morphogenetic Protein (BMP) signaling cascade, leading to the induction of the tumor suppressor genes p16, p107, and p130\textsuperscript{10}. Renaud et al. showed that in ovarian cancer cell lines, MIS induced a transduction pathway which resulted in expression of varying levels of p16, p130 and p107\textsuperscript{10}. p107 and p130 act as G1 checkpoints during mitosis\textsuperscript{10}. When they are hypophosphorolated, they inhibit activity of the E2F proteins, transcription factors which promote cell division\textsuperscript{10}. Renaud et al. discovered that increasing levels of MIS treatment on OVCAR-8 cells increased expression of p16, causing ovarian cancer cells to undergo apoptosis\textsuperscript{10}.

2.2.2. Cervical Cancer
Cervical cancer is cancer of the cells lining the cervix, which connects the uterus to the vagina\textsuperscript{6}. MIS binds to the type II and type I receptors in cervical cancer cells inducing a signaling pathway similar to that of the TGF-\beta superfamily\textsuperscript{11}. Both normal and cancerous cervical cells express the MIS type II receptor\textsuperscript{11}. Barbie et al. found that the MIS type II receptor was present in three cervical cancer cell lines CaSki, SiHa and C33A\textsuperscript{11}. For cells that express the MIS Type II receptor, the binding of MIS to the receptor inhibits cell growth. In cervical cancer cell line C33A, it was shown that MIS inhibited the growth of the cells by inducing transcription of proteins p130 and 107, which act as G1 check points in mitosis, and inducing transcription of tumor suppressor protein p16\textsuperscript{12}. When the p16 is induced, the cervical cancer cells undergo apoptosis\textsuperscript{12}.
2.2.3. Endometrium Cancer

The endometrium consists of the inner lining of the uterus. There are six types of endometrial cancers including adenocarcinoma, carcinosarcoma, squamous cell carcinoma, undifferentiated carcinoma, small cell carcinoma, and transitional carcinoma\textsuperscript{13}. The most common endometrial cancer is the adenocarcinoma, also known as endotroud cancer. Both normal and cancerous human endometrium cells express the MIS type II receptor\textsuperscript{10}. Because some endometrial cells express this receptor, MIS is able to inhibit the growth and differentiation of these cells. MIS signaling induces gene transcription of two members of the Rb family, p107 and p130\textsuperscript{12}. These proteins regulate cell division by regulating the E2F family of transcription factors\textsuperscript{12}. MIS treatment of endometrial stromal cells down regulated E2F1 transcription factor levels, which is known to cause cell arrest and apoptosis\textsuperscript{12}.

2.2.4. Breast Cancer

MIS receptors are expressed in mammary tissue and breast cancer cells\textsuperscript{14}. In mammary cells, MIS uses a NF-κβ signaling pathway to regulate gene expression of tumor suppressor proteins\textsuperscript{14}. It up-regulates the early gene IEX-1S through an NF-κβ dependent mechanism\textsuperscript{4}. When this gene is overexpressed, it inhibits growth of mammary cells, indicating a regulatory pathway for MIS\textsuperscript{4}. Gupta et al. demonstrated that mammary tumors arising in the C (3) T antigen expressed the MIS type II receptor, and in vitro MIS inhibited the growth of these tumor cells\textsuperscript{14}. MIS also induces transcription the IRF-1 gene, resulting in apoptosis of breast cancer cells\textsuperscript{15}.

2.2.5. Prostate Cancer

The prostate gland is found exclusively in males. Anatomically, it is below the bladder and in front of the rectum. The prostate secretes fluid that nourishes sperm cells in semen and while
many forms of prostate cancer exist, the most common cancer is adenocarcinoma, cancer in the prostate gland cells\textsuperscript{16}.

The presence of MIS in the adult male reveals that MIS has a role post development. Tissues and cell lines from male prostates express both the MIS Type II and type I receptor, suggesting that the prostate cells are a target for MIS\textsuperscript{17}. Unlike ovarian, cervical and endometrium cancers, MIS in the prostate does not signal for transcription of genes through the TGF-β family pathway\textsuperscript{17}. Instead, MIS exclusively induces the NF-κβ pathway leading to gene transcription for p65 and p50. It also induces the transcription of the early gene iEX-1, which aids in apoptosis and inhibition of LNCap cells\textsuperscript{17}.

2.3. Müllerian Inhibiting Substance (MIS) and its Receptors

MIS is a 140-kDA glycoprotein disulfide containing homodimer with 15% carbohydrate\textsuperscript{10}. The human gene for MIS is located on chromosome 19 and consists of two identical disulfide-linked subunits of 535 amino acids each\textsuperscript{12}. The protein structure of MIS makes it a part of the Transforming Growth Factor Beta Polypeptide Family (TGF-β)\textsuperscript{10}. Members of the TGF-β family include activins, inhibins and the Bone Morphogenetic Proteins (BMP)\textsuperscript{4}. Figure 2 depicts the various proteins that fall under the TGF-β super family. Because MIS undergoes the BMP pathway, it is further classified as a BMP. This family of proteins regulates growth and differentiation in a variety of tissues by utilizing a receptor-mediated signaling to induce gene transcription in cells\textsuperscript{18}. MIS contains a bioactive C-terminus domain and an inactive N-terminus domain\textsuperscript{3}. Figure 3 shows the comparative homology between the active- C-terminus domains of MIS in animals to humans. The large percent of homology between various animals suggests that the bioactive C-terminus domain of MIS is evolutionarily conserved.
MIS utilizes a two-receptor system mechanism that requires the Type II and type I receptor. The specific MIS Type II receptor is required for MIS to induce a signaling cascade. The Type II receptor is expressed in the urogenital ridge and the mesenchymal cells surrounding the Müllerian Duct, fetal and adult gonads, as well as the coelomic epithelium. Both type I and Type II receptors are serine-threonine kinases which span the cellular membrane. The gene for the MIS type II receptor is located on chromosome 12q13. It is comprised of 11 exons which code for a 63-kDa protein. The type I receptor gene has 9 exons. Both receptors are located on the surface of mesenchymal cells. The type II receptor primarily binds to MIS while the type I receptor is involved in the BMP signaling cascade. Figure 4 depicts the homology comparison for the MIS type II receptor between humans and other animals. The large percent of homology between the Type II receptors in various animals to humans suggests that the Type II receptor is evolutionarily conserved.

2.4. Pathways Influenced by MIS

There are two known pathways that are influenced by the presence of MIS including the BMP and NF-κβ pathway. Within the NF-κβ pathway, there are two sub pathways known as Canonical (classical) pathway and Non-Canonical (Alternative). The Canonical pathway has been studied extensively and is less specific. There is less known about the Non-Canonical pathway, but it is theorized to be more specific and less common.

2.4.1. Bone Morphogenetic Protein (BMP) Pathway

In Müllerian Duct derived tissues, MIS signaling follows the BMP pathway characterized by the use of type I and Type II serine/threonine kinase receptors, shown in Figure 5. The specific MIS Type II receptor is required for the proper binding of MIS and is constitutively active. The binding of MIS to the MIS Type II Receptor (MISTIIR) activates the Type II
receptor leading to the activation of type I receptor by phosphorylation of the Gly-Ser domains in the type I receptor\textsuperscript{21}. The type I receptor is less specific and can be one of following three receptors used in the BMP pathway; activin receptor-like kinase (ALK) -2, ALK-3(BMPR-IA) and ALK-6(BMPR-IB)\textsuperscript{22}. The type I receptor phosphorylates the human homolog to the Mothers Against Decapentpleic (MAD) protein found in the Drosophila and the Sma protein in Caenorhabditis elegans called the SMAD protein\textsuperscript{23}. The three types of human SMAD include: The Receptor-Regulated SMAD (R-SMAD), the Common Partner SMAD (Co-SMAD), and the Inhibitory SMAD (I-SMAD). Each SMAD has two homology domains located at the N and C terminus referred to as MH1 and MH2\textsuperscript{24}. These 40-62kDa proteins are bound by a proline-rich connection\textsuperscript{24}. The R-SMADs (SMAD-1, SMAD-5 and SMAD-8) are the active SMADs and are phosphorylated by the type I receptor in the SSXS (Ser-Ser-Val/Met-Ser) motif located at the C-terminal end of the R-SMAD\textsuperscript{25,10,26}. Once phosphorylated, the R-SMAD binds to the Co-SMAD or an I-SMAD. If bound to a Co-SMAD (SMAD-4) the signal cascade will continue. If bound to an I-SMAD, SMAD-6 or SMAD-7, the signaling cascade is halted\textsuperscript{27}.

After binding of MIS to the MISTIIR, the type I receptor is activated and phosphorylates SMAD-1. SMAD 1 then binds SMAD 4, the only Co-SMAD, causing a conformational change within the unit and creates a SMAD-1/SMAD-4 complex\textsuperscript{25}. The complex translocates into the nucleus and binds to the Bone-Morphogenetic Response Element (BRE) on the DNA at a GCCG sequence\textsuperscript{26}. Because multiple GCCG sequences exist in the BRE gene, there are multiple binding sites for the SMAD-1/SMAD-4 complexes\textsuperscript{29}. In ovarian cells, the complex binds to the BRE inducing transcription of genes coding for of p16, p130 and p107\textsuperscript{10}. These proteins facilitate apoptosis of the cell by inhibiting E2F1, a transcription factor involved in the promotion of division\textsuperscript{10}.
2.4.2. Nuclear Factor that Binds to the Enhancer element of the Immunoglobulin Kappa Light-Chain of Activated B cells (NF-κβ)

Additionally, MIS induces the NF-κβ pathway. A nuclear factor that binds to the enhancer element of the immunoglobulin kappa light-chain of activated B cells (NF-κβ) is a family of transcription factors that play an essential role in inflammation, immunity, and cancer\textsuperscript{28}. Proteins of the NF-κβ family exist in almost all cell types and regulate different genes with a variety of functions\textsuperscript{28}. There are a total of five known members of this family including RelA (p65), RelB, c-Rel, NF-κβ 1 (p105), and NF-κβ 2 (p100)\textsuperscript{28}. p105 and p100 are later processed into shorter forms known as p50 and p52\textsuperscript{28}. All members of the NF-κβ family contain an N-terminal Rel homology domain (RHD)\textsuperscript{29}. The RHD is the dimerization and DNA-binding site for these proteins, contains nuclear localization sequence (NLS), and is the binding site of NF-κβ inhibitors\textsuperscript{29}. Members RelA, RelB, and c-Rel also have C-terminal transactivation domains that are necessary for transcription of DNA\textsuperscript{28}. These proteins are located in the cytoplasm in the inactive state\textsuperscript{29}.

The NF-κβ proteins are bound to inhibitors of the Iκβ family, making the NF-κβ proteins transcriptionally inactive\textsuperscript{28}. This Iκβ family consists of four members including IκBα, IκBβ, IκBε and BCL-3\textsuperscript{28}. Members of the Iκβ family are characterized by their ankryin repeats which allow binding of the IκBs to the NF-κβ proteins\textsuperscript{28}.

This signaling cascade is dependent on the IKK kinase complex\textsuperscript{30}. This complex is comprised kinases IKKα, IKKβ and a regularity subunit known as IKKγ (NEMO)\textsuperscript{30}. IKKα and IKKβ phosphorylate the IκB inhibitors, resulting in polyubiquitination and destruction by a proteasome and NF-κβ proteins to be released and able to enter the nucleus for transcription\textsuperscript{31}. The NF-κβ pathway is composed of two signaling pathways, canonical and non-canonical.
2.4.2.1. Canonical (Classical)

The canonical pathway (classical pathway relies on signaling mediated by the Toll-like receptors (TLRS). These TLRS are located on the cellular membrane and include Interleukin-1 receptor (IL-1R), tumor necrosis factor receptor (TNFR) and antigen receptors. Signaling molecules for these receptors include, inflammatory cytokines, pathogen-associated molecules, and antigen receptors. There are three canonical NF-κβ proteins, RelA, c-Rel and p50, which form dimers. These dimers remain inactive in the cytoplasm when bound to the IκB family of proteins. Once these receptors are stimulated, the IκB kinase complex becomes activated. The IκB proteins contain serine residues where the IKKβ binds to and phosphorylates. IKKβ phosphorylate IκBα on serine residues S32 and S36. The phosphorylated IκB proteins, primarily IκBα, are then modified by K48-linked ubiquitin chains through ubiquitin ligases. Ubiquitination of the IκB proteins leads to their degradation by the proteasome, allowing the NF-κβ dimers to translocate to the nucleus and induce activation of gene transcription. Figure 6 depicts the key events in the NF-κβ canonical pathway.

2.4.2.2. Non-Canonical (Alternative)

The non-canonical (alternative) pathway relies on signaling mediated by receptors including B-cell activation factor (BAFFR), lymphotoxin β-receptor (LTβR), CD40, receptor activator for nuclear factor kappa B (RANK), TNFR2 and Fn14. This pathway relies on IKKα, but not IKKβ or IKKγ. When the receptors are stimulated, it leads to activation of IKKα by the NF-κB-inducing kinase (NIK). IKKα then phosphorylates p100 on serine residues S866 and S870. Phosphorylation of the p100 results in polyubiquitination of p100 at Lys855. It is then processed by a proteasome
to p52. The p52-RelB heterodimers are then able to translocate into the nucleus and induce gene transcription\textsuperscript{28}. Figure 6 depicts the key events in the NF-κβ non-canonical pathway.

2.5. Bioactivity of C- MIS

Once generated, the MIS molecule undergoes post-translational modification. There are two components to this hormone, the N-Terminus and the C-terminus. The protein sequence of monomer MIS contains an amino acid sequence of 20 residues and a monomeric protein component consisting of 535 amino acids\textsuperscript{33}. When the 535 amino acid sequences are glycosylated at two glycosylation sites, the overall weight of the protein becomes 70kda\textsuperscript{3}. Cleavage by a protease at amino acid residue 427 shows the “breakage” of the components into two respective parts; the 55kDa N-terminus and the 12.5 bioactive C-terminus\textsuperscript{3}. Although the function of the N-terminus MIS is unknown, it has been suggested by pervious research to act as a stabilizing component for the mature, bioactive C-terminus MIS. Clemente et al.’s research indicated that the cleaved C-terminus MIS binds to the MIS Type II receptor and induces a signaling cascade resulting in apoptosis cells\textsuperscript{33}. Purification of the bioactive C terminus MIS has been attempted, but it has been shown to degrade during the process. This resulted in difficulties with mass production of the C-terminus, preventing it from progressing to clinical trials.

Scientists have been investing means to produce active MIS for clinical trials for three decades. The potential for cancer therapeutics has been remarkable, however each trial has gone unsuccessful due to the impossible nature of producing MIS. At best only a few mg/ml has been produced and for therapeutics, kilograms would be needed yearly as patients using this treatment may be on it for life. This poses a problem that needs to be remedied. MIS has the biological properties to potentially end drug resistant recurrent Ovarian Cancer all that needs to happen is a production of MIS greater then past attempts.
Bioactive MIS has been unable to be produced, therefore, SP600125 has been used as an alternative to testing. SP600125 is a small molecule that has been shown to inhibit c-Jun N-Terminal Kinase (JNK) pathways by competitively binding to the ATP binding site\textsuperscript{34}. Though it is not confirmed that SP600125 works specifically on the MISRII, it has been shown to activate a BRE-Luc plasmid in cells containing this receptor. When used on MISRII positive cells, such as MOV CAR-7 and COS-7 cells transfected with the MISRII receptor, SP600125 was shown to inhibit proliferation, and it has not been shown to activate other TGF-\(\beta\) receptors\textsuperscript{35}. Because MIS has not been able to be purified in large quantities for testing, SP600125 was determined to be a good substitute for MIS when complete luciferase assays.

3. Materials and Methods

3.1. Cell Culture

Human ovarian cancer cell lines, OVCAR-8, were grown in Roswell Parke Memorial Institute-1640 media (RPMI-1640) with 10% Fetal Bovine Serum (FBS), 100U/mL penicillin and 100U/mL streptomycin. OVCAR-8 cells transfected with BRE-Luciferase plasmid had 10mg/ml puromycin added to media. Transfected monkey kidney cells, COS-7, were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS, 100mg/mL hygromycin, and 10mg/mL puromycin All cell lines were incubated in 5% CO\(_2\) at 37\(^\circ\)C. All experiments were conducted with low passage cells.

3.2. Plasmid Construction

In order to transfec into mammalian cells the following plasmids were created by BlueSky Biosciences of Worcester MA for the use by Nemucore Medical Innovations.
3.2.1. rhMIS N-Terminus Plasmids

Two constructs containing rhMIS N-terminus, referred to as Construct 7 and Construct 12 (Figures 7 and 8), were created for transfection and production in COS-7 cells (monkey kidney cells). To select for cells successfully transfected with rhMIS N-Terminus plasmid, hygromycin resistance was added to the plasmid. In order for secretion of the rhMIS N-terminus, signal sequences IL2 and CD33 were incorporated in constructs 7 and 12 respectively. To facilitate binding on the rhMIS N-terminus to Ni\(^{2+}\), both constructs contained a sequence coding for 8 histidine residues. For later removal of the histidines from the rhMIS N-terminus, a TEV cleavage site was incorporated into both constructs. Promotion of rhMIS N-terminus dimerization was facilitated by the addition of a HulG/I FC Plus Linker in construct 12.

3.2.2. rhMIS C-Terminus Plasmids

For production of rhMIS C-terminus in COS-7 (monkey kidney) cells, an rhMIS C-Terminus plasmid was created for co-transfection with rhMIS N-Terminus plasmids (construct 7 or construct 12). The details of this plasmid can be seen in Figure 9 in order for secretion of the rhMIS C-terminus, the signal sequence CD33 was incorporated into the plasmid construct. To select for cells successfully co-transfected with rhMIS N-terminus and rhMIS C-terminus, puromycin resistance was added to the rhMIS C-terminus plasmid.

3.2.3. BRE-Luc- Puro Plasmid

In order to create a stable luciferase assay using OVCAR-8 cells, a BRE-Luciferase plasmid was created using the pGL 17.Luc/Puro plasmid from Promega. Through research it was determined the sequence of the BRE was determined and cloned between the NheI restriction sites:

TCAGACCGTTAGACGCCAGGACGGGCTGTCAGGCTGGCGCCGCGCCAGCCTGA
To select OVCAR-8 cells successfully transfected with BRE/Luc plasmid, puromycin resistance gene was chosen. The details of the BRE plasmid are depicted in Figure 10.

3.3. Purification of COS-7 Construct Media

3.3.1. His-Tag Nickel Column

A Ni2+ affinity column was attached to a Fast Protein Liquid Chromatography (FPLC) machine (AKTA Prime) to detect and isolate the His-tagged rhMIS N-terminus and His-tagged rhMIS N-C-terminus heterodimer from media produced by COS-7 Construct 7 C-MIS, COS-7 Construct 12 C-MIS, and COS-7 Construct 12. The Ni2+ affinity column was washed with 140mL of deionized H2O. Next, 50mL of 50mM Imidazole binding buffer was injected into the FPLC and allowed to run through the column. Once a baseline level was reached, 100mL of media produced by COS-7 cell constructs mentioned above was injected. The media was then allowed to pass through the column until binding buffer baseline was again reached. Next, a 250mM Imidazole elution buffer was injected into the FPLC. The elution buffer was allowed to run through the column until a peak containing the proteins of interest was detected. Fractions containing samples with proteins of interest were then further analyzed via gel electrophoresis and western blot analysis.

3.3.2. Gel Electrophoresis

To complete gel electrophoresis on samples containing the proteins of interest, a SureLock X-Cell system was used with Novex Tris-Glycine SDS Running Buffer (10x) with Novex 4-20% Tris-Glycine gels. Samples containing the proteins of interest were prepared with 1% reducing agent and 25% Sample buffer and 15uL of the prepared samples were added into each well.
3.4. Western Blots

To confirm/deny the presence of the rhMIS N-terminus and rhMIS C-terminus in samples analyzed by the gel electrophoresis, western blots were completed. The gel electrophoresis analyzed by western blot was composed of 18% acrylamide, Tris-Glycine and completed by BlueSky Biosciences. For detection of his-tagged rhMIS N-terminus in samples, a western blot was probed with Mouse Anti-His mAb. To detect rhMIS C-terminus, a western blot was probed with Rabbit Anti-C-MIS mAb.

3.5. Transfection of OVCAR-8 with BRE-Luciferase Plasmid

OVCAR-8 cells were plated in 4 wells of a 6-well plate at 150k cells/well. The cells were allowed to attach overnight. OPTI-MEM was used as the transfection media. Increasing amounts of Lipofectamine 2000 was added to 150uL OPTI-MEM for each well in the following amounts of Lipofectamine: 1uL 2uL 4uL and 8uL. A solution of plasmid was made with 600uL OPTI-MEM and enough plasmid to make a 3ug/mL solution. 150uL of the plasmid solution was added to each Lipofectamine dilution and incubated for 10 minutes. The cells were removed from the incubator and washed with 2mL PBS. 250uL of the Lipofectamine/plasmid media was added to the well-intended for the respective concentration of Lipofectamine. 250uL OPTI-MEM was then added to each well to make the total volume 500uL. After 2 hours of incubation, 1500uL of the RPMI 1640 with 10%FBS was added to the wells to allow for full growth of the cells. The transfected cells were then allowed to grow for 48 hours. They were then treated with the resistant marker in the plasmid and allowed to grow for 72 hours. After this time, the media was changed with the resistance marker and colonies were picked for future growth.
3.6. Luciferase Assay

BRE-Luciferase plasmid was transfected into low passage OVCAR-8 cells. These cells were then plated alongside non-transfected OVCAR-8 cells in one opaque 96-well plate. The plated cells were treated with SP600125 24 hours after plating in the following concentrations of SP600125: 0uM, 0.1uM, 10uM and 100uM. This was completed in the following FBS percent concentrations: 10%, 0.1% and 0%. 24hours after treatment, media was removed and cells were washed with PBS. 100uL of Glo-Lysis Buffer (Promega) was added to each well and incubated at room temperature for 5 min. 100uL of Bright-Glo (PROMEGA) was added to each well after lysis buffer incubation. The plates were then read for emission at 1sec/well by a Synergy plate reader.

4. Results

4.1. Purification Background

Previously used purification methods of MIS have only been successful in purification of extremely small quantities of the hormone. The lack of a method for large scale MIS production has prevented MIS from moving to clinical trials for ovarian cancer therapy. Researchers attempted to produce the bioactive C-terminus MIS, but were unsuccessful because rhMIS C-terminus degraded after production. Pervious research has indicated that stabilization of the C-terminus MIS could be achieved through non-covalent binding with the N-terminus MIS. To produce rhMIS in large quantities, Nemucore attempted to develop a new and innovative approach to purification of rhMIS in mammalian cells. The intention of this project was to co-transfect rhMIS C-terminus plasmid with rhMIS N-terminus plasmid in mammalian cells with the hypothesis that the rhMIS N-terminus and rhMIS C-terminus produced would bind together
to form a heterodimer. To determine constructs with the highest protein production, Nemucore and BlueSky created six rhMIS N-terminus plasmids and six rhMIS C-terminus plasmids.

Incorporated into the twelve constructs were one of five different mammalian cell secretion signal sequences. In nature, full length MIS is present in dimer form, therefore to facilitate production of the dimer form, Construct 6 (rhMIS C-terminus) and Construct 12 (rhMIS N-terminus) contained a HulgGI FC Plus Linker. All rhMIS N-Terminus plasmids contain a His-tag consisting of eight histidines to allow binding of the rhMIS N-terminus to the Ni$^{2+}$ Affinity Column. In order to be purified using a strep-tactin purification method, all rhMIS C-terminus plasmids contained strep-tag. Additionally, all plasmids contained hygromycin resistance for selection. These plasmids were then transfected into six different mammalian cell lines. Initial testing showed that monkey kidney cells (COS-7) were the best cell line for expressing proteins and only two rhMIS N-terminus constructs were able to be produced in high enough quantities for purification; Construct 7 and Construct 12 (Figures 11 and 12). The rhMIS C-terminus plasmids were unable to express confirmed stable rhMIS C-terminus.

Since rhMIS C-terminus was unable to be produced through transfection of the initial plasmids, a secondary rhMIS C-terminus plasmid containing a puromycin resistance marker was created with the intention of co-transfection with the rhMIS N-terminus plasmid (Figure 13). The rhMIS C-terminus contained a puromycin resistance marker rather than a hygromycin resistance marker to ensure that only cells successfully co-transfected would survive cell selection. The three following construct combinations were transfected into COS-7 cells and the cell media was purified: COS-7 Construct 7- rhMIS C-terminus, COS-7 Construct 12- rhMIS C-terminus, and COS-7 Construct 12 rhMIS N-terminus.
4.2. Affinity Column and FPLC Purification

The eight histidine tag in the rhMIS N-terminus plasmid was transfected in three different construct combinations mentioned above to allow for the media produced by the COS-7 cells to be purified via Ni\(^{2+}\) affinity column. The transfected COS-7 cells were allowed to grow for 72 hours until media became saturated, which was then collected. The collected media was spun down to remove cell debris, then purification was initiated. These media samples were then analyzed by a Fast Protein Liquid Chromatography (FPLC) machine, allowing for UV detection of protein eluted from the affinity column. To interfere with other proteins that exhibit weak binding to the Ni\(^{2+}\) affinity column, each run used a binding buffer containing 50mM imidazole. Elution buffer containing 250mM imidazole was used to facilitate elution of strongly bound His-tagged rhMIS N-terminus from the column. FPLC peaks exhibited during binding buffer wash were determined to not contain the His-tagged rhMIS N-terminus. Fractions produced in FPLC peaks exhibited after elution buffer wash were collected and analyzed in gel electrophoresis because they most likely contained the His-tagged rhMIS N-terminus. For COS-7 cells co-transfected with the rhMIS C-terminus plasmid, it was hypothesized that the rhMIS C-terminus would bind with the rhMIS N-terminus resulting in the elution of the heterodimer.

4.2.1. COS-7 Cells Transfected with Construct 7-rhMIS C-Terminus

In order to determine if proteins could be produced without facilitated dimerization, COS-7 cells were co-transfected with Construct 7 (no HulgGI FC Plus Linker) and rhMIS C-Terminus plasmids. The FLPC graph of purified media produced by these cells is shown in Figure 14. Peak 4 eluted with the binding buffer and it can therefore be concluded that this peak contained weakly bound proteins that did not contain the His-tag. Peak 6 was formed after the addition of the elution buffer and therefore the fractions within this peak (20-25) were
determined to contain the His-tagged rhMIS N-terminus and the rhMIS N-C terminus heterodimer. Fractions 20-25 were analyzed for protein content using gel electrophoresis. All samples in the coomassie gel in Figure 14 appear to contain the rhMIS N-terminus monomer (49.5kDa), samples 21-24 appear to contain rhMIS N-terminus homodimer (99 kDa), samples 21-23 appear to contain the rhMIS C-terminus monomer (11.5 kDa), and samples 21-23 to have the rhMIS N-C terminus heterodimer (61 kDa).

4.2.2. COS-7 Cells Transfected with Construct 12-rhMIS C-Terminus

To determine if proteins could be produced in dimer form, COS-7 cells were co-transfected with Construct 12 and rhMIS C-Terminus plasmids. The HulgGI FC Plus Linker (FC Linker) helps to facilitate dimerization in Construct 12 and it was therefore theorized to facilitate dimerization of rhMIS N- C-terminus heterodimer in produced media. The FLPC graph of the purified media produced by these cells is shown in Figure 15. Since Peak 3 was formed during the wash with the binding buffer, it can be concluded that the proteins in this peak did not bind strongly enough to the Ni$^{2+}$ affinity column and did not contain the His-tag. Peak 4 was formed after the elution buffer and fractions compromising this peak (23-25) were therefore concluded to contain his-tagged rhMIS N-terminus and rhMIS N-C- terminus heterodimer. These samples were analyzed in the coomassie gel seen in Figure 15. Samples 23-25 appear to contain rhMIS N-terminus +FC linker homodimer (152 kDa), rhMIS N-terminus +FC linker monomer (76 kDa), and the rhMIS N-C-terminus +FC linker heterodimer (87.5 kDa). Sample 24 appears to contain rhMIS C-terminus monomer (11.5 kDa).

4.2.3. COS-7 Cells Transfected with Construct 12-rhMIS N-Terminus

For a control that could be used in later experiments, COS-7 cells were transfected with only Construct 12. The FLPC graph of media produced by these cells is shown in Figure 16.
Peak 3 was formed during the wash with binding buffer and therefore proteins eluted during this peak can be concluded to be weakly bound to Ni$^{2+}$ affinity column and not contain the His-tag.

Peak 4 was formed after the elution buffer and theretofore fractions comprising this peak (12-16) were determined to contain the His-tagged rhMIS N-terminus+ FC linker. Fractions 12-16 were analyzed on a coomassie gel shown in Figure 16 and the following analysis was made: samples 13-16 appear to contain the rhMIS N-terminus+FC linker homodimer (152 kDa), and samples 13-15 appear to contain rhMIS N-terminus+FC linker monomer (76 kDa). Sample 14 contains a band in the 11.5 kDa range. Since these cells were not co-transfected with the rhMIS C-terminus, there should not be a band in this range. The presence of this band suggests the possibility that the proteins in the 11.5kDa range in Figures 14 and 15 were not the rhMIS C-terminus.

Appearance of multiple laddering bands in all coomassie gels indicate, that overall, purification was not specific enough.

4.2.4. Western Analysis of Purified Media

To determine if the proteins of interest in the samples tested in gel electrophoresis were actually purified, western blot analysis was completed. Figure 17 shows two westerns completed to determine if the His-tagged rhMIS N-terminus and rhMIS C-terminus were present in the fractions analyzed by the coomassie gel.

The top western blot in Figure 17 was probed with an Anti-HIS mAb to detect rhMIS N-terminus. The presence of bands at the expected size ranges in this western blot indicates that the rhMIS-N-terminus was successfully purified by the Ni$^{2+}$ affinity column in both monomer and dimer form. The rhMIS- N-terminus+ FC linker monomer (76 kDa) is indicated by the green box. Boxed in Blue appears to be the rhMIS N-terminus+ FC linker homodimer (152 kDa). Boxed in red appears to be the rhMIS N-terminus homodimer (90kDa). Boxed in orange appears
to be the rhMIS N-terminus monomer (49.5kDa). The lack of protein bands in lanes 2, 3 and 11 indicates that fractions in these lanes did not contain the rhMIS N-terminus in any form.

Fractions in lanes 21, 23 and 25 were taken directly from the media produced and not from FPLC fractions. The lack of protein bands present in these lanes suggests that the samples were too dilute and therefore the His-tagged rhMIS N-terminus was not able to be detected by the mouse anti-His mAb.

The bottom western blot in Figure 17 was probed with rabbit anti-C-MIS to detect rhMIS N-C-terminus heterodimer and rhMIS C-terminus monomer in all samples tested in gel electrophoresis. The lack of protein bands where rhMIS N-C-terminus heterodimer (61 kDa) and rhMIS C-terminus monomer (11.5 kDa) would be expected in all samples indicates that neither rhMIS N-C-terminus heterodimer nor rhMIS C-terminus was purified. Additionally, this could suggest that either rhMIS C-terminus was degraded in the process or that the relative affinity of rhMIS N-terminus was not high enough to be used as an affinity ligand for rhMIS C-terminus. Bands present in lanes 7-9 correspond with samples from purified COS-7 cells transfected with Construct 12, however this media did not contain rhMIS C-terminus plasmid. The presence of protein bands in these lanes lead to the conclusion that the Rabbit anti-C MIS antibody had non-specific binding. Furthermore, lanes 20-25 contained fractions taken directly from media and were considered to be dilute and protein detection was not expected. Bands in these lanes, therefore, further support the claim that the Rabbit anti-C MIS antibody had non-specific binding.

The purifications of COS-7 cells transfected with Construct 7, Construct 12 and rhMIS C-Terminus plasmids was determined inconclusive. These samples were intended to be used on the luciferase assay being developed in ovarian cancer cells, however because they had not been
purified successfully, an alternative molecule was looked into. SP600125, a small molecule found to activate the BMP pathway in a similar way to MIS, was therefore used as a control for the Luciferase assay.

4.3. Luciferase Assays

4.3.1. Treatment of SP600125 at 4 and 24 hours in 10% FBS

To develop a new analytical method to measure the activity of MIS on the BMP pathway, an initial luciferase model was developed. The BRE-Luciferase report plasmid (Figure 10) was transfected into ovarian cancer cells (OVCAR-8) which would luminesce when the BMP pathway was activated. To account for potential background luminescence, un-transfected OVCAR-8 cells were used as controls. The intent was to use purified media samples of rhMIS as treatments for the assay, however because the protein purification was found to be unsuccessful, SP600125 was used instead. Previous research had discovered that SP600125, which is a small particle molecule, behaves like MIS to activate the BMP pathway and was therefore used to mimic MIS in this assay. This molecule was found to cause heart failure in mice and was therefore terminated as a potential ovarian cancer therapeutic. The following concentrations of SP600125 were used to create a standard curve of activated luminescence: 0uM, 0.1uM, 1uM, 10uM and 100uM. To determine the effect of the time on luminescence, transfected OVCAR 8 and control cells were treated for 4 and 24 hours in in 10% Fetal Bovine Serum (FBS). The graphed results can be seen in Figure 18.

OVCAR-8 cells transfected with BRE-Luciferase plasmid treated at 4 and 24 hours showed higher luminescence than the control cells at these times. For this reason, it was concluded that the BRE-Luc gene increased the levels of luminescence in OVCAR-8 cells. Since both OVCAR-8 BRE and the control cells treated for 24 hours showed greater luminescence than
cells treated for 4 hours, it was determined that 24 hours of treatment increased the luminescence. It was hypothesized that increased concentrations of SP600125 would increase binding activity of SP600125 and therefore increased production of luciferase. The decrease in luminescence on OVCAR-8 BRE-Luc treated with 100uM SP600125 indicates that this concentration may have been causing cells to undergo apoptosis, which could have resulted in the lower luminescence.

The increased luminescence seen in controls suggests that a ligand in the FBS was binding to the MIS Type II receptor initiating the BMP pathway. Therefore, the luciferase assay was redone in OVCAR-8 cells transfected with BRE-Luc plasmid in serum starved conditions.

4.3.2. Treatment of SP600125 for 24 hours in 0%, 0.1% and 10% FBS Conditions

In order to determine the effects of FBS on the luciferase assay, OVCAR-8 cells transfected with BRE- Luc plasmid were plated in 0% and 0.1% FBS starved conditions and also in 10% FBS as a control. The following concentrations of SP600125 were used: 0uM, 0.1uM, 1uM and 10uM. 100uM was removed from the testing treatments because it showed a decrease in luminescence in the initial testing of OVCAR- 8 cells transfected with BRE-Luc plasmid and the control cells. 0uM SP600125 treatment of OVCAR- 8 cells transfected with the BRE-Luc plasmid was conducted to determine background luminescence and serve as negative control.

The graphed results can be seen in Figure 19.

Untreated cells showed the highest luminescence in both 0% and 0.1% FBS. No pattern for luminescence was observed with the different treatments of SP600125. Increased concentrations of SP600125 did not correlate with increased levels of luminescence. The cells plated in 0% FBS showed lower levels of luminescence when compared to the high levels at 0.1% and 10% FBS, leading to the conclusion that increased levels of FBS positively correlates
with increased luminescence. Since no ligand should have been present in the untreated cells plated in 0% FBS, it was concluded it the BMP pathway in OVCAR-8 cells is constitutively active. Therefore OVCAR-8 cells were deemed unreliable for future testing of this luciferase assay.

5. Discussion

The development of cancer therapeutics that induce apoptosis of cancer cells and cancer stem cells is crucial for cancers that have high reoccurrence rates and exhibit high resistance to chemotherapy. Müllerian Inhibiting Substance (MIS) has the potential to be used as a cancer localizing therapeutic for ovarian cancer. The bioactive C-terminus MIS binds specifically to the MIS Type II receptor, causing a BMP signal cascade which induces transcription of apoptotic proteins. MIS induces apoptosis in cells positive for the Type II receptor, while leaving cells negative for the Type II receptor unaffected. Although the function of the N-terminus component of MIS is unknown, previous research has indicated that it acts as a stabilizing component to the bioactive C-terminus. This was suggested partly because proteosomal cleavage dissociating the N-terminus from the C-terminus occurs upon the hormone binding to the MIS Type II receptor. While MIS shows great potential to be a therapeutic agent for ovarian cancer stem cells because of its specificity, it has never been produced in a large scale, preventing it from moving to clinical trials. This project aimed to develop initial analytical and developmental methods to purify recombinant human MIS N-terminus and recombinant human MIS C-terminus via Ni²⁺ affinity column in congruence with the development of a reliable luciferase assay to detect MIS activity on ovarian cancer cells.
After researching the bioactive component of C-terminus MIS and the potential stabilizing effect of the N-terminus MIS, Nemucore sought to develop an innovative method to purify the two components. Plasmid constructs for production of rhMIS N-terminus and rhMIS C-terminus were constructed by BlueSky Biosciences and Nemucore. They were then co-transfected into COS-7 cells for media purification. It was hypothesized that the cells would produce the rhMIS N-terminus and rhMIS C-terminus and the two components would covalently bind in media, which would then be purified. Results from purification of COS-7 construct 7- C-MIS, COS-7 Construct 12 C-MIS, and Cos-7 Construct 12 via Ni\(^{2+}\) show that purification of the HIS-tagged rhMIS N-terminus was possible. This was evident on the western blot probed with the mouse anti- His mAb. The results, however, also indicate non-specific binding of proteins to the column as shown by the multitude of proteins present on the coomassie gels. In theory, after the addition of binding buffer, protein elution should show multiple peaks from the FPLC because binding affinities of proteins differ. The FPLCs obtained from all purifications show a single broad peak after the addition of the binding buffer, further suggesting non-specific binding. For this reason, it is suggested that the binding solution be made with an increased amount of imidazole. This can help with increased elution of proteins that are weakly bound, leaving only the His-tagged rhMIS N-terminus on the column and minimizing non-specific binding. The proof that the His-tagged rhMIS N-terminus was able to be purified indicates that purification using this technique is viable. It is suggested that before further purification be completed, optimizations to imidazole concentrations be made until only the proteins of interest appear on the coomassie gel.

Although gel electrophoresis suggests the presence of the rhMIS N-C-terminus heterodimer, rhMIS-N-C-terminus+ FC linker heterodimer, and the rhMIS C-terminus monomer,
western blots of gel probed with rabbit anti-C-MIS mAb show that the rhMIS C-terminus was not present in either form. It is unclear why this is the case. It could have degraded after production in COS-7 cells, degraded during the purification process, or it could have never bound to the rhMIS N-terminus. To better understand this, it is recommended that an rhMIS C-terminus plasmid be constructed with the addition of a HIS-tag. This plasmid should then be transfected into COS-7 cells for individual purification and to better understand its binding capabilities to the Ni²⁺ affinity column. To determine if the addition of the HIS-tag could aid in the rhMIS N-C-terminus purification through the Ni²⁺ column, the His-tagged C-terminus plasmid could additionally be co-transfected with both rhMIS N-terminus plasmids in COS-7 cells. Since the western probed with anti-C-MIS showed non-specific binding, a new anti-C-MIS mAb should be utilized.

MIS activates a pathway known as the Bone Morphogenetic Protein (BMP) pathway. After binding to the MIS Type II receptor, a type I receptor is recruited and the SMAD pathway is induced. This pathway induction leads to the transcription of apoptotic proteins, resulting in cell death. To develop an analytical method to detect the activity of this pathway, ovarian cancer cells (OVCAR-8) naturally containing the MIS Type II receptor were transfected with a plasmid containing the Bone Morphogenetic Response Element and Luciferase gene. Since MIS is unable to be produced in large scales, SP600125 was used to mimic MIS in these assays. This small particle molecule is known to activate the BMP pathway upon binding to the MIS type II receptor and is not known to bind to other receptors in the TGF-β superfamily. It was hypothesized that binding of the SP600125 to Type II receptor of OVCAR-8 cells transfected with the BRE-Luc plasmid would induce the BMP pathway which could be measured by reading luminescence. Increased levels of luminescence would indicate increased activation of the BMP
pathway. Higher concentrations of SP600125 were theorized to result in greater luminescence of the transfected OVCAR-8 cells. Untransfected OVCAR- 8 cells, the control cells, were theorized to have little to no luminescence regardless of the concentration of SP600125. Results from luciferase assays completed on transfected and the control OVCAR-8 cells indicate that, although the transfected OVCAR-8 cells did have increased levels of luminesces, the BMP pathway may be constitutively active in these cells. This was further supported by the high levels of luminescence shown in untreated transfected OVCAR-8 cells in 0% FBS. Comparison between luminescence levels of transfected OVCAR-8 cells plated in 0%, 0.1% and 10% FBS indicate a positive correlation between increased FBS levels and luminance. This suggest that serum proteins in FBS are activating the BMP SMAD pathway. The luciferase assay in FBS starved conditions was completed on transfected OVCAR-8 cells, but not untransfected OVCAR-8 cells. For this reason, the effect of serum starvation on levels of luminescence on starved non-transfected OVCAR-8 cells is not known. It is recommended that this assay be reproduced using untreated, non-transfected OVCAR- 8 cells as a control.

While speculation of constitutively active SMAD pathway is a possible explanation of increased luminescence, there is also a possibility that the BRE-Luciferase plasmid transfected into the OVCAR-8 cells was constitutively active. Since OVCAR-8 cells are ovarian cancer cells, optimization of this assay using these cells has comparative benefits to understanding the BMP pathway in relation to ovarian cancer. Therefore, it is suggested that before termination of this cell line in this assay, optimization techniques be tested. To determine if the BRE-luciferase plasmid is constitutively active, it is recommended that OVCAR-8 cells be transfected with a luciferase plasmid that does not contain the BRE gene. FBS starved Luciferase assays conducted on OVCAR-8 cells transfected with only the luciferase gene could potentially help to determine
if the SMAD pathway or the Luciferase plasmid is constitutively active. If this plasmid is not constitutively active, then luminesce levels should be similar to untransfected OVCAR-8 cells. If luminescence levels, however, are comparable to levels of luminescence in OVCAR-8 cells transfected with the BRE-luciferase gene, then an alternative plasmid construct should be designed.

If suggested modifications to the luciferase assay using OVCAR-8 cells do not produce conclusive results, it is suggested that an alternative mammalian cell line be used to understand the correlation between MIS and luminescence as well as the correlation between the SMAD pathway and luminescence. One cell line that could be utilized is monkey kidney (COS-7) cells because these cells do not naturally have the MIS type II receptor. It is first suggested that the following stable transfection pools of COS-7 cells be made: COS-7 cells transfected with the BRE- Luciferase plasmid, COS-7 cells transfected with the MIS Type II receptor, and COS-7 cells co-transfected with the BRE- Luciferase gene plasmid and the MIS type II receptor. It is also suggested that untransfected COS-7 cells be used both for comparison and as a control. All luciferase assays are suggested to be conducted in 0% FBS serum because a positive correlation between increased FBS and increased luminescence was shown in luciferase assays conducted in this project. Concentrations of SP6000125 treatment should remain consistent to allow for accurate comparison of results between the OVCAR-8 luciferase assays and the COS-7 luciferase assays. Twenty-four hour treatment of COS-7 cells with SP600125 should be implemented before measuring luminescence because luciferase assays completed in this project indicate greater differences in luminescence levels after 24 hours compared to four hours. Luminescence levels of untransfected COS-7 cells treated with SP600125 would show how these cells naturally react to SP600125 and luminescence levels could be used as background
luminescence. The luciferase assay completed on COS-7 cells transfected with the BRE-luciferase gene plasmid would shed light on both the binding specificity of SP600125 to alternative receptors as well as the level of activity of the BRE-luciferase plasmid. Since these cells do not contain the MIS Type II receptor, it can be theorized that luminescence levels remain consistent with luminescence levels produced by untransfected COS-7 cells. If luminescence levels increase with increased concentrations of SP600125, it could indicate binding of the SP600125 to alternative receptors. Luciferase assays conducted on COS-7 cells transfected with only the MIS Type II receptor would serve as another negative control and could show the correlation of binding of SP600125 to MIS Type II receptor and luminescence. Since there is no BRE- Luciferase gene, luminescence levels should remain consistent with luminescence levels of untransfected COS-7 cells. These levels could be then subtracted from luminescence levels produced by SP600125 treated COS-7 cells co-transfected with the BRE-Luciferase gene and the MIS Type II receptor. It is theorized that co-transfected COS-7 cells would have both high levels of luminescence as well as be the only cells to have differences in luminescence with varying concentrations of SP600125. Provided this assay was successful, it would provide initial analytics for purified rhMIS samples.

Ovarian cancer proves to be one of the deadliest of genealogical cancers in women. The symptoms associated with ovarian cancer reflect those of menopause, making the cancer difficult to detect until it has metastasized beyond the ovaries. Once detected, the cancer has progressed throughout the body, requiring harsh chemotherapy treatments. These treatments target not only cancerous cells, but also non-cancerous cells causing patients undergoing these treatments to suffer many uncomfortable side effects. These side effects include, but are not limited to, low white blood cell count, fatigue, anemia, nausea, and abdominal pain. Late detection and the lack
of ability for chemotherapy to destroy ovarian cancer stem cells contributes to its high mortality and high reoccurrence rate. The harshness of ovarian cancer therapy the first time could affect if a patient is physically able to undergo a second round of therapy if the cancer returns. Patients will also often develop resistance to the chemotherapeutics, adding to the difficulty of treatment and permanent elimination of the cancer. Although therapies including surgical removal of the tumor and chemotherapy are available to patients with ovarian cancer, no drug has been successfully developed to treat only ovarian cancer cells. With its ability to induce apoptosis in only ovarian cancer cells, MIS shows great potential as new ovarian cancer therapeutic. When treated with a combination of surgical removal, moderate chemotherapy, and MIS, patients with ovarian cancer potentially can be cured of ovarian cancer with minimal effects of harsh chemotherapy. MIS’ apoptotic effects on ovarian cancer stem cells could suggest that patients with ovarian cancer would take MIS for a large portion of their life in order to remain cancer free. The lack of effect of MIS on cells not containing the MIS Type II receptor eliminates the painful side effects of chemotherapy and provides more localized ovarian cancer cell treatment.

Scientists have purified MIS in small quantities, but never in a large enough scale for testing in clinical trials. For this reason, investigation of high-scale purification of this protein should be a topic of ongoing research.

Purification of MIS has the potential for many broader implications of other localized gonadal cancer cell treatments. Gonadal cancers including cervical, endometrium, breast, and prostate cancers have been shown to be affected by MIS. In these cancers, MIS induces transcription of apoptotic proteins either through the BMP or NF-κβ. Large scale purification of MIS could encourage further research into development and incorporation of MIS into treatment options for patients with these cancers.
6. Table of Figures

Figure 1: Reproductive Organs Formed in the Presence of the SRY Gene.
Presence of the SRY gene dictates formation of reproductive organs the embryo will have leading to the production/lack of production of the Müllerian Inhibiting Substance (MIS) \(^{36}\).

Figure 2: The TGF-β Superfamily.
Figure 3: Human C-terminus Müllerian Inhibiting Substance Homology.
The human C-terminus MIS is compared with the C-terminus MIS of different animals commonly used in clinical trials in order of decreasing homology: monkey (100%), dog (97%), mouse (93%), pig (94%), and rat (88%). The 109 amino acid sequences are color coded to show cysteine residues (Red) and variations in the amino acid sequence (Blue Underlined). All C-terminus MIS shown have 100% homology of cysteine residues (Cys).
Figure 4: Human Müllerian Inhibiting Substance Type II Receptor Homology.
The human MIS Type II receptor is compared with the C-Terminus MIS of different animals commonly used in clinical trials in order of decreasing homology: monkey (99%), dog (89%), rat (73%), mouse (72%), and pig (48.2%). The 145 amino acid sequences are color coded to show Variations in the Amino Acid sequence (Blue Underlined).
Figure 5: Bone Morphogenetic Pathway.

1. Full Length MIS is cleaved by protease (2) allowing C-Terminus MIS to bind to the MIS Type II Receptor. (3) Once bound, a second MISRII is recruited and (4) the N-Terminus MIS dissociates. (5) The Type I receptor is then recruited by the activated MISRII as theorized by Clemente et al. 2010. (6) SMAD 1 is recruited and (7) Phosphorylated by the Type I receptor causing a conformational change in SMAD 1. (8) The Conformational change of SMAD 1 allows for SMAD 4 to bind to SMAD 1 (9) creating the SMAD 1 / 4 Complex. (10) The SMAD 1 / 4 complex translocates into the nucleus and binds with the BRE gene causing transcription tumor suppressor genes p16, p107 and p130.
Figure 6: NF-κB Canonical (Classical) and NF-κB Non- Canonical (Alternative) Pathway.  
Canonical. (1) MIS binds to a Toll- like Receptor (TLR). (2) Binding of MIS to the receptor results in the recruitment of IKK complex, comprised of IKKγ, IKKα (attached are proteins p50 and p65), and IKKβ. (3) The IKK complex then phosphorylates the IKKβ. (4) Phosphorylation of IKKβ results in its dissociation and (5) ubiquitin ligases polyubiquitates the IKKα resulting in proteosomal degradation of the IKKα- Ubiquitin complex. (6) p50-p65 heterodimer dissociates from the IKKα+Ubiquitin complex and (7) translocates into the nucleus and induces gene transcription of apoptotic proteins.  
Non- Canonical (Alternative). (1) MIS binds to a Toll- like Receptor (TLR). (2) Binding of MIS to the receptor results in the recruitment and activation of the IKK complex, which is comprised of IKKγ and 2 IKKαs (attached are proteins RelB and p100). (3) IKKα phosphorylates p100 causing (4) dissociation from the complex and polyubiquitination of the IKKα-RelB-p100-Phosphate complex. (5) Polyubiquitination leads to proteosomal degradation resulting in (6) RelB and p100 dissociation from the IKKα- Ubiquitin complex. This dissociation allows p100 to be processed by a proteasome to p52. (7) The RelB-p52 heterodimer translocates into the nucleus and induces gene transcription of apoptotic proteins.
Figure 7: Construct 7 Plasmid Map.
Plasmid containing CD33 signal sequence, a poly-HIS tag, and the rhMIS N-Terminus gene.
Figure 8: Construct 12 Plasmid Map.
Plasmid containing IL2 signal sequence, a poly-HIS tag, the N-Terminus MIS gene, and hIgGLFc Linker gene.
Figure 9: rhMIS C-terminus Plasmid Map.
Plasmid containing CD33 signaling sequence and gene for rhMIS C-terminus
Figure 10: Bone Morphogenetic Response Element (BRE) And Luc2 Gene Plasmid.
Plasmid containing Luc2 gene, SV40 promoter, and BRE gene
<table>
<thead>
<tr>
<th>CD33 Signal</th>
<th>HIS</th>
<th>TEV</th>
<th>N-Terminus</th>
</tr>
</thead>
</table>

*Figure 11: Construct 7: N-Terminus MIS and CD33 Signaling Sequence.*

<table>
<thead>
<tr>
<th>IL2 Signal</th>
<th>HIS</th>
<th>TEV</th>
<th>N-Terminus</th>
<th>HulgGI FC Plus Linker</th>
</tr>
</thead>
</table>

*Figure 12: Construct 12: N-Terminus MIS with HulgGI FC Plus Linker and IL2 Signaling Sequence.*

<table>
<thead>
<tr>
<th>CD33 Signal</th>
<th>C-Terminus</th>
</tr>
</thead>
</table>

*Figure 13: C-Terminus MIS and CD33 Signaling Sequence.*
Figure 14: FPLC and Coomassie Gel Results for Media Produced by Cos-7 Construct 7-C-terminus MIS.

**FPLC.** (1) Baseline 50mM of Imidazole ran through column at 0.5ml/min. (2) At 40 minutes, Cos-7 Construct 12 N-C terminus MIS media was added at flow rate 0.5mL/min. (3) At 260 minutes the flow rate was changed to 2mL/min. (4) At approximately 270 minutes, unattached proteins eluted. (5) At 300 minutes, 250mM imidazole was run through column and fraction size changed to 1.0mL/ min at 305 minutes. (6) Fractions 20-25 were collected.

**Gel.** Samples 20-25 were collected because proteins in these samples eluted after addition of the elution buffer and were tested using gel electrophoresis. The desired proteins are labeled on the gel with arrows. The arrows correspond with rhMIS N-Terminus homodimer (Red-90kDa), rhMIS N-terminus monomer (Orange- 49.5kDa), rhMIS N-C-terminus heterodimer (purple- 61kDa), and rhMIS C-terminus monomer (Blue- 11.5kDa)
Figure 15: FPLC and Coomassie Gel Results for Media produced by COS-7 Construct 12 N-C-terminus MIS.

FPLC. (1) Baseline 50mM of Imidazole was run through the column at 0.5ml/min. (2) At 60 minutes, Cos-7 Construct 12 N-Terminus MIS media was added at flow rate 0.5mL/min. (3) Between 215 and 225 minutes, unattached proteins eluted. (4) At 250 minutes, 250mM imidazole was run through column and fraction size changed to 1.0mL/min.

Gel. Samples 23-25 were collected because proteins in these samples eluted after addition of the elution buffer and were tested using gel electrophoresis. The desired proteins are labeled on the gel with arrows. The rhMIS N-terminus+Fc Linker monomer (Gray-76.5 kDa), rhMIS N-terminus+FC linker homodimer (yellow-152kDa) the rhMIS N-C-terminus + FC-linker heterodimer (Green-160kDa) rhMIS C-terminus monomer (Blue-11.5kDa)
**Figure 16: FPLC and Coomassie Gel Results for Media Produced by Cos-7 Construct 12 N-terminus MIS.**

**FPLC.** (1) Baseline 50mM of Imidazole was run through the column at 0.5ml/min. (2) At 45 minutes, Cos-7 Construct 12 N-terminus MIS media was added at flow rate 0.5mL/min. (3) Between 200 and 220 minutes, unattached proteins eluted. (4) At 230 minutes, 250mM imidazole was run through column and fraction size changed to 1.0mL/ min.

**Gel.** Samples 13-16 were collected because proteins in these samples eluted after addition of the elution buffer and were tested using gel electrophoresis. The desired proteins are labeled on the gel with arrows. The rhMIS N-terminus+Fc Linker monomer (Gray-76.5 kDa), rhMIS N-terminus+ FC linker homodimer (yellow-152kdA), and the rhMIS N-C-terminus + FC-linker heterodimer (Green-160kDa)
Figure 17: BlueSky Biosciences completed Western Blots with Mouse anti-His mAb and Rabbit anti-C-MIS mAb.

Westerns were completed by BlueSky Biosciences in 18% acrylamide, Tris-Glycine. Three different medias were tested: COS-7 Construct 12 N-C Terminus MIS (Lanes 2-6), COS-7 Construct 12 N Terminus MIS (Lanes 7-9), and COS-7 Construct 7 N-C Terminus MIS (Lanes 11-15). Lanes 21, 23, and 25 contained direct media samples of the 3 medias tested. The top western was probed with Mouse anti-His mAb which binds to polyHis tagged proteins. The bottom western was probed with Rabbit Anti-C MS mAb.
Figure 18: Luminescence of OVCAR-8-BRE-Luc cells in 10%FBS Treated with SP600125 at 4 and 24 Hours.
OVCAR-8 Cells and OVCAR-8 cells transfected with BRE-Luc Plasmid were treated with four different concentrations of SP600125. Luminescence was taken at 4 hours and 24 Hours. Concentrations of SP600125 were: 0.1uM, 1uM, 10uM, and 100uM.
Figure 19: Luminescence of OVCAR-8 BRE-Luc cells in 0% FBS vs 0.1% FBS vs 10% FBS. BRE- Luciferase assay was completed on OVCAR-8 BRE- Luc cells in 0% and 0.1% FBS with the following concentrations of SP600125: 0mM, 0.1mM, 1uM, and 10uM. Untreated OVCAR-8 BRE- Luc cells were read in 10% FBS as a control.
7. References


Cells through a Nuclear Factor- B-Dependent and Smad1-Dependent Mechanism. Cancer Research, 67(6), 2747-2756. doi:10.1158/0008-5472.CAN-06-2312


