The Effect of Potential Anticancer Gene Products on Breast Cancer Cell Lines

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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October 2013

Acknowledgements

I’d like to thank Dr. Teresa Rose-Hellekant, Dr. Edward Perkins, Dr. Jon Holy, and Dr. Amy Greene for their unwavering support throughout my time at the University of Minnesota Duluth.

Abstract

Cellular or viral delivery of anti-cancer gene therapeutics typically has been investigated as single agents. More recently combinatorial agents have been investigated for which some combinations have shown synergism. The present study was designed to evaluate three different potential anticancer factors, decorin, interferon beta (IFNb), and tumor necrosis factor (TNF) related apoptotic inducing ligand (TRAIL). The overall hypothesis was that combinatorial treatment of two of anti-cancer factors would be more efficacious and exhibit less toxicity than single treatments in breast cancer cell lines.

In order to be able to test this hypothesis, the construction of these anticancer genes was required as well as engineering and expression of transgenes in a cellular production system. Therefore, my thesis project required the isolation of anti-cancer genes from either human genomic or cDNA libraries, verification of sequence fidelity, construction of transgene expression cassettes, and transient transfection of the transgenes in Chinese hamster ovarian (CHO) cells. Secreted cellular protein production was verified in culture media and the anti-cancer effects of conditioned media containing single proteins on growth of human and mouse breast cancer cell lines was measured and compared to commercially available agents.

Mesenchymal stem cells (MSCs) have been shown to home to tumors and are under investigation as vehicles for delivering anticancer gene therapeutics. MSC-based anti-cancer gene therapeutics are typically investigated as single agents. The present study was designed to assess the anti-cancer properties of the selected gene products as a prelude to their production from MSCs. This study specifically evaluated decorin, interferon beta (IFNb), and tumor necrosis factor (TNF) related apoptotic inducing ligand

(TRAIL) as growth inhibitors *in vitro* as previous studies have demonstrated their efficacy as growth inhibitors in multiple cancer cell lines including breast cancer in *in vitro* and *in vivo* studies. My experimental results indicated that commercially available human and mouse TRAIL inhibited MDA-MB-231, MCF-7, and WM793 human cancer cell lines. TRAIL produced by CHO cells were able to decrease WM793 cancer cell growth, but not illicit a response in MDA-MB-231 or MCF-7 breast cancer cell lines. Further studies are needed to discover the relationship between the combination of these gene products.

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Chapter 1: Introduction

1.1 - Cancer Background

Cancer is a group of diseases that encompass a diverse set of phenotypic and genotypic mutations to specific cell types. This group of diseases is characterized by an uncontrolled growth and spread of abnormal cells (American Cancer Society 2013). Cancer can be caused by extrinsic and intrinsic factors such as environmental or inherited conditions. These factors may interact together to initiate and proliferate cancer events. Generally speaking, cancer can be treated by various combinations of surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapy. In 2013, it is estimated that 1,660,290 new cancers will be diagnosed and 580,350 people will die from cancer (American Cancer Society 2013). In 2008, the cost of cancer was 201.5 billion USD (77.4 billion USD in direct healthcare costs) (American Cancer Society 2013).

Specific to breast cancer, 230,480 new invasive and 57,650 *in situ* cases occurred in 2011 (American Cancer Society 2012). The median age for developing breast cancer is 61 years old and it is estimated that one in eight women will develop breast cancer by the age of 70. Being a female, being above 65 years of age, possessing BRCA1 and/or BRCA2 mutations, having more than two 1st degree relatives diagnosed with breast cancer at an early age, having a personal history of breast cancer, having high breast tissue density, or having a confirmed biopsy showing atypical hyperplasia increase the relative risk of developing breast cancer by more than 4 fold increase. Having one first degree relative with breast cancer, a prior history of high dose radiation to the chest, or a high bone density post menopause increases the relative risk of developing breast cancer

by 2.1-4 fold increase. Late age at first full term pregnancy, early menarche, late menopause, no full term pregnancies, not having breastfed, recent oral contraceptive use, recent/long term use of estrogen and progestin, or postmenopausal obesity increases the relative risk of developing breast cancer by 1.1- 2.0 fold increase. Personal history of endometrial or ovarian cancer, elevated alcohol consumption, increased height, higher socioeconomic status, or Ashkenazi Jewish lineage are also factors that increase the risk of developing breast cancer (American Cancer Society 2012). Besides grades and stage of breast cancers, breast cancer has been further classified into different types depending on the expression of specific mitogenic proteins including estrogen receptor (ER), progesterone receptor (PR), and HER2 (ErbB2 in mouse), as well as morphology. Cancers expressing ER and/or PR yields a more favorable prognosis than ER-/PR- breast cancers due largely to the likelihood of their response to selective estrogen receptor modulators that block estrogen effects. A subset of breast cancers that express HER2 respond to the specific anti-cancer agents such as trastuzumab. Other classifications are triple negative, which is ER-/PR-/HER2- cancers that are associated with poor clinical outcomes. Efficacy of established therapeutics is dependent on the grade, stage, and ER/PR/HER2 status of breast cancers, therefore the design and testing of novel therapeutics must be evaluated in models that represent these varied types.

1.2 - TNF-Related Apoptosis-Inducing Ligand

TNF-related apoptosis-inducing ligand (TRAIL), mediated through a FAS independent pathway, induces apoptosis in many transformed cell lines (Wiley *et al.* 1995 and Pitti *et al.* 1996). TRAIL is characterized as a type II membrane protein with

homology to the TNF ligand family that interacts with specific receptors for TRAIL (Wiley *et al.* 1995 and Pitti *et al.* 1996). TRAIL receptor and TRAIL form a hexameric complex resulting from a stable homotrimer TRAIL ligand with 3 receptor units (Hymowitz *et al.* 1999). TRAIL ligand, at amino acid residues 119 through 281 and TRAIL receptor at amino acid residues 21 though 130 were found to be the point of interaction between the two proteins (Hymowitz *et al.* 1999). The first receptor identified for TRAIL was the DR4 or TRAIL-R1. The resultant apoptosis from the interaction of the TRAIL-R1 receptor and ligand pair were abrogated with the use of caspase inhibitors, indicating that caspases are involved in mediating the TRAIL/TRAIL receptor mediated apoptosis (Pan *et al.* 1997a). The second receptor mediating a TRAIL induced apoptotic response was found after the discovery of the first receptor and named DR5 (Pan *et al.* 1997b) or TRAIL-R2 (Sheridan *et al.* 1997). TRAIL-R2 showed a 66% extracellular region amino acid identity with TRAIL-R1 (Pan *et al.* 1997b). An over-expression of TRAIL-R2 induced cell death by apoptosis through the activation of FADD and caspases (Walczak *et al.* 1997). A decoy receptor TRAIL-R3, originally identified as DcR1 (Sheridan *et al.* 1997) or TRID (Pan *et al.* 1997b), showed a 69% and 52% amino acid homology with the extracellular regions of TRAIL-R1 and TRAIL-R2 respectively. An additional decoy receptor, TRAIL-R4 was identified by Degli-Esposti *et al.* (1997). The extra cellular domain identity of TRAIL-R4 was 58%, 57%, and 70% to TRAIL-R1, TRAIL-R2, and TRAIL-R3 respectively (Degli-Esposti *et al.* 1997). Various experiments attempting to identify the relationship between the receptors and their susceptibility to TRAIL have been conducted using various means.

TRAIL-R3 and TRAIL-R4 were discovered to block the apoptotic effect of TRAIL. Cells that originally displayed sensitivity to TRAIL mediated apoptosis conferred resistance when transfected with TRAIL-R3 (Sheridan *et al.* 1997) or TRAIL-R4 (Degli-Esposti *et al.* 1997). It was reported that TRAIL-R3 was found in normal tissues but not present in many tumors (Sheridan *et al.* 1997). TRAIL-R4 was found to have high tissue expression (Degli-Esposti *et al.* 1997). For tumors expressing TRAIL-R3, the transcript level of the R3 receptor did not correlate to the sensitivity of TRAIL administration (Keane *et al.* 1999). TRAIL-R4 differs from TRAIL-R3 in that it can activate the NFκb pathway but cannot induce apoptosis (Degli-Esposti *et al.* 1997). The fifth TRAIL receptor, Osteoprotegerin, was found to be a soluble antagonist receptor for TRAIL, potentially blocking the effects of TRAIL (Emery *et al.* 1998). Little is known about the relationship of the fifth receptor and TRAIL and it might be of practical and clinical significance to further clarify its role in TRAIL signaling. Although TRAIL-R1 and TRAIL-R2 have been identified as critical components in the mediation of the apoptotic signal from TRAIL, studies investigating knockouts and knockdowns of either TRAIL-R1 or TRAIL-R2 indicate that TRAIL-R2 is more important in mediating the apoptotic signal from the administration of TRAIL (Goda *et al.* 2008 and Rahman *et al.* 2009). A TRAIL-R2 knockdown had the same effect as a TRAIL-R1 and TRAIL-R2 knockdown, whereas a TRAIL-R1 knockdown had no effect on reducing apoptosis after the administration of TRAIL to TRAIL sensitive cells (Goda *et al.* 2008). Rahman *et al.* (2009) used MDA-MB-231 breast cancer cells (triple negative breast cancer subtype) to create knockouts for TRAIL-R1 and TRAIL-R2. It was found that the TRAIL-R1 knockout in these cells had no effect on inhibiting apoptosis after a TRAIL

administration *in vitro*. However this study found that TRAIL-R2 knockout in MDA-MB-231s inhibited apoptosis mediated by TRAIL.

Several initial studies of TRAIL receptors measured transcript alone instead of cellular surface expression. It was later found that mRNA of TRAIL-R1 and TRAIL-R2 does not correlate to TRAIL sensitivity of cell lines (Zhang *et al.* 2008). It was shown that TRAIL-R1 linked to a green fluorescent protein reporter (GFP) was located in the cytoplasm for TRAIL resistant cells and receptors located on the cellular surface for TRAIL sensitive cells (Zhang *et al.* 2008). Endocytosis of TRAIL receptors may contribute to TRAIL resistance as shown by sensitization of resistant cell lines to TRAIL after the administration of endocytosis inhibitors (Zhang et *al.* 2008). The administration of endocytosis inhibitors resulted in MDA-MB-231 cells that were sensitive to TRAIL, a greater sensitivity to TRAIL, and greater apoptosis *in vitro* (Zhang *et al.* 2009). Ganten *et al.* (2009) analyzed 311 tumors from breast cancer patients and found that the presence of TRAIL-R2 and TRAIL-R4 correlated with bad clinical prognostic markers for overall survival while the presence of TRAIL-R1 correlated with good prognostic markers. Immunohistochemistry was used in the 311 breast cancer samples and TRAIL-R1 was found to be located in both the cytoplasm and membrane regions while TRAIL-R2 was seen mostly in the cytoplasm (Ganten *et al.* 2009). TRAIL-R3 was not found to be a predictor of clinical outcome (Ganten *et al.* 2009). TRAIL-R3 may however play in important role with the molecular involvement of TRAIL signaling. This data highlights an area of clinical importance where the use of TRAIL may be beneficial.

MDA-MB-231 cells, which are highly sensitive to TRAIL, can become resistant to TRAIL after a prolonged exposure to sub-toxic doses of TRAIL resulting in

irreversible resistance to TRAIL (Yoshida *et al.* 2009). During the sub-toxic administration of TRAIL, transcripts for TRAIL-R1, -R2, -R3, and -R4 all remained the same as compared to untreated cells however the surface expression of TRAIL-R1 and TRAIL-R2 decreased significantly (Yoshida *et al.* 2009).

While TNF and FasL have shown to be excellent at causing apoptosis in cancer cells, they have however shown to elicit severe toxicity to normal hepatocytes and other cells (Ashkenazi *et al.* 1999). TRAIL, did not show initial toxicity to normal cells, but had an apoptotic effect on 32 of 39 cell lines in colon, lung, breast, kidney, brain, and skin cancer (Ashkenazi *et al.* 1999). TRAIL did not cause toxicity in primates and the addition of TRAIL caused apoptosis in solid tumors, suppressed tumor progression, and improved survival (Ashkenazi *et al.* 1999). These results indicate that TRAIL has the potential to be an effective method for the treatment of various cancers in diverse tissues. Further studies involving TRAIL highlighted potential issues with TRAIL causing hepatocyte cell death (Ozoren *et al.* 2000). However, Walczak *et al.* (1999) had found that TRAIL had not shown any signs of toxicity to liver, brain, and other tissue. Lawrence *et al.* (2001) were able to show that the hepatic apoptosis was caused by His-tagged TRAIL (polyhistidine epitope tagged TRAIL). His-tagged TRAIL was found to be unstable in solution and had formed insoluble aggregates at 37°C. Not only was His-tagged TRAIL less potent than inducing apoptosis in tumor cell lines than normal TRAIL, but apoptosis seen in His-tagged TRAIL with human hepatocytes was not seen from normal TRAIL at a concentration 1000X that of His-tagged TRAIL (Lawrence *et al.* 2001). TRAIL was administered to nonhuman primates and had all tissues analyzed for

apoptotic or pathologic effect (Lawrence *et al.* 2001). These results indicate that TRAIL is safe on human hepatocytes and nonhuman primate models as well.

Besides active TRAIL-R1 and TRAIL-R2 transcriptional activity and functioning proteins expressed on the cellular surface, signaling proteins that mediate the propagation of both extrinsic and intrinsic apoptotic pathways are needed. FADD (Fas-associated death domain) and caspase 8 are recruited to the receptor and induce apoptosis through caspase activation (Suliman *et al.* 2001). The pathway was verified by testing knockouts of either FADD or caspase 8. Cells deficient in FADD or caspase 8 blocked TRAIL mediated apoptosis (Suliman *et al.* 2001). Caspase 8 was found to activate caspase 3 indicating the major contributors to TRAIL mediated apoptosis (Suliman *et al.* 2001).

FLIP (Flice Inhibitory Protein) inhibits FADD, therefore inhibits apoptotic signaling (Ricci *et al.* 2004). C-myc binds to the FLIP gene promoter resulting in a repressed FLIP transcription and protein expression (Ricci *et al.* 2004). C-myc levels in tumor lines formed a linear relationship between responsiveness to TRAIL mediated apoptosis. The more c-myc that is present results in lower amounts of FLIP. A lower amount of FLIP results in a lower inhibition to FADD. Through an engineering knock down of sensitive cell lines and a molecular knock in to resistant cell lines, TRAIL mediated apoptosis could be controlled by amounts of c-myc present (Ricci *et al.* 2004). The higher c-myc levels resulted in the greater sensitivity to TRAIL (Ricci et al. 2004).

The initial experiments related to TRAIL mediated apoptosis indicate the importance of and dependence to several cellular characteristics and the protein expression of certain genes. Alone, TRAIL represents a strong candidate for the treatment of breast cancer. The aforementioned studies indicate TRAIL as a potentially efficacious

and safe treatment for breast cancer. Other studies have attempted to use the apoptotic properties of TRAIL as a combinatorial or adjuvant therapy along with other established anticancer treatments. TRAIL has increased apoptosis of various cancers with the addition of trastuzumab (Cuello *et al.* 2001), tamoxifen (Ricci *et al.* 2004 and Lagadec *et al.* 2008), ionizing radiation (Shankar *et al.* 2004), interferons (Chawla-Sarkar *et al.* 2002 and Kumar-Sinha *et al.* 2002), and a wide variety of chemotherapies that influenced mediator caspases of TRAIL induced apoptosis (Thai *et al.* 2006). Several chemotherapeutics have shown a synergistic interaction with TRAIL causing apoptosis in various cancer lines *in vitro* and *in vivo*. Keane *et al.* (1999) showed this synergistic relationship was related only to chemotherapeutics that activated similar caspases that are involved in TRAIL mediated apoptosis. Not only has chemotherapy shown to increase caspase activity, but it has also been shown to upregulate TRAIL-R1 and TRAIL-R2 after 24 and 48 hours *in vivo* (Singh *et al.* 2003). A chemotherapeutic pretreatment 24 hours prior to the administration of systemically delivered TRAIL in a mouse model of breast cancer illustrated a significant survival increase of the animals over TRAIL or chemotherapy alone without the observation of liver or brain toxicity (Singh *et al.* 2003). This data supports the use of TRAIL used in combination with treatments efficacious for certain types of cancers resulting in potential synergistic apoptosis effects of malignant cells.

Tamoxifen has shown to be efficacious in the treatment of ER positive breast cancer in humans which occurs in 60 to 70% of primary breast cancer incidences (Osborne 1998). A mouse model using MDA-MB-231 or MCF7 human breast cancer cells were shown to exhibit synergistic apoptotic effects from the use of TRAIL and

tamoxifen (Lagadec *et al.* 2008). While tamoxifen alone did not influence apoptosis in ER negative breast cancer cells, with the combination of hTRAIL, synergistic apoptosis was observed. The combination of tamoxifen and TRAIL increased caspase 3 synergistically (Ricci *et al.* 2004). These results indicate that tamoxifen, with TRAIL are able to induce synergistic apoptosis in breast cancer cells in vivo regardless of ER status.

TRAIL along with trastuzumab caused down regulation of ErbB2 and enhanced apoptosis more than either therapeutic alone in breast cancer cells expressing high levels of ErbB2 (Cuello *et al.* 2001). Ionizing radiation has shown to increase TRAIL-R2, caspase 8, and sensitivity to hTRAIL (Shankar *et al.* 2004). The in vivo study involving ionizing radiation and TRAIL resulted in complete tumor regression and 100% survival, an outcome not seen from TRAIL or ionizing radiation alone (Shankar *et al.* 2004).

Clinically, a method used to deliver TRAIL locally as opposed to systemically would be a preferable method of delivery. MSCs have been used to deliver TRAIL to gliomas and MDA-MB-231 tumors. Yang *et al.* (2009) found that full length TRAIL expressed in MSCs resulted in soluble TRAIL secreted while naive MSC cells did not have any secreted TRAIL. MSCs home to some tumor cells, but not to other cancer cells (Loebinger *et al.* 2009). MSCs expressing TRAIL significantly reduced metastatic tumor burden, significantly killed lung, breast, squamous cell, and cervical cancer lines (Loebinger *et al.* 2009). These results make the delivery of TRAIL or other therapeutics by MSCs as an optimal method for delivery.

Murine TRAIL (mTRAIL) appears to function similarly to human TRAIL (hTRAIL), likely due to sequence homologies. The open reading frame of human TRAIL consists of 846 base pairs while mouse TRAIL consists of 876 base pairs. Human and

mouse TRAIL share 65% amino acid identity and have shown similar effects to TRAIL sensitive cells, regardless of whether or not the cells were of human or mouse origins (Wiley *et al.* 1995 and Pitti *et al.* 1996). mTRAIL was found to induce apoptosis, promote cell death, and increase the doubling time of 4T1 murine mammary carcinoma cells *in vitro* and *in vivo* (Ganai *et al.* 2009). From the observations seen in other cell lines, both human and mouse TRAIL should affect TRAIL sensitive cells. MCF7 cells have caspase 8 and FADD and were found to be susceptible to TRAIL administration (Suliman *et al.* 2001). MDA-MB-231 (Yoshida *et al.* 2009) and WM793 melanoma cells are highly susceptible to TRAIL administration. These features suggest that testing of human or mouse TRAIL in cancer cells of human or mouse origin will reveal cell specific sensitivities and mechanisms of action.

1.3 - Decorin

Decorin is a small secreted chondroitin/dermatan sulfate proteoglycan from the family of small leucine rich proteoglycans (SLRP). Decorin has been localized to the 12q21 region of the human genome and mRNA has been found highest in connective tissue such as aorta, lung, skin, kidney, smooth muscle, placenta, skeletal muscle, spleen, and adrenal gland (Pulkkinen *et al.* 1992). Decorin is produced and excreted into the media when full length decorin cDNA is transfected into CHO cell line (Yamaguchi and Ruoslahti 1988) or the WiDr human colon carcinoma cell line (Santra *et al.* 1995). The produced decorin caused a reduction in CHO density and slowed the growth of CHO cells in culture (Yamaguchi and Ruoslahti 1988), indicating that decorin may have an impact on cell proliferation. Yamaguchi *et al.* (1990) were able to show that decorin,

isolated from bovine skin as well as recombinant decorin produced by CHO cells, were able to inhibit TGF-β1 dependent cell growth through decorin inhibition of TGF-β1 binding. The decorin experiments conducted by Yamaguchi *et al.* (1988 and 1990) provided initial data indicating decorin's potential impact on inhibiting cellular growth.

Carcinoma cell lines not affected by TGFβ growth were found to also be suppressed by decorin, indicating that decorin interacts with other mechanisms involving suppression and reduced growth rate *in vitro* other than the TGFβ pathway (Santra *et al.* 1995). The observed decline and suppression of growth rate correlated to a shift of cell population into G1 phase of the cell cycle as compared to control groups not receiving decorin (Santra *et al.* 1995). After observing the antiproliferative effects of decorin on the human colon carcinoma cell line, Santra *et al.* (1995) hypothesized that decorin deletions may increase the incidence of tumor formation. Several neoplastic cell lines from various tissues exhibited suppressed growth from decorin expressed as a proteoglycan or protein core (Santra *et al.* 1997). The cell lines that showed suppressed cell growth had an increase of cells in the G1 phase and experienced an up regulation of p21 mRNA and protein (Santra *et al.* 1997). Several additional findings indicate that neoplastic cells that contain a disrupted p21 gene failed to respond to decorin. Decorin acts from outside of the cell in a paracrine manner, and that decorin is absent in transformed cell lines (Santra *et al.* 1997). Decorin was also found to bind to the EGFR in the soluble ectodomain region (Iozzo *et al.* 1999). Full length and core decorin protein activates MAPK, increases p21, and prolongs cells in G1 (Iozzo *et al.* 1999). The direct effect of prolonging the cells in the G1 phase indicates that decorin has the ability exert an antiprolifertive effect on cancer cells.

The majority of the antiproliferative effects of decorin was identified at 96 hours and later in *in vitro* experiments using MDA-MB-231 cells, indicating that decorin may require a longer period of incubation with the cancer cells of interest than TRAIL or IFNb to mediate a measureable effect (Araki *et al.* 2009). Decorin expressing MDA-MB-231 cells exhibited growth suppression, reduced motility, and lowered metastasis to bone as compared to untreated MDA-MB-231 cells *in vivo* (Araki *et al.* 2009).

Several breast cancers exhibit higher expression levels of ErbB2/HER2 and HER2 is thought to play a significant role in negatively influencing outcome and therefore breast cancer prognosis. Decorin inhibits ErbB2 phosphorylation and overexpression in breast carcinomas cells *in vitro* (Santra *et al.* 2000). Decorin has been found to reduce ErbB2/HER2 through the ErbB4 receptor interactions that resulted in an inhibition of primary growth and metastasis in breast cancer cells during systemic administration of decorin (Goldoni *et al.* 2008).

Decorin was found to act as a paracrine inhibitor of cell growth; decorin suppressed EGFR Kinase activity in co-culture experiments involving decorin expressing cells (Csordas *et al.* 2000). Systemic administration of decorin was also shown to down regulate EGFR and thereby tumor growth suppression (Hu *et al.* 2009).

Decorin has also been shown to trigger apoptosis (Hu *et al.* 2009 and Goldoni *et al.* 2009). This apoptotic activity was mediated through capsase 3 and 7 (Goldoni *et al.* 2009). Decorin, is also a novel inhibitor of the Met Receptor; decorin, dose dependently blocked the MET receptor as measured by physical and functional down regulation of MET mediated activities (Goldoni *et al.* 2009). Decorin antagonizing effects on TGFβ1

and receptors for MET, EGF, ErbB2 indicate the strong potential for decorin to be used for the treatment of breast cancer.

1.4 - Interferon Beta

Interferon Beta (IFNb), as a member of the interferon gene family, has been shown to interfere with viral replication, affect cell motility, inhibit cell proliferation, and modulate the immune system (Lengyel 1982). Initial studies had demonstrated interferon to be safe to use in human therapy and illustrated the potential to increase estrogen and progesterone receptor content in isolated tumor tissue from advanced breast cancer metastases (Pouillart *et al.* 1982). Fierlbeck *et al.* (1996) measured CHO cell derived IFNb levels that had been administered to melanoma patients and found that the IFNb level had returned to baseline by 48 hours after subcutaneous administration. This finding illustrates the relatively short half-life of IFNb *in vivo* and the need for a continual delivery method to achieve the antiproliferative effects of IFNb on malignant cells.

Mesenchymal stem cells (MSCs) have been used as vectors in the delivery of genes to malignant cells in vivo. Studeny *et al.* (2002) exhibited the ability of MSCs expressing human recombinant interferon beta (hrIFNb) to home to sites of tumor formation. The MSCs expressing hrIFNb inhibited growth of human malignant cells *in vitro* and *in vivo* (Studeny *et al.* 2002). MSCs expressing hrIFNb has been shown to inhibit growth of MDA-MB-231 tumors formed in lung tissue more than systemically administered hrIFNb or naive MSCs had inhibited tumors alone (Studeny *et al.* 2004). IFNb derived from purified MSCs cells engineered with hrIFNb inhibited proliferation of MDA-MB-231 cells *in vitro* (Studeny *et al.* 2004).

IFNb enhances the cytotoxicity caused by chemotherapeutics *in vitro* (Brickelmaier *et al.* 2002). Several human breast cancer cell lines along with other cancer cell lines displayed a variation in susceptibility with different concentrations of hIFNb indicating that not all breast cancers will be equally susceptible to IFNb (Brickelmaier *et al.* 2002). The effects seen in the cancer lines from hrIFNb treatment were increased with the use of chemotherapeutics (Brickelmaier *et al.* 2002). MCF-7 human breast cancer cells displayed an EC50 of 900 IU/uL of hrIFNb while the EC50 of hrIFNb was 100 IU/uL in MDA-MB-468 cells (Brickelmaier *et al.* 2002). With MCF-7 cells showing some resistance to hrIFNb administration, it required 1000IU/ml to induce significant apoptosis (Kaynor *et al.* 2002). It has been shown that mIFNb does not exert the same effect as hIFNb on tumors created from human MDA-MB-231 cells (Qin *et al.* 2001). Mice treated with hIFNb showed a higher antiproliferative effect on the MDA-MB-231 cells along with a higher survival rate, than mice treated with mIFNb (Qin *et al.* 2001). These studies indicate hrIFNb exhibits an antiproliferative effect on MDA-MB-231 and other breast cancer cell lines making it a good anticancer agent candidate that may prove particularly valuable in combination with other gene products. Furthermore these studies suggest species specificity of recombinant IFNb.

Chapter 2: Material and Methods

2.1 - Engineering Methods

The initial stage of the project involved identifying genes of interest, designing primers for specific genes, amplifying genes from DNA libraries, ligation of the genes into plasmid, amplification of plasmids, sequence verification of genes, ligation of genes into mammalian expression vectors, and finally producing gene products to be used in further experiments.

2.1.1 - Introduction

TRAIL, IFNb, and decorin were identified as having potential mediating effects on various cancer cells. The primers (Table 1) were designed to have sequence homology within the human genome towards the specific genes of interest, a Kozak sequence, a *BamH*I cut site, similar melting temperatures, and start or stop codons in order to express the respective open reading frames. The melting temperatures were calculated by OligoAnalyzer (Integrated DNA Technologies, Coralville, Iowa, USA).

Table 1- Primer sequences for anticancer factors

	5'	3'
hrIFNbFOR	GGA TCC ACC ATG ACC AAC AAG TGT CTC CTC C	
hrIFNbREV	GGA TCC TCA GTT TCG GAG GTA ACC TGT AAG	
hrTRAILFOR	GGA TCC ACC ATG GCT ATG GCT ATG ATG GAG GTC CAG	
hrTRAILREV	GGA TCC TTA GCC AAC TAA AAA GGC CCC	
hrDECORINFOR	GGA TCC ACC ATG AAG GCC ACT ATC ATC CTC C	
hrDECORINREV	GGA TCC TTA CTT ATA GTT TCC GAG TTG AAT GGC A	

2.1.2 - PCR protocol for hrDecorin

Human recombinant decorin (hrDecorin) was amplified using human ovarian or brain cDNA library obtained from Dr. Edward Perkins. The PCR tube contained 1 uL Pfx 50 PCR buffer, 0.33 uL 10mM dNTPs, 0.33 uL Ovarian cDNA, and 0.2 uL of the Pfx 50 polymerase (Invitrogen, Carlsbad, California, USA) and 0.25 uL of both the forward and reverse primers for the hrDecorin gene were added to water in 10 uL final volume. The PCR program consisted of a denaturing step of 94°C for 2 minutes followed by 40 cycles of 94°C for 15 seconds, 53°C for 30 seconds, 68°C for 1 min, a final 68°C for 5 minutes, and a holding temperature of 4 °C. The PCR products were separated by loading product on a 0.7% agarose gel with 2uL 10x Orange G and 2uL 10x Sybr Green fluorescent dyes. The PCR product sizes were compared to a 1kb ladder (New England Biolabs, Inc., Ipswich, Massachusetts, USA) to estimate fragment size. The isolated band weighed 0.080gm and the QIAEX II Agarose Gel Extraction protocol was followed as described in the section below. The isolated hrDecorin PCR product was measured using a Nanodrop spectrometer (Thermo Scientific, Wilmington, Delaware, USA). The concentration of 3.74 ng/uL, a 260/280 ratio of hrDecorin was 1.49, and a 260/230 ratio of 0.78.

2.1.3 - PCR protocol for hrIFNb

Human recombinant IFNb was amplified using a NT2D1 genomic DNA sample obtained from Dr. Edward Perkins. The PCR tube contained 1 uL pfx 50 PCR buffer, 0.3uL 10mM dNTPs, 0.5 uL NT2D1 DNA, and 0.25 uL of both the forward and reverse primers for hrIFNb were added to water to a final volume of 10uL. The mixture was run

using the program as described in section above and separated using electrophoresis as described for hrDecorin. The isolated band weighed 0.160gm and the QIAEX II Agarose Gel Extraction protocol was used to isolate hrIFNb. The concentration of isolated hrIFNb PCR product was determined to be 12.86 ng/uL, and the product had a 260/280 ratio of 1.32, and a 260/230 ratio of 1.32.

2.1.4 - PCR protocol for hrTRAIL

Human recombinant TRAIL was amplified from a human testicular cDNA library obtained from by Dr. Edward Perkins. The reaction tube contained 1.6 μL dNTPs, 0.5 μL testes cDNA, 0.1 μL LATaq (Takara, Shiga, Japan), 0.3 μL forward and reverse primers for hTRAIL, 1 μL LA Taq buffer, 1 μL betaine, and 5.2 μL dH_20 for a total of 10 μL. The solution was run on a different PCR program that consisted of a denaturing step of 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 54°C for 60 seconds, 68°C for 1 min, a final 68°C for 5 minutes, and a holding temperature of 4 °C. The products were run on a 1.5% agarose gel with 1 μL of 10x Sybr Green and 1 μL 10x Orange G dye added to each tube. The resultant bands were compared to 1kb and a low molecular weight ladder (New England Biolabs, Inc., Ipswich, Massachusetts, USA). Also run on the gel was 10 μL of the identical PCR reaction, however the contents of the tube were digested using 1 μL K buffer and 0.3 μL *ScaI* (Promega, Madison, Wisconsin, USA) restriction enzyme prior to electrophoresis. The digested products conformed to the expected band fragment sizes which indicated probable successful amplification. The isolated and undigested band of interest weighed 0.093gm and the QIAEX II Agarose Gel

Extraction protocol was followed. The purified hTRAIL PCR product was measured to be 12.3ng/ μL with a 260/280 ratio of 1.1.

2.1.5 - QIAEX II Agarose Gel Extraction

The QIAEXII agarose gel extraction (Qiagen, Valencia, California, USA) protocol was used to isolate DNA from agarose gels. The procedure began by using a scalpel to remove bands dyed with Sybr Safe that had undergone gel electrophoresis to separate DNA fragments of interest. The isolated bands were further processed by removing excess agarose away from the fluorescent portion of the bands. The isolated gel fragments were weighed to calculate the correct amount of QX1 buffer to add to the gel fragment. A ratio of 3 μL of QX1 to 1mg of agarose gel was added to a 1.5 mL microcentrifuge tube. Before adding 10uL of QIAEXII to the QX1 gel fragment solution, the QIAEXII was resuspended by votexing for 30 seconds. The QIAEX II, QX1, and gel fragment were incubated in a 50-60°C sand bath for 6 minutes while inverting and votexing the solution every 2 minutes. The solution was centrifuged for 30 seconds at 13,000RPM and the supernatant was removed. The remaining pellet was washed and resuspended in 500 μL of buffer QX1 and recentrifuged at 13,000RPM for 30 seconds. The subsequent pellet was then washed twice with 500 μL PE buffer. With each wash, the pellet was resuspended and the solution was centrifuged at 13,000RPM for 30 seconds. Following the second wash with PE, the pellet was air dried for 10 to 15 minutes or until the pellet became white. The pellet was resuspended in a two staged approach to yield maximum DNA collection. The first step, 20 μL of autoclaved 10mM Tris-Cl, pH 8.5 was used to resuspend the dried pellet, the solution was votexed and centrifuged for

30 seconds at 13,000RPM and the supernatant was transferred to a 0.5mL microcentrifuge tube. The next step repeated the prior DNA extraction step replacing 20 μL 10mM Tris-Cl with 10 μL 10 mM Tris-Cl. The two supernatants were added together to yield 30 μL of the isolated DNA solution. Subsequent Nanodrop readings were conducted to determine concentration, amount, and possible contaminants with the DNA extracted from agarose gel.

2.1.6 – *E. coli* Transformation Protocol

Competent cells were stored in -70°C freezer and used when needed. The plasmid DNA was placed in 50uL of 1x KCM (100mM KCl, 30mM $CaCl_2$) in a 14mL round bottom tube on ice. The 50uL of competent cells were thawed on ice and mixed gently with the DNA and KCM mixture. This solution was kept on ice for 10 to 20 minutes. Following the ice incubation, the cell mixture was heat shocked at room temperature for 10 minutes. After being heat shocked, the cells were brought up in 400 μL SOC medium. Cultures were then incubated at 37°C for 1 hour in a shaker at 225 RPM. A portion of the culture was plated using a spreader bar on LB or YT agar along with various selection markers. LB Kanamycin (Kan) plates were used to grow up transfected *Escherichia coli* (*E. coli)* with pZErO 2.1 (Invitrogen, Carlsbad, California, USA) and LB or YT Ampicillin (Amp) plates were used to grow up transfected *E. coli* with pcDNA 3.1 (Invitrogen, Carlsbad, California, USA) and pGEM-T Easy vectors (Promega, Madison, Wisconsin, USA). Plates were incubated at 37°C overnight and colonies were isolated and expanded the following day. Plates with probable colonies containing the vector with the insert of interest were sealed with parafilm and placed in 4°C for storage.

2.1.7 - hrIFNb and hrDecorin Ligation and Transformation

hrDecorin and hrIFNb were ligated into pZero2.1 using T4 DNA Ligase (New England Biolabs, Inc, Ipswich, Massachusetts, USA). pZErO 2.1, prior to its use in the ligation procedure, and cDNA was cut using the restriction enzyme blunt cutter *EcoRV*. Because this was a blunt end ligation, a higher insert to vector ratio was required. Each ligation reaction contained 5ng of cut pZErO 2.1 and 14.36 ng and 19.074 ng DNA (an insert:vector ratio of 8.5:1for hrIFNb and 10.4:1 for hrDecorin). 5.1 uL of hrDecorin or 1.5 uL of hrIFNb, 1uL of digested pZero2.1, 2uL 10x Ligase buffer, 1/3 uL T4 DNA Ligase, and an amount of dH_20 to bring the final volumes to a total of 20uL (11.56 uL for the hrDecorin tube and 15.16 uL for hrIFNb) were placed into a thermal cycler tube, ligated at room temperature for 2 hours, and subjected to 65 degrees for 10 minutes to heat kill T4 DNA Ligase. 5uL of each ligation mix was transformed into GC5 competent cells (Sigma Aldrich, Inc., St. Louis, Missouri, USA) using the transformation protocol listed above.

2.1.8 - hrTRAIL Ligation and transformation

The ligation of hrTRAIL into a vector was done using the PGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA). 1.5 µL purified hTRAIL PCR product (12.3ng/ µL), 2.5 µL 2x rapid ligation buffer, 0.5 µL PGEM-T Easy Vector, and 0.5 µL T4 DNA Ligase were incubated at 4°C overnight and transformed into DH10B cells. Five and 10 µL of transformation mix along with 50 µL SOC were spread onto X-gal/ampicillin LB agarose plates. From the two plates, a total of 4 colonies were selected as potential clones containing hrTRAIL.

2.1.9 - Plasmid DNA Isolation

A 5 mL culture was grown from selected colonies from either a LB Kan agarose plate and placed into a LB Kan broth for pZErO 2.1 plasmid constructs or a LB Amp plate and placed into a LB Amp broth for pGEM-T Easy and pcDNA3.1/Hygro+ plasmid containing cells. These 5 mL cultures were grown overnight at 37°C. 1.5 mL of volume was spun down, the liquid was aspirated and an additional 1.5 mL of culture was spun down atop the existing pellet. The second volume was also aspirated leaving a double pellet of bacteria that potentially had incorporated the transformed plasmid of interest. The pellet was resuspended in 100 uL of lysis buffer. The lysis buffer consisted of 10mM Tris-HCl with a pH of 8.0, 1mM EDTA, 15% wt/vol sucrose, 2mg/mL lysozyme, 0.2 mg/mL pancreatic RNase, and 0.1 mg/mL bovine serum albumin. The resuspended pellet was placed in a room temperature microcentrifuge shaker for 5 minutes. Following the shaking, the tubes were placed into a rack with a cover and submerged in boiling water for 1 minute, the rack was quickly transferred to an ice water bath for 1 minute. The tubes were spun at 13,000xg for 20 minutes in room temperature. Approximately 90 uL of supernatant was transferred to a clean microcentrifuge tube to await restriction enzyme digest testing to determine whether or not the correct PCR product had been inserted into the plasmid.

2.1.10 - Plasmid Verification of hrIFNb

Five uL of each of the 6 hrIFNb easy preps (all from the NT2D1 template library), isolated from cultures grown from random white colonies grown on LB plates containing Kan and X-gal, were digested with *Pst*I (New England Biolabs, Inc., Ipswich,

Massachusetts, USA). The reactions were incubated for 2 hours at 37°C. One uL of 10X Orange G was added to each 10uL digestion reaction, the contents of the tubes were lightly vortexed and spun. Ten uL of each sample was loaded into an ethidium bromide (EtBr) gel for electrophoresis. The expected bands from a *Pst*I digestion of pZErO 2.1, with the hrIFNb fragment inserted into the *EcoR*V site in the forward orientation, were roughly 200 and 3600bp. Band fragments measured indicate that a correct fragment had likely been inserted. The plasmids were regrown and purified using the Sigma Miniprep Plasmid Isolation Kit. Once regrown and purified, the clean pZErO 2.1 + suspected hrIFNb insert were digested with *Pst*I in 10uL volumes. Digests were run on EtBr with 1uL of Orange G dye added to each digest. Plasmids exhibiting the expected restriction fragment sizes were subsequently purified and sequenced to verify fragment identity.

2.1.11 - Plasmid Verification of hrDecorin

Five uL of each of the 12 hrDecorin easy preps (6 samples from ovarian and 6 samples from brain template cDNA libraries) isolated from cultures grown from random white colonies grown on LB plates containing Kan and X-gal were cut using *Hind*III (New England Biolabs, Inc., Ipswich, Massachusetts, USA). The reactions were incubated for 2 hours at 37°C. One uL of 10X Orange G was added to each reaction and the contents of the tubes were lightly vortexed and spun. Ten uL of each sample was loaded into an EtBr gel. The expected bands from a *Hind*III digestion of pZErO 2.1 with the hrDecorin fragment inserted into the *Eco*RV site were roughly 850 and 3500bp if inserted in the forward direction (correct orientation). For clones exhibiting the expected and correct digestion pattern, plasmids will be regrown and purified using the cleaner

Genelute kit (Sigma-Aldrich, Inc., St. Louis, Missouri, USA). Once regrown and purified, the clean pZErO 2.1 with the suspected hrDecorin insert were again digested with *Hind*III. Digests were run on a EtBr agarose (0.7%) gel. Bands occur slightly under the 1kb band from the 1log ladder standard (New England Biolabs, Inc., Ipswich, Massachusetts, USA), resulting in a banding pattern predicted by the digestion of pZErO 2.1 + hrDecorin. Purified plasmids were sequenced to verify and confirm fragment identity.

2.1.12 - hrTRAIL Colony Selection

Four colonies, labeled hrTRAIL 3.1-1 through 3.1-4 were grown in LB broth in the presence of ampicilin and purified using the *E. coli* miniprep protocol. Each sample was cut in separate reactions with either *Sca*I or *EcoR*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA). For each sample, restriction enzyme digestions were performed in 10uL volumes. The digests were incubated for 48 hours at 37°C. The products were run on 0.6% agarose gel with EtBr along with the 1 kb ladder (New England Biolabs, Inc., Ipswich, Massachusetts, USA). Comparing the bands from the gel, hrTRAIL samples 3.1-3 and 3.1-4 had exhibited the proper banding pattern expected for a hrTRAIL insert.

2.1.13 – Preparation and analysis of restriction verified fragments

Once there was sufficient evidence that a transformed clone potentially incorporated the plasmid with the correct insert, a fresh culture of 4 to 5 mL was grown in either 2XYT or LB broth with the correct antibiotic (Kan for pZero 2.1 and Amp for

PGem-T Easy and pZero 3.1) and plasmid was isolated with the GenElute Plasmid Miniprep kit (Sigma-Aldrich, Inc., St. Louis, Missouri, USA). From the culture, 1.5 mL was spun at 12,000 to 13,000xg for 1 minute in a microcentrifuge tube. The supernatant was removed and another 1.5 mL of culture was placed atop the pellet, spun for another minute at 12,000 to 13,000xg. The supernatant was again removed. The double pellet was resuspended in 200 uL of the resuspension solution. Two hundred uL of lysis solution was placed into the resuspended solution and immediately inverted 6 to 8 times. Three hundred and fifty uL of neutralizing/binding solution was placed into the mixture before 5 minutes had elapsed. Subsequently, the new solution was inverted 4 to 6 times. This solution was spun for 10 minutes at 12,000xg to 13,000xg at room temperature. During this time, the spin columns were inserted into the supplied microcentrifuge tubes and prepared by adding 500uL of column preparation solution to each column and spun for 1 minute at 12,000xg to 13,000xg. The flow through was discarded and the cleared lysate from the lysed solution was transferred to the prepared column carefully to limit the amount of cellular debris and other precipitate that was transferred to the column. Once in the column, the solution was spun for 1 minute at 12,000xg to 13,000xg. The flow through was discarded and 750 uL of wash solution was placed into the column and the tube was spun again for 1 minute at 12,000xg to 13,000xg. Again, the flow through was discarded and the column was spun again for 2 minutes at 12,000xg to 13,000xg to ensure that all of the wash solution was spun out of the column. The dry column was placed into a clean tube and 100 uL of RNAse and DNase free H_20 was placed into the column. The tube was spun for 1 minute at 12,000xg to 13,000xg and the flow through

contained the eluted plasmid. The isolated plasmid was placed on ice and measured using the Nanodrop spectrometer.

Table 2- DNA readings following a Sigma Miniprep Plasmid Isolation for hrDecorin and hrIFNb

	Reading	ng/uL	Ave ng/uL	260/280	Ave 260/280	260/230	Ave 260/230
hrDecorin 1	1	96.25	94.85	1.8	1.81	1.86	1.835
	2	93.45		1.82		1.81	
hrDecorin 2	1	91.75	90.415	1.84	1.85	1.91	1.92
	2	89.08		1.86		1.93	
hrIFNb 21	1	120.2	119.29	1.9	1.875	2	2
	2	118.38		1.85		2	
hrIFNb 24	1	97.18	99.88	1.87	1.865	1.97	1.85
	2	102.58		1.86		1.73	

2.1.14 - Purifying easyprep isolations using Qiagen miniprep purification protocol from "other methods"

The easy miniprep isolations of the potentially correct ligated vector+insert hrTRAIL 3.1-3 and hrTRAIL 3.1-4 that needed to be purified more completely for use in the DNA sequencer were estimated to have a volume of 90uL. The Qiagen protocol required a volume of PB solution 5X more than the purified plasmid volume. The samples were then placed into the prepared spin columns and the protocol was continued. The resultant concentration and purity of the DNA is indicated in Table 3. These samples were then sequenced using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Brea, California, USA).

Table 3- DNA readings following a Sigma Miniprep Plasmid Isolation for hrTRAIL

	ng/uL	Average ng/uL	260/280	260/230
TRAIL 3.1-3	202	206	1.9	3.05
TRAIL 3.1-3 2nd read	210		1.9	2.3
TRAIL 3.1-4	274	261.5	1.83	2.27
TRAIL 3.1-4 2nd read	249		1.88	2.29

2.1.15 - Sequencing of Isolated Constructs

The purified GenElute plasmid miniprep constructs were sequenced using a CEQ8000 (Beckman Coulter, Inc., Brea, California, USA). The presence of human recombinant factor inserts within a plasmid was verified by using SP6, M13 reverse, and M13 forward primers. The sequencing primer binding sites occur flanking the insert on pZErO-2.1 and pGEM T Easy. The reagents and protocol were obtained from The Beckman Coulter Genome Lab Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit. Sequencing primers were diluted to 1.6uM prior to beginning the experiments. One hundred and thirty ng of plasmid DNA (Table 1)was brought up to a final volume of 6 μL with molecular grade H_2O in a thermalcycle tube and treated at 96°C for 1 minute. Following DNA treatment, 8 μL of Master Mix, 2 μL of SP6/M13 Rev primer or 2 μL of M13For, and 4 μL of molecular grade H_2O were added to each tube. Tubes were votexed and the contents were pooled by brief centrifugation. The tubes were run on the PCR program CEQSER. This program consisted of 31 cycles of 96°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 min, and a holding temperature of 4°C. Following the PCR amplification, the amplified DNA solutions were pipetted into a 0.5mL tube containing a stop solution consisting of 2 μL of 3M sodium acetate, 2 μL 100mM Na2-EDTA, and 1 μL of glycogen per sample. The tubes were then vortexed thoroughly and the contents

were lightly spun. In a refrigerated environment, 60 μL of ice cold 95% ethanol was placed into each tube, mixed thoroughly, and spun at 14,000 RPM for 15 minutes. The supernatant was removed and the pellet was washed 2 times with 200 μL 70% ethanol. Special care was taken to not dislodge the pellet. Each wash was completed by spinning the pellet and 70% ethanol at 14,000RPM at 4°C for 5 minutes and discarding the supernatant. After the final wash, the pellet was spun at max speed for 1 minute to remove any residual supernatant. At room temperature, the tube caps were left open to allow evaporation of the remaining ethanol. Following complete evaporation, the dried pellets were resuspended into 40 μL of the sample loading solution. The solution was allowed to sit for 10 minutes, and then the solution and pellet were votexed and spun. The sample plate was loaded with 40 μL along topped with one drop of mineral oil. The buffer plate was loaded with 10 drops of separation buffer to each well in the buffer plate. Both the sample and buffer plates were then loaded into the CEQ8000. The sequencer was run in "LFRc mode" and the following options were selected options menu. In the analysis section, the perform analysis box was checked. In the export section, the export data box was selected and the export options were edited to ensure that raw data, results data, results output, quality parameters, alignment results, and alignment accuracy boxes were checked to ensure proper data collection. These settings were saved and the output file was assigned a name corresponding to the DNA sequence project of interest. Once the file was saved, capillary array and the gel cartridge were installed, manifold purge was performed, and the wetting tray had been filled, the trays were loaded and samples were sequenced. The output was analyzed using several methods, but the primary

sequences were analyzed using Vector NTI versions 10 and 11 (Invitrogen, Carlsbad, California, USA) to align raw sequences to NCBI reference sequences.

2.1.16 - Strategy of Isolating hrDecorin inserts from pZErO 2.1

Following sequence verification, pZErO 2.1 hrDecorin 1 and pZErO 2.1 hrDecorin 2 were double digested with *BamH*I (New England Biolabs, Inc.) and *Xho*I (Promega, Inc.). Digestions were done in a 37°C incubator.

2.1.17 - Strategy of Isolating hrIFNb inserts from pZErO 2.1

Following sequence verification, pZErO 2.1 hrIFNb21 and pZErO 2.1 hrIFNb24 were double digested with *Hind*III and *Xba*I (New England Biolabs, Inc.). Digestions were done in a 37°C incubator.

2.1.18 - Double Digest Fragment Isolation

Double digests of pZErO 2.1 hrDecorin and pZErO 2.1 hrIFNb were designed to isolate the human recombinant genes of interest from the plasmid pZErO 2.1. The digested products were run on an agarose gel following loading with Sybr Safe. The Qiaex II gel extraction protocol was performed on the 4 bands (2 bands for hrDecorin and 2 bands for hrIFNb) containing the genes of interest. pcDNA 3.1/Hygro+ (Invitrogen, Carlsbad, California, USA) was also double digested in a manner that would leave sticky ends to facilitate proper incorporation and ligation of the gene of interest into pcDNA 3.1. These plasmid digests were separated using electrophoresis on agarose gel following

addition of Sybr Safe. The fragments were isolated from the gel and the DNA was extracted using the Qiaex II gel extraction protocol.

2.1.19 - Antarctic Phosphatase

pcDNA3.1 Hygro+ (Invitrogen, Carlsbad, California, USA) following double digestion in preparation for the insertion of human recombinant factors was treated with Antarctic Phosphatase (New England Biolabs, Inc., Ipswich, Massachusetts, USA) to remove 5' phosphates on the free ends of the cleaved plasmid inhibiting the religation to itself without the incorporation of the human recombinant inserts.

2.1.20 - Double Digest of pcDNA 3.1 for hrDecorin

pcDNA 3.1/Hygro+ (Invitrogen, Carlsbad, California, USA) was cut using *Xho*I (Promega, Madison, Wisconsin, USA), and *BamH*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA). The digests were left overnight at 37°C to ensure the vector was completely digested. Following the digest, 1uL of 10X Antarctic Phosphatase buffer and 1 uL of Antarctic Phosphatase were added to the overnight digests to remove the free phosphates on the cut plasmid to prevent religation. The solution was run on the thermal cycler program PWANTPHO. This program consisted of 37°C for 15 minutes followed by 65°C for 5 minutes. Sybr Safe and Orange G were added to the digest and the reactions were run on an agarose gel. The resultant band was isolated and the DNA was extracted using the Qiaex II gel extraction protocol.

2.1.21 - Double Digest of pcDNA 3.1 for hrIFNb

pcDNA 3.1 was cut using *Hind*III and *Nhe*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA) in a 10uL final volume. The digests were left overnight at 37°C to ensure the vector was completely digested. Following the digest, the vector was treated with Antartic Phosphase (New England Biolabs, Inc., Ipswich, Massachusetts, USA) to prevent vector religation. The reaction was carried out using the thermal cycler program PWANTPHO. Sybr Safe and Orange G were added to the digest and the sample was subjected to agarose gel electrophoresis. The resultant vector band was isolated and the DNA was extracted using the Qiaex II gel extraction protocol.

2.1.22 - Ligation of Double Digest hrDecorin and hrIFNb

hrDecorin and hr IFNb were ligated into cut pcDNA 3.1 plasmids using T4 DNA Ligase (New England Biolabs, Inc., Ipswich, Massachusetts, USA). The insert to vector ratio was 10 to 1, 9 to 1, 14.5 to 1 and 16.5 to 1 for hrDecorin1, hrDecorin2, hrIFNb 21, and hrIFNb 24b respectively. hrDecorin1 and hrDecorin2 were ligated into the digested pcDNA3.1 digested specifically for the incorporation of hrDecorin fragments. hrIFNb21 and hrIFNb24 were ligated into the digested pcDNA3.1 digested specifically for directional cloning of the hrIFNb fragments. Control ligations for pcDNA 3.1 cut for hrDecorin and pcDNA 3.1 cut for hrIFNb without human recombinant DNA were conducted using the same quantity as the ligation without human recombinant DNA. All ligations were performed in 10 uL final volume. Three uL of each ligation were transformed using the modified *E. coli* transformation protocol. Four colonies from hrDecorin1, 3 colonies from hrIFNb21, and 4 colonies from hrIFN24 were grown in LB

Amp cultures overnight and the plasmid DNA was isolated using the Sigma Miniprep Plasmid Isolation Kit (Sigma-Aldrich, Inc., St. Louis, Missouri, USA).

Table 4- Values from the various parameters for DNA isolation

	band weight	QX1 Buffer Vol	ng/uL	260/280	260/230
hrDecorin 1 DD	92 mg	276 mL	5.97	1.25	0.76
hrDecorin 2 DD	94 mg	282 mL	5.24	1.27	0.8
hrIFNb 21 DD	116 mg	348 mL	19.72	1.4	0.84
hrIFNb 24 DD	120 mg	360 mL	10.98	1.46	0.79
pcDNA 3.1 - DCN cut	88 mg	264 mL	5.9	1.65	0.65
pcDNA 3.1 - IFNb cut	114 mg	342 mL	12.83	1.41	0.6

2.1.23 - Digest Verification of hrDecorin and hrIFNb Insertion into pcDNA3.1

The hrDecorin samples (5 uL of each) were digested with *BamH*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA) and *Xho*I (Promega, Madison, Wisconsin, USA) in 10uL final volume. hrIFNb samples (5 uL of each) were digested with *Hind*III and *Nhe*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA) in a 10uL final volume. All digestions were carried out overnight at 37°C. Digestion of isolated pcDNA3.1+ insert and insert free uncut pcDNA3.1/Hygro+ were conducted to verify the correct insertion of the fragments into the pcDNA3.1 plasmid. The uncut naive pcDNA 3.1/Hygro+ and pcDNA3.1 hrDecorin1 isolated plasmid were digested with the *Hind*III enzyme. The uncut naive pcDNA 3.1/Hygro+ and pcDNA3.1 IFNb21 and pcDNA3.1 IFNb24 isolated plasmids were digested with the *Pst*I enzyme. The agarose gel bands representing all of the digests were compared with undigested, uncut, and naive pcDNA 3.1/Hygro+ vector and the 1kb ladder (New England Biolabs, Inc., Ipswich,

Massachusetts, USA). Both hrDecorin and hrIFNb containing vectors exhibited the expected band fragments.

2.1.24 - Transformation of Restriction Verified Fragments

Aliquots (0.5uL) of pcDNA3.1 + hrDecorin1, pcDNA3.1 + hrIFNb 21, or pcDNA3.1 + hrIFNb 24 were transformed into GC5 cells using the modified *E. coli* transformation protocol. One hundred uL of each transformation mix was plated on LB/Amp plates. A colony was isolated from each transformation plate, the colony was grown up and stored in 50% glycerol and stored in a -70°C freezer.

2.1.25 - TRAIL Construction from Sequenced Fragments Containing Errors

The sequence results for hrTRAIL 3.1-3 and hrTRAIL 3.1-4 both displayed a few DNA sequence errors. The sample hrTRAIL 3.1-3 was halted because the errors in hrTRAIL 3.1-4 contained fewer sequence errors. The 5' end of hrTRAIL 3.1-4 was identical to the reference sequence obtained from the NBCI gene sequence database (www.ncbi.nlm.gov). The 3' end of hrTRAIL 3.1-4 had problems within the primer region and a single nucleotide polymorphism in a "wobble" base position in an amino acid directly adjacent to the primer leading to a silent mutation. hrTRAIL 3.1-4 was used as the template for amplification. A PCR reaction consisting of 1.6uL 10mM dNTPs, 5ng hrTRAIL 2.1-4, 0.1 uL LATaq (5UuL: Takara, Shiga, Japan), 0.3uL hrTRAIL forward primer (10 uM), 0.3uL hrTRAIL reverse primer (10 uM), 1uL LATaq Buffer, 1uL Betaine, 5.2uL dH_2O was performed using the PCR program "Paul 2." Following the PCR amplification, the DNA was electrophoresed on an agarose gel using 1uL of Sybr

Safe added directly to the PCR sample. The expected 850 bp bands were isolated. The DNA was isolated using the Qiaex gel extraction protocol (Qiagen, Valencia, California, USA). The first gel fragment weighed 246 mg, requiring 738 uL of QX1 solution to be used. The second gel fragment weighed 91 mg, requiring 273 uL of QX1 solution to be used. The remainder of the protocol follows the Qiaex II Agarose Gel Extraction protocol previously listed in this manuscript. The concentration and purity values for the isolated DNA are listed on Table 5.

Table 5- DNA Values for Isolated hrTRAIL

	ng/uL	260/280	260/230
hrTRAIL 3.2a	38.9	2.05	1.88
hrTRAIL 3.2a 2nd elution	9.89	2.31	1.56
hrTRAIL 3.2b	39.6	1.9	1.94
hrTRAIL 3.2b 2nd elution	13.8	1.62	1.05

The new PCR product was ligated into the pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA) using 0.56uL Purified hrTRAIL v3.2a or hrTRAIL v3.2b, 2.5uL 2X rapid ligation buffer, 0.5 uL pGEM-T Easy Vector, 0.5uL T4 DNA Ligase, and 0.94uL dH_2O. The contents of each tube were briefly votexed and spun to ensure proper mixing of all elements. The tubes were incubated overnight at 4°C. The ligation mixes were transformed using the One Shot Top 10 kit (Invitrogen, Carlsbad, California, USA). One uL of hrTRAIL v3.2a or hrTRAIL v3.2b was transferred into 1 vial of Top10 cells that had thawed on ice and was tapped to gently mix the DNA. The mixture was incubated on ice for 30 minutes. The cells were then incubated for 30 seconds in a 42°C water bath and immediately placed back in the ice bath for 2 additional minutes. Two hundred and fifty uL of SOC media was placed into the tubes of both

pGEM-T Easy + hrTRAIL v3.2a and pGEM-T Easy + hrTRAIL v3.2b. Both tubes were then placed on a shaking incubator at 37°C for 60 minutes. One hundred uL of both hrTRAIL v3.2a and hrTRAIL v3.2b were plated on LB AMP plates and grown for 20 hours at 37°C. Four colonies were selected from the hrTRAIL v3.2a plate and six colonies were selected from the hrTRAIL 3.2b plate and expanded in LB+AMP broth. The plasmids were isolated using the Easy Quick Prep protocol. The plasmids were digested using either *EcoR*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA) or *Sca*I (Promega, Madison, Wisconsin, USA) on the hrTRAIL plasmids in 10 uL final volumes. The digests were run on an EtBr agarose gel. From the digests, samples 3.2 A1 and 3.2 A2 conferred the expected fragment sizes. The hrTRAIL v3.2a1 and 3.2 a2 were transformed into GC5 cells. Four colonies were selected from both hrTRAIL v3.2A1 and v3.2A2 and identified as hrTRAIL v3.2A1-1 through 4 and hrTRAIL v3.2A2-1 through 4. The colonies were grown in LB AMP and the plasmids were purified using the Sigma Miniprep Plasmid Isolation Kit (Sigma-Aldrich, Inc. St. Louis, Missouri, USA). The same *EcoR*I and *Sca*I digests were conducted and all samples exhibited proper band fragment sizes. Samples hrTRAIL v3.2A1-1 and hrTRAIL v3.2A2-1 were saved and sequenced. pGEM-T Easy hrTRAIL 3.2A1-1 was measured at a concentration of 43.6ng/uL and pGEM-T Easy hrTRAIL 3.2A2-1 was measured at a concentration of 48.6 ng/uL. During the DNA sequence process 130ng of each hrTRAIL plasmid was used. The samples were sequenced as described in Sequencing of Isolated Constructs section (see above). Sequencing results indicated that the 5' end of hrTRAIL v3.2A1-1 was an exact match however 5' hrTRAIL v3.2A2-1 had two errors in comparison to the reference code. The 3' end of hrTRAIL v3.2A2-1 differed from the reference sequence

by encoding a different stop codon and a wobble position change for the 8th codon or 7th terminal amino acid from the end of the sequence. This single nucleotide polymorphism matched NCBI reference sequence rs1131532 in the database. From this point, the strategy was to splice the 2 correct portions of the gene to yield one complete and correct version of the gene.

To carry out the ligation of the 5' region of hrTRAIL v3.2A1-1 with the 3' region of hrTRAIL v3.2A2-1, the *Pst*I site within the hrTRAIL gene along with the *Pst*I site on the pGEM-T Easy, was chosen for correcting the sequence errors. Digesting hrTRAIL v3.2A1-1 with *Pst*I (New England Biolabs, Inc., Ipswich, Massachusetts, USA) yielded 2 fragments, one fragment containing the correct 5' portion of the hrTRAIL gene measuring roughly 100bp and the other fragment containing plasmid vector backbone along with nearly 750bp representing the remaining 3' portion of the gene. Digesting hrTRAIL v3.2A2-1 with *Pst*I yielded 2 fragments, one fragment containing the correct 3' portion of the hrTRAIL gene along with the complete pGEM-T Easy and the other fragment was roughly 100bp containing the incorrect 5' region. The *Pst*I digests were done overnight at 37°C to ensure complete digestion. The completed digestion of the vector containing the correct 3' end of the gene in pGEM-T Easy was treated with Antarctic Phosphatase (New England Biolabs, Inc., Ipswich, Massachusetts, USA) to prevent religation. The reaction was carried out for 30 minutes and following the phosphatase reaction, 2.5uL of Sybr Safe and 2.5uL of Orange G loading gel were added to the digest. The products were evaluated on an agarose gel. The resultant band running near 4kb was isolated.

Because the 5’ fragment from the *Pst*I digestion of hrTRAIL v3.2A1-1 was so small in relation to the size of the other fragment created from the *Pst*I digestion, the current DNA concentration was not high enough to yield a high amount of the 5’ region of hrTRAIL v3.2A1-1, the original hrTRAILv3.21-1 was retransformed into GC5 cells to purify larger and higher concentrated amounts of this DNA. Two colonies were isolated from the retransformation of hrTRAIL v3.2A1-1. After performing the Sigma Miniprep Plasmid isolation, a higher concentration of plasmid was isolated. The concentration of hrTRAIL v3.2A1-1 DNA is listed in Table 6.

Table 6- Purified DNA plasmid values from the sequence verified TRAIL v3.2A1-1 regrown cultures

	ng/uL	260/280	260/230
TRAIL v3.2A1-1 Regrow 1	140	1.92	2.37
TRAIL v3.2A1-1 Regrow 2	136	1.90	2.42

A large amount of higher concentrated hrTRAIL v3.2A1-1 from both purified colonies was cut to isolate 100bp *Pst*I fragment containing the correct 5’ region. Eighty uL of either hrTRAIL v3.2A1-1 R1 or hrTRAIL v3.2A1-1 R2 (R= regrown plasmid) containing v3.2A1-1 were digested with *Pst*I overnight. The Orange G loading buffer and Sybr Safe were added to each digest, and each digest was split into two lanes due to volume. The digests were run along with a 1kb and a 100bp ladder (New England Biolabs, Inc., Ipswich, Massachusetts, USA). Two distinct bands were seen at near 100 bp and several thousand bp. The 100bp band for all four lanes was isolated, purified, and quantified using the Nanodrop. The results for the Nanodrop are listed in Table 7.

Table 7- DNA values for ATG fragments from sequence verified hrTRAIL v3.2A1-1 regrown colonies

	ng/uL	260/280	260/230
ATG fragment from R1-1	7.76	1.55	0.74
ATG fragment from R1-2	4.10	1.74	0.62
ATG fragment from R2-1	9.08	1.90	0.76
ATG fragment from R1-2	9.03	1.26	0.51

The 3' region of hrTRAIL v3.2A2-1, containing the complete pGEM-T Easy Vector, was ligated with the 5' segment isolated from hrTRAIL v3.2A1-1 using T4 DNA Ligase (New England Biolabs, Inc., Ipswich, Massachusetts, USA) in 10 uL final volume. One point one uL (10.6ng) of digested hrTRAIL v3.2A2-1 (containing plasmid and 3' region), 1uL (9.08ng) of the hrTRAIL v3.2A1-1 R2-1 fragment were used for ligations. The ligation control used 1.1uL (10.6ng) of digested hrTRAIL v3.2A2-1 (containing plasmid and 3' region) only. The ligations were run using the thermal cycler program "LigKill." Three uL of the ligation and the ligation control were transformed into 50uL of GC5 competent cells.

Twenty five uL of the transformation mix was plated on LB Amp plates. Colony PCR was conducted on 24 colonies that had grown on the LB Amp plates. Using a 10uL pipet tip, a colony was picked off the plate and the tip was rubbed into the bottom of a PCR tube, resulting in plasmid DNA ready for amplification. In addition, the tips were each placed into LB broth with Amp to expand the selected colonies that had been picked from the ligation transformation plate. In each PCR tube, 5 uL of 2x GoTaq mix (Promega, Madison, Wisconsin, USA), 0.5uL hrTRAIL For primer (10 uM), 0.5uL hrTRAIL Rev Primer (10 uM) in a final volume of 10 uL. The PCR reactions were carried out using the "Paul2" PCR program and the samples were loaded on an EtBr

agarose gel and subjected to electrophoresis. hrTRAIL v3.2A1/A2 samples 1, 4, 5, 7, 10, 11, 15, 17, and 18 all displayed DNA products occurring near hrTRAIL's expected 846 bps. These correct plasmid samples were isolated from the overnight cultures. Only the cultures from hrTRAIL 3.2 A1/A2 1 and hrTRAIL 3.2 A1/A2 4 were purified using the Sigma Miniprep Plasmid Isolation protocol. These two samples were sequenced to verify a proper ligation of the two previously verified portions of the hrTRAIL gene. One hundred and thirty ng of both hrTRAIL 3.2 A1/A2 1 and hrTRAIL 3.2 A1/A2 4 were sequenced using the sequencing protocol described previously. hrTRAIL 3.2A1/A2 1 was found to match the reference sequence for hTRAIL.

hrTRAIL 3.2 A1/A2 1was removed from pGEM-T Easy by a double digest of *Spe*I and *Apa*I restriction enzymes (New England Biolabs, Inc., Ipswich, Massachusetts, USA). pcDNA 3.1/Hygro+ was cut using *Nhe*I and *Apa*I restriction enzymes (New England Biolabs, Inc., Ipswich, Massachusetts, USA). The overhang created from the *Spe*I digest of pGEM-T Easy hrTRAIL v3.2 A1/A2 4 was compatible with the overhang created from a *Nhe*I digest of pcDNA3.1/Hygro+. One uL of pcDNA 3.1/Hygro+ vector was digested using *Nhe*I and ApaI in 10 uL final volume. Five uL of pGEM-T Easy hrTRAIL v3.1 A1/A2 1 was digested using *Apa*I and *Spe*I in a 10uL final volume. The digests were run with 1uL Orange G loading dye and 1uL Sybr Safe. The hrTRAIL digest yielded two fragments, one fragment contained hrTRAIL and the other band contained pGEM-T Easy (vector backbone). The pcDNA3.1/Hygro+ digest contained a single band representing the linearized plasmid. The DNA in the gel fragments was isolated using the QiaexII DNA gel extraction kit (Qiagen, Valencia, California, USA). Spectrophotometric results are shown in Table 8.

Table 8- DNA values for digested and isolated hrTRAIL and pcDNA3.1/Hygro+ from agarose gel

Double Digested DNA		ng/uL	260/230	260/280
hrTRAIL 3.2 A1/A2 1		8.66	1.2	1.78
pcDNA 3.1/Hygro+		25.5	0.76	1.64

The cut vector and isolated hrTRAIL were ligated using T4 DNA Ligase. The control ligation consisted of cut pcDNA3.1/Hygro+ vector alone. The ligation and control were ligated using the "LigKill" program as described previously. The ligation samples were transformed into 50uL of GC5 competent cells using 3uL of each ligation following the modified *E. coli* transformation protocol. Six colonies were isolated, grown overnight in LB Amp broth, and the plasmids were purified using the Sigma Miniprep Plasmid Isolation Kit. The plasmids were verified by performing a *Pst*I digestion comparing the pcDNA3.1 hTRAIL v3.2 A1/A2 1 ligation transformations with cut and uncut pcDNA 3.1 vectors. All of the plasmid isolates appears to have correctly inserted the hrTRAIL fragment into the pcDNA 3.1/Hygro+ vector. The GC5 culture transformed with the plasmid containing the pcDNA3.1 hTRAIL v3.2 A1/A2 1 colony 1 was expanded and stored as the culture containing the mammalian expression vector with hrTRAIL ligated into it.

2.2 – Experimental Protocol using Sequence Verified DNA Sequences

2.2.1 - Media Preparation, Cancer Cells, Splitting of Cells, and Cell Culture Management

DMEM media was prepared by pipetting 50mL of Fetal Clone 3 (FC3: Thermo Fisher Scientific, Waltham, Massachusetts, USA), 5mL Glutamax (Invitrogen, Carlsbad, California, USA), and 5mL of 100X penicillin plus streptomycin stock (Invitrogen, Carlsbad, California, USA) into 500mL of DMEM. 50/50 is a media mixture consisting of 50 percent F12 (Hyclone, Inc.) and 50 percent DMEM. Two hundred mL of the 50/50 media was made using 100mL of enriched DMEM with antibiotics, 90mL of F12 media, 10mL FC3, 1mL of 100X penicillin and streptomycin stock, and 0.5mL Glutamax. The cancer cell lines used in this study were: MDA-MB-231 obtained from ATCC (Manassas, VA), UMD227 obtained from the Rose-Hellekant Laboratory (Duluth, MN), Hs578T obtained from ATCC, WM793 obtained from Meenhard Herlyn Laboratory (Philadelphia, PA), 4T1 obtained from ATCC, and MCF-7 obtained from ATCC. When culturing cells in 10cm dishes, 1.5mL of trypsin (Invitrogen, Carlsbad, California, USA) was used to remove cells from the dish, 4mL of media was used to neutralize trypsin, and 10mL of media was used to culture the cells. With 6cm dishes, 500uL trypsin was used, 1.5mL of media was used to neutralize trypsin, and 5mL of media was used to culture cells in 6cm dishes. When cells were removed from a single well in a 24 well plate, 125 μL trypsin was used and 375 μL of media was used to neutralize the trypsin.

2.2.2 - Cell Counting

Cells of interest that need to be counted were first trypsinized, spun, and resuspended in 1mL of 50/50 culture media. Ten μL of suspended cells were pipette into a 1.5 microcentrifuge tube along with 40 μL of media and 50 μL of trypan blue (0.4% solution, Invitrogen, Carlsbad, California, USA). The tube was then votexed and 10 μL of the mixture was plated onto a hemocytometer. The four corners were counted and added together, multiplied by the dilution factor (10x), and multiplied by 2500. The resulting value indicates the number of cells per mL.

2.2.3 - LTX Transfectamine of CHO cells in 10 cm plates

One day before the cells were transfected, CHO-K1 cells (ATCC, Manassas, Virginia, USA) were trypsinized and counted. Cell passages were kept below 30 to lower the possibility that the cells would diverge from the parent population. To achieve a cell density of 60 to 80% confluency after 24 hours of growth, 1 to $2x10^6$ cells were plated the previous day. Prior to the transfection, the complete media was replaced with 8mL of 50/50 media lacking antibiotics and serum. In a 15mL tube, 2 mL of 50/50 media lacking antibiotics and serum were added. 10ug of DNA isolated from the Sigma Midiprep kit was pipetted into the 2mL of media. 10 μL of resuspended PLUS Reagent (Invitrogen, Carlsbad, California, USA) was added to the media/DNA mixture, mixed gently, and the mixture was incubated for five minutes at room temperature. Following the incubation, 25 μL of a resuspended Lipofectamine LTX (Invitrogen, Carlsbad, California, USA) was pipetteted into the PLUS/DNA complex, mixed gently through inversion, and incubated for 30 minutes at room temperature. Following the incubation period, the tubes were spun

down briefly to pool the samples. The media along with the DNA, LTX, and Plus reagents, were pipetted into the 10cm dishes containing CHO-K1 cells with 8mL of 50/50 media lacking antibiotics and serum. The cells were gently rocked back and forth several times to facilitate proper and even distribution of the DNA complexes. The dishes were placed back into the 37°C incubator 5% CO_2 for 6 hours. Following 6 hours of incubation time, the transfection media was removed and 10mL of complete growth media was placed back on the transfected CHO-K1 cells. Following 42 hours, the media that had been conditioned by the transfected CHO-K1 was collected. The collected media was spun at 1400RPM for 7.5 minutes. The conditioned media was removed, placed into a clean tube, and the pellet was left undisturbed. The media was enriched with 300 μL of FC3 and 50 μL of Glutamax per 10mL of conditioned media. The conditioned media was immediately placed onto the cells of interest and the remainder of the conditioned media was stored in 4°C.

2.2.4 - Sulforhodamine B Assay

Individual wells that were analyzed via SRB method, were washed with 1mL of 1% PBS two times after growth media had been removed. Following a proper washing, the cells were allowed to dry for 24 hours to ensure a complete desiccation. To a dry 24 well dish being processed, 300 μL of 0.5% sulfurhodamine B (Sigma-Aldrich, Inc., St. Louis, Missouri, USA) in 1% acetic acid was placed in each well. The sulforhodamine B was swirled and incubated at 37C for at least 45 minutes. The sulforhodamine B was emptied from the plate and was washed 3 times with 1% acetic acid to remove excess stain. The plates were allowed to dry completely and were stored in a dark environment.

One mL 10mM Tris pH10 was added to each dried well in a 24 well plate and placed on a plate rocker for 30 minutes to facilitate complete release of the sulforhodamine dye. One hundred μL of the dye was transferred to a well on a 96 well plate and measured at 540nm. For wells that had reached confluency, a 1:10 dilution was made to prevent an incorrect reading due to limited range of the plate readers accuracy.

2.2.5 - R&D Quantikine Human TRAIL ELISA

The expression of human recombinant TRAIL in conditioned media that had been manufactured by CHO-K1 cells (ATCC, Manassas, Virginia, USA) was measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, Minnesota, USA). Samples were obtained from hrTRAIL conditioned media, fresh media, media obtained from CHO cultures, and green fluorescent protein (GFP) conditioned media. Dilutions of 1:1 and 1:9 were utilized to ensure the detection values coincided within the standard curve for proper calculation of TRAIL concentration in media types. The standard curve was created by reconstituting the TRAIL standard in 1.0mL DNase/RNase free H_2O. This reconstituted standard represents a concentration of 10,000pg/mL. Because the hrTRAIL analysis involves cell culture supernate, 1x calibrator diluent RD5-33 was prepared by diluting 5mL of the calibrator diluent RD5-33 concentrate with 15mL DNase/RNase H2O. Nine hundred uL 1x RD5-33 was placed in the 100pg/mL tube and 500uL 1x RD5-33 was pipette into the remainder microcentrifuge tubes used in making the standards. One hundred μL from the 10,000pg/mL TRAIL standard was pipette into the 100pg/mL tube that contained 900 μL 1x RD5-33. The contents were votexed and 500 μL of this solution was pipette into the 500pg/mL tube. A two-fold serial dilution was carried out

until 15.6pg/mL was achieved. In each well 100 μL of assay diluent RD1S is placed into each well being used in both standard and sample wells. Fifty μL of a standard, sample, or a diluted sample is pipette into a corresponding well. An adhesive cover was applied to ensure that wells would not contaminate adjacent wells and the plate was placed on a horizontal orbital microplate shaker set at 500 RPM for 2 hours. Each well was emptied by shaking the contents out of the wells and washed by pipetting in 400 μL of wash buffer 4 times. Each wash phase was completed by shaking the 400 μL wash buffer out, and lightly hitting the plate flat on a dry paper towel ensuring maximum removal of wash buffer. Two hundred μL of TRAIL conjugate was added to each well and a new adhesive cover was attached to prevent cross well contamination. The plate was put back on the horizontal orbital microplate shaker for 2 hours at 500RPM. The wash step as previously described was repeated with a total of 4 additional washes. The substrate solution was mixed 5 minutes before being used and was added in a 1:1 ratio of solution A and solution B. Two hundred μL of this A+B substrate solution was pipetted into each well and allowed to incubate for 30 minutes at room temperature in a light protected environment. Following 30 minute incubation, 50 μL of the stop solution was put in each well and mixed gently for 5 minutes by gentile tapping, ensuring proper and thorough mixing. It was observed that the more TRAIL in a well resulted in longer time for an effective mixing of the stop solution. Premature reading without proper mixture of the stop solution resulted in a well consisting of both blue and yellow conditions, mixing must continue until a solid yellow persists in each well. Within 30 minutes of placing the stop solution into each well, the plate was read. The plate was read at 450nm on a SpectraMax plate reader (Molecular Devices, Sunnyvale, California, USA). To correct

for the plate, a 540nm reading was also taken and this value was subtracted from the 450nm reading. The subsequent value represents the absorbance accounting for the plate imperfections. Microsoft Excel was used to form a fit line from the standard values and for statistical analysis. The unknowns were compared to the standard fit line to determine how much hTRAIL exists in a particular sample.

Chapter 3: Results

3.1 - Sequence Verified plasmids

hrTRAIL, hrIFNb, and hrDecorin genes were amplified into plasmids and the sequences were verified via DNA sequencing (Appendix A). Once verified, the genes were ligated into the mammalian expression vector pcDNA 3.1/Hygro+. The proper gene orientation and gene product were verified by restriction enzyme digestion.

3.2 - MDA-MB 231, MCF-7, and WM793 cells are susceptible to the apoptotic effects of commercially available TRAIL

A 24 well plate was seeded with WM793, MDA-MB-231, or MCF-7 cells using the 50:50 media as described in the Materials and Methods. WM793, MDA-MB-231, and MCF-7 cells were shown to be susceptible to apoptosis in both hrTRAIL and mrTRAIL experiments in a dose dependent manner. Specifically, a concentration of 62.5 ng/mL hrTRAIL or mrTRAIL administered to seeded cells for a 3 day period demonstrates TRAIL's ability to induce apoptosis (using serial dilutions) in WM793 (Figure 1A and 1B), MDA-MB-231 (Figure 2A and 2B), and MCF-7 (Figure 3A and 3B) cell lines as compared to cells not given TRAIL. UMD227, Hs578T, and 4T1 cell lines did not show a change in cell density when an administered human or mouse TRAIL (results not shown). Cell densities were determined using the SRB protocol described in section 2.2.4 of this manuscript. Cell density on day 3 was determined in the following way: a mean cell density was corrected for by subtracting the mean cell density of all day zero values obtained for each cell type (i.e. all WM793 day 0 values used in the mouse experiment) before the treatment means and standard deviations were calculated. The correction was

made this way to account for inter-day seeding variation that could occur as a result of plating the same cell line on different days.

The results clearly show that recombinant TRAIL had an effect on human cell lines regardless of the species of origin. MDA-MB-231, MCF-7, and WM793 cell lines displayed reduced cell number after 3 days of treatment with the positive control commercially available recombinant TRAIL derived from either, human or mouse sequences. Susceptibility for MDA-MB-231, MCF-7, and WM793 cell lines were seen at 62.5ng/mL commercial TRAIL after a 3 day period. Individual experiments are presented in Tables 9-14 and summarized results in Figures 1-3 (Tables 9-14; Figures 1-3) Not only were cell densities greatly reduced as measured by SRB assays, but floating debris was visible microscopically indicating cell death.

Table 9- Corrected values obtained from Commercial human TRAIL on WM793

	Day 0	0ng/mL	0.625	6.25	62.5
Experiment 1	0.000	0.490	0.490	0.186	0.075
Experiment 2	0.000	0.399	0.368	0.164	0.037
Experiment 3	0.000	0.384	0.433	0.414	0.180
Experiment 4	0.000	0.569	0.584	0.400	0.205

Table 10- Corrected values obtained from of Commercial mouse TRAIL on WM793

	Day 0	0ng/mL	0.625	6.25	62.5
Experiment 1	0.000	0.611	0.464	0.077	0.025
Experiment 2	0.000	0.683	0.408	0.003	0.000
Experiment 3	0.000	0.495	0.406	0.105	0.024
Experiment 4	0.000	0.318	0.341	0.200	0.004
Experiment 5	0.000	0.462	0.443	0.193	0.048
Experiment 6	0.000	0.511	0.489	0.188	0.068

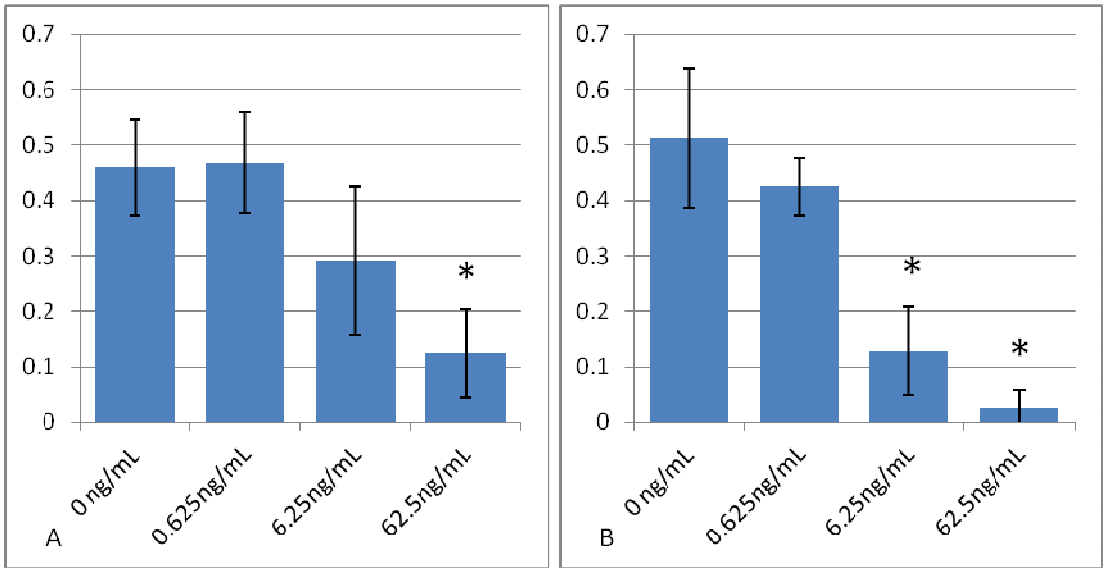


Figure 1 – Commercially available human (Fig. 1A) and mouse (Fig. 1B) recombinant TRAIL and the effect of varying concentrations on WM793 melanoma cancer cells over a 3 day period as compared to cells not receiving the TRAIL protein. Plates were analyzed using SRB protocol and data from Tables 9 and 10 are graphed as mean ± s.d. (Human n=4 and Mouse n=6)

Table 11- Corrected values obtained from Commercial human TRAIL on MDA-MB 231

	Day 0	0ng/mL	0.625	6.25	62.5
Experiment 1	0.000	0.489	0.579	0.405	0.039
Experiment 2	0.000	0.461	0.466	0.276	0.016
Experiment 3	0.000	0.405	0.411	0.389	0.140
Experiment 4	0.000	0.582	0.607	0.556	0.206

Table 12- Corrected values obtained from Commercial mouse TRAIL on MDA-MB 231

	Day 0	0ng/mL	0.625	6.25	62.5
Experiment 1	0.000	0.355	0.398	0.292	0.075
Experiment 2	0.000	0.598	0.568	0.340	0.046
Experiment 3	0.000	0.574	0.542	0.415	0.078
Experiment 4	0.000	0.521	0.521	0.483	0.214
Experiment 5	0.000	0.434	0.421	0.417	0.248
Experiment 6	0.000	0.595	0.615	0.579	0.289

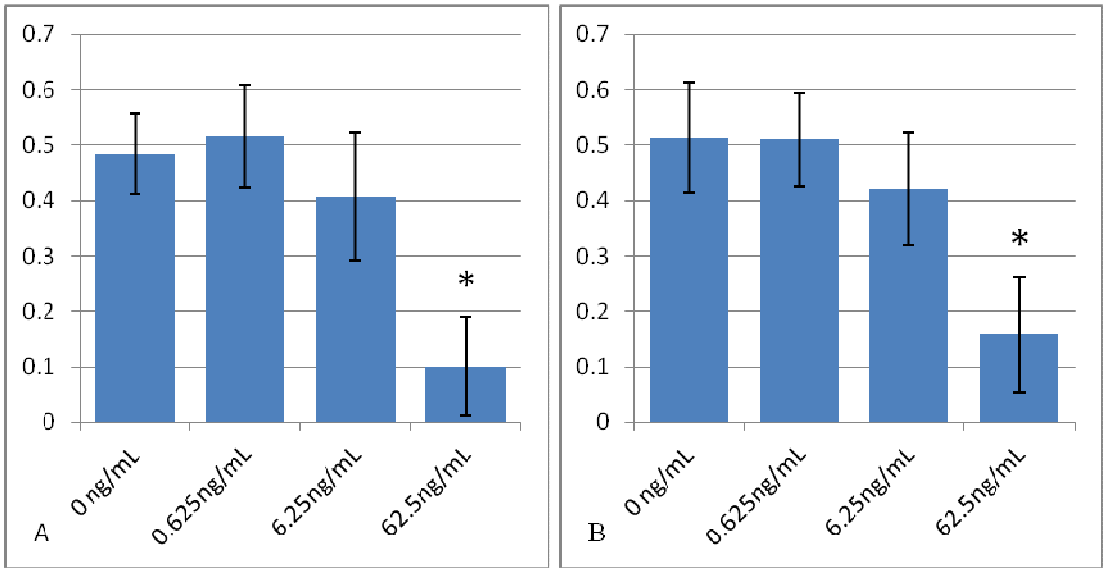


Figure 2 – Commercially available human (Fig. 2A) and mouse (Fig. 2B) recombinant TRAIL and the effect of varying concentrations on MDA-MB 231 Human Breast Cancer cells over a 3 day period as compared to cells not receiving the TRAIL protein. Plates were analyzed using SRB protocol and data from Tables 11 and 12 are graphed as mean ± s.d. (Human n=4 and Mouse n=6)

Table 13- Corrected values obtained from Commercial human TRAIL on MCF-7

	Day 0	0ng/mL	.625	6.25	62.5
Experiment 1	0.000	0.945	1.009	0.789	0.264
Experiment 2	0.000	1.221	1.179	1.030	0.396
Experiment 3	0.000	1.069	1.113	1.030	0.604
Experiment 4	0.000	1.015	1.030	0.983	0.587

Table 14- Corrected values obtained from Commercial mouse TRAIL on MCF-7

	Day 0	0ng/mL	.625	6.25	62.5
Experiment 1	0.000	0.796	0.833	0.651	0.381
Experiment 2	0.000	1.363	1.382	1.004	0.694
Experiment 3	0.000	1.030	0.964	0.674	0.388
Experiment 4	0.000	1.219	1.146	1.053	0.755
Experiment 5	0.000	1.135	1.130	1.079	0.767
Experiment 6	0.000	1.077	1.059	0.987	0.640

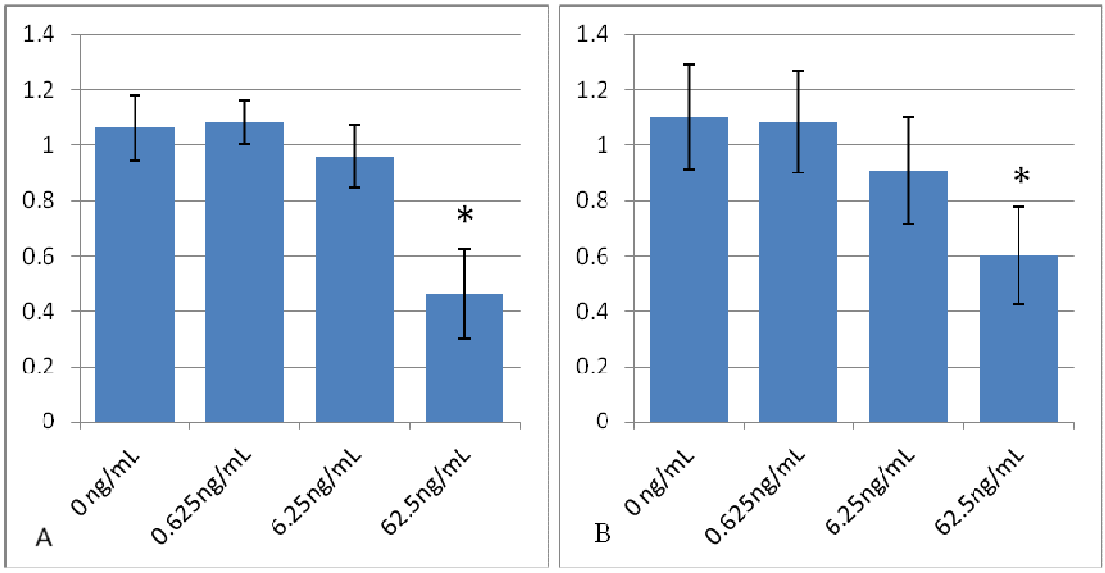


Figure 3 – Commercially available human (Fig. 3A) and mouse (Fig. 3B) recombinant TRAIL and the effect of varying concentrations on MCF-7 Human Breast Cancer cells over a 3 day period as compared to cells not receiving the TRAIL protein. Plates were analyzed using SRB protocol and data data from Tables 13 and 14 are graphed as mean ± s.d. (Human n=4 and Mouse n=6)

The sensitivity of several cancer lines to TRAIL encouraged the concept of engineering hrTRAIL in mesenchymal stem cells for use as a means to deliver anti-cancer gene therapy to specific sites. Below I describe the in vitro biological activity of hrTRAIL which I engineered and expressed in CHO cells.

3.3 - Biological activity of hrTRAIL

Engineered hrTRAIL was produced by transfected CHO cells. The quantity of TRAIL production was determined by measuring the secreted TRAIL in "conditioned" media using enzyme-linked immunosorbent assay (ELISA). Specifically, media was collected from CHO cells that were transfected with hrTRAIL as well as media from several control CHO lines which included transfected CHO cells, GFP transfected CHO cells, and cell free 50% fresh/ 50% aged media were below level of detection. The

TRAIL containing media collected from transfected CHO cells was processed as described in the Materials and Methods of this manuscript. The concentration of hrTRAIL in transfected CHO cells was 4.6 ng/mL while levels were below detection in all control CHO cell groups.

Thereafter the effect of hrTRAIL conditioned media on cancer cell line growth was measured using SRB assays. Cancer cell lines tested for effects of 100% and 50% hrTRAIL conditioned media included MDA-MB-231, MCF-7, and WM793 cells. Neither MDA-MB-231 nor MCF-7 cells responded to hrTRAIL conditioned medium but cell density after 96h of cell culture was reduced in WM793 cells grown in the presence of 100% conditioned medium containing hrTRAIL.

Table 15- SRB data on WM793 cells following treatment from media collected from transfected CHO cell producing GFP, hrTRAIL, or a media composed of a 50:50 ratio of the GFP and hrTRAIL conditioned media

	GFP media	50:50	100% hrTRAIL media
Day 0	0.076	0.079	0.080
Day 1	0.160	0.150	0.127
Day 3	0.305	0.215	0.192
Day 4	0.379	0.244	0.199

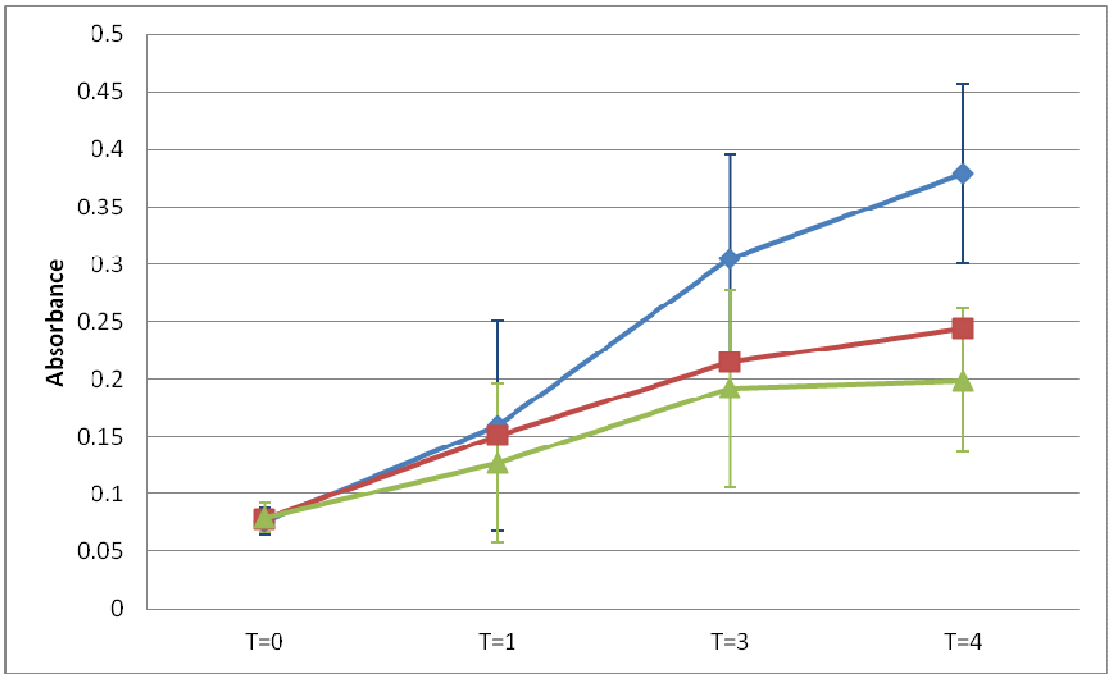


Figure 4 - The conditioned media from CHO cells transfected with GFP or hrTRAIL affected the final SRB results in a dose response relationship. Values were collected daily starting at day 0 (T=0), 24 hours after seeding and seeded with fresh 50:50 F12:DMEM media. (▲ = 100% hrTRAIL media ● = 100% GFP media, and ■ = 50% GFP/50% hrTRAIL media) Data points = mean ± s.d. (n=3).

3.4 - Engineered IFNb conditioned media may decrease cell number of MDA-MB 231 cells

MDA-MB-231 cells showed a significant reduction in cell number when cultured in 100% media obtained from CHO cells producing hrIFNB as compared to media obtained from CHO cells producing GFP (Table 16). Statistical analysis using one tailed T-test was used with p-value <0.05 considered significant.

Conditioned media containing IFNb was also used in cell lines that did not show a response to TRAIL in the initial commercial TRAIL studies (UMD 227 (murine mammary tumor cells), Hs578T (human breast tumor cells), and 4T1 (murine mammary

tumor cells)) as a possible sensitization step in a combinational approach to testing these cell lines. There was no change in the cell densities following the cells treated with conditioned media IFNb and commercial TRAIL as compared to treatments involving GFP media with TRAIL, TRAIL alone, fresh media alone, and GFP media alone following 4 days (data not shown).

Table 16 –IFNb condition media experiment testing the effect of IFNb on MDA-MB 231 Cells

Experiment		GFP	50%	100%
1	T=0	0.052	0.054	0.055
	T=1	0.062	0.076	0.069
	T=3	0.203	0.174	0.163
	T=4	0.71	0.71	0.67
2	T=0	0.050	0.051	0.054
	T=1	0.094	0.074	0.069
	T=3	0.273	0.167	0.142
	T=4	0.81	0.74	0.63
3	T=0	0.050	0.049	0.049
	T=1	0.064	0.058	0.054
	T=3	0.154	0.110	0.108
	T=4	0.65	0.52	0.49

4. Discussion

The cause of TRAIL susceptibility was not determined in this study for the cell lines of interest, but previous literature suggests that TRAIL receptor level surface expression of TRAIL-R1, TRAIL-R2, and the absence of decoy receptors (Sheridan *et al.* 1997, Pan *et al.* 1997b, and Emery *et al.* 1998) and the presence of downstream apoptotic mediators as major determinants of TRAIL induced apoptotic response (Walczak *et al.* 1997).

This study demonstrated that a concentration of 62.5 ng/mL of human or mouse rTRAIL was able to significantly reduce the cell concentrations of MDA-MB-231, WM793, and MCF-7 cell lines over a 3 day period (Figures 1-3). Cell lines UMD227, Hs578T, and 4T1 that did not show a change in cell density to either mouse or human rTRAIL during the testing protocol of 62.5 ng/mL for 3 days also were subjected to a one time test of 1000 ng/mL environment for 3 days. In all cases, there appeared to be no apoptosis by observation with microscopy or reduction in cell number as assessed by the SRB assay (results not shown).

The outcome regarding the MCF-7 cell line in this study differs from Rahman *et al.* (2009) in that they did not find a growth inhibition to MCF-7 cells with the administration of 1000ng/mL of TRAIL. Rahman *et al.* (2009) conducted western blot data for the presence of TRAIL-R1, -R2, -R3, -R4, and downstream mediators of TRAIL induced apoptosis. The western data for the TRAIL-R1 and -R2 receptors in the cell line Hs578T appear to be in lower quantity than obtained for MDA-MB-231 and MCF-7. Based on Rahman *et al.* (2009) data, my data suggests the functional presence of TRAIL-

R1 and -R2 since the MCF-7 cells in my studies have the potential to be responsive to TRAIL (both human and mouse rTRAIL).

WM793, a melanoma cell line, was shown to be the most susceptible to TRAIL out of the cell lines tested. It also demonstrated a susceptibility to TRAIL in a dose response manner with the TRAIL secreted from CHO cells that had been transfected with a mammalian expression vector containing the full length human TRAIL gene. This demonstrated our ability to synthesize TRAIL which is functionally similar to commercial products. ELISA studies confirmed that the 50:50 (F12:DMEM) media that was used, media taken from CHO cells, and media taken from CHO cells following a transfection with GFP did not contain hrTRAIL. These controls were used to make certain that TRAIL was not present in the media, produced by untransfected CHO cells, or produced in the media as a result of transfecting a plasmid into CHO cells. It is suggested from this data that if concentrations of hrTRAIL were to be increased, that it would have an effect on MDA-MB-231 cells, MCF-7, and any other cell line not studied in this experiment that is susceptible to TRAIL related apoptosis signaling.

Conditioned media experiments using media from CHO cells that have been transfected with either hrIFNb or hrDecorin were also conducted. Although sequencing data indicates a successful amplification of both hrIFNb and hrDecorin, the results did not show a measurable result after four days of treatment using the SRB protocol to measure cell density after treatment. The absence of any significant response to the treatment of the cell lines used in this study with conditioned media of either hrIFNb or hrDecorin for four days indicate either an absence of functional protein produced, the cell types used are not susceptible to the antiproliferative or apoptotic effects of hrIFNb or

hrDecorin, or the experimental protocol described here was not sufficient to elicit the effects of hrIFNb or hrDecorin (i.e. low production levels of functional protein being produced in our CHO cell line). Future experiments with the engineered constructs should involve the use of protein specific ELISA tests to determine protein presence within the conditioned media and to correlate responses with highly purified commercially available proteins. Furthermore, my results suggest that the CHO-based production of these factors would be of benefit with the use of a regulatable and inducible protein production system in future *in vivo* studies.

Many cancer treatments rely on the combination of surgery, radiation, and chemotherapy. Many times, a chemotherapy approach relies on a combination of drugs to be delivered at a particular time during a treatment cycle in the attempts to shrink or pretreat a cancer site prior to surgery, radiation, or the addition of another chemotherapeutic drug. Adjuvant, neoadjuvant, and combination chemotherapy regiments pose a significant potential for the future of the treatment of breast cancer and cancer as an entire disease entity. Cell based treatments represent an exciting future for the treatment of cancer as they have the possibility of delivering many gene products at the local level.

Bibliography

American Cancer Society. *Cancer Facts & Figures 2013*. Atlanta: American Cancer Society; 2013. Print.

American Cancer Society. *Breast Cancer Facts & Figures 2011-2012*. Atlanta: American Cancer Society; 2012. Print.

Araki, Kentaro, Hiroki Wakabayashi, Ken Shintani, Joji Morikawa, Akihiko Matsumine, Katsuyuki Kusuzaki, Skihiro Sudo, and Atsumasa Uchida. "Decorin Suppresses Bone Metastasis in a Breast Cancer Cell Line." *Oncology* 77 (2009): 92-99. Print.

Ashkenazi, Avi, Roger C. Pai, Sharon Fong, Susan Leung, David A. Lawrence, Scot A. Marsters, Christine Blackie, Ling Chang, Amy E. McMurtrey, Andrea Hebert, Laura DeForge, Iphigenia L. Koumenis, Derf Lewis, Louise Harris, Jeanine Bussiere, Hartmut Koeppen, Zahra Shahrokh, and Ralph H. Schwall. "Safety and Antitumor Activity of Recombinant Soluble Apo2 Ligand." *The Journal of Clinical Investigation* 104.2 (1999): 155-62. Print.

Brickelmaier, Margot, Amie Carmillo, Susan Goelz, James Barsoum, and Xiao-Qiang Qin. "Cytotoxicity of Combinations of IFN-b and Chemotherapeutic Drugs." *Journal of Interferon and Cytokine Research* 22 (2002): 873-80. Print.

Chawla-Sarkar, Mamta, Douglas W. Leaman, Barbara S. Jacobs, and Ernest C. Borden. "IFN-b Pretreatment Sensitizes Human Melanoma Cells to TRAIL/Apo2 Ligand-Induced Apoptosis." *The Journal of Immunology* 169 (2002): 847-55. Print.

Csordas, Gyorgy, Manoranjan Santra, Charles C. Reed, Inge Eichstetter, David J. McQuillan, Danielle Gross, Matthew A. Nugent, Gyorgy Hajnoczky, and Renato V. Iozzo. "Sustained Down-regulation of the Epidermal Growth Factor Receptor

by Decorin." *The Journal of Biological Chemistry* 275.42 (2000): 32879-2887. Print.

Cuello, Mauricio, Seth A. Ettenberg, Amy S. Clark, Maccon M. Keane, Reuben H. Posner, Marion M. Nau, Phillip A. Dennis, and Stan Lipkowitz. "Down-Regulation of the ErbB-2 Receptor by Trastuzumab (Herceptin) Enhances Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated Apoptosis in Breast and Ovarian Cancer Cell Lines That Overexpress ErbB-2." *Cancer Research* 61 (2001): 4892-900. Print.

Degli-Esposti, Mariapia A., William C. Dougall, Pamela J. Smolak, Jennifer Y. Waugh, Craig A. Smith, and Raymond G. Goodwin. "The Novel Receptor TRAIL-R4 Induces NF-kB and Protects against TRAIL-Mediated Apoptosis, Yet Retains an Incomplete Death Domain." *Immunity* 7 (1997): 813-20. Print.

Emery, J. G., P. McDonnell, M. B. Burke, K. C. Deen, S. Lyn, C. Silverman, E. Dul, E. R. Appelbaum, C. Eichman, R. DiPrinzio, R. A. Dodds, I. E. James, M. Rosenberg, J. C. Lee, and P. R. Young. "Osteoprotegerin Is a Receptor for the Cytotoxic Ligand TRAIL." *J Biol Chem* 273.23 (1998): 14363-4367. Print.

Fierlbeck, G., A. Ulmer, T. Schreiner, W. Stroebel, U. Schiebel, and J. Brzoska. "Pharmacodynamics of Recombinant IFN-/3 During Long-Term Treatment of Malignant Melanoma." *Journal of Interferon and Cytokine Research* 16 (1996): 777-81. Print.

Ganai, S., R. B. Arenas, and N. S. Forbes. "Tumour-targeted Delivery of TRAIL Using Salmonella Typhimurium Enhances Breast Cancer Survival in Mice." *British Journal of Cancer* 101 (2009): 1683-691. Print.

Ganten, Tom M., Jaromir Sykora, Ronald Koschiny, Emanuela Batke, Sebastian Aulmann, Ulrich Mansmann, Wolfgang Stremmel, Hans-Peter Sinn, and Henning Walczak. "Prognostic Significance of Tumour Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Receptor Expression in Patients with Breast Cancer." *J Mol Med* 87 (2009): 995-1007. Print.

Goda, A. E., T. Yoshida, M. Horinaka, T. Yasuda, T. Shiraishi, M. Wakada, and T. Sakai. "Mechanisms of Enhancement of TRAIL Tumoricidal Activity against Human Cancer Cells of Different Origin by Dipyridamole." *Oncogene* 27 (2008): 3435-445. Print.

Goldoni, Silvia, Daniela G. Seidler, Jack Heath, Matteo Fassan, Raffaele Baffa, Mathew L. Thakur, Rick T. Owens, David J. McQuillan, and Renato V. Iozzo. "An Antimetastatic Role for Decorin in Breast Cancer." *The American Journal of Pathology* 173.3 (2008): 844-55. Print.

Goldoni, Silvia, Ashley Humphries, Alexander Nystrom, Sampurna Sattar, Rick T. Owens, David J. McQuillan, Keith Ireton, and Renato V. Iozzo. "Decorin Is a Novel Antagonistic Ligand of the Met Receptor." *The Journal of Cell Biology* 185.4 (2009): 743-54. Print.

Hu, Yunping, Haiguo Sun, Rick T. Owens, Jiansheng Wu, Yong Q. Chen, Isabelle M. Berquin, Donna Perry, Joseph T. O'Flaherty, and Iris J. Edwards. "Decorin Suppresses Prostate Tumor Growth through Inhibition of Epidermal Growth Factor and Androgen Receptor Pathways." *Neoplasia* 11.10 (2009): 1042-053. Print.

Hymowitz, Sarah G., Hans W. Christinger, Germaine Fuh, Mark Ultsch, Mark O'Connell, Robert F. Kelley, Avi Ashkenazi, and Abraham M. De Vos. "Triggering Cell Death: The Crystal Structure of Apo2L/TRAIL in a Complex with Death Receptor 5." *Molecular Cell* 4 (1999): 563-71. Print.

Iozzo, Renato V., David K. Moscatello, David J. McQuillan, and Inge Eichstetter. "Decorin Is a Biological Ligand for the Epidermal Growth Factor Receptor." *The Journal of Biological Chemistry* 274.8 (1999): 4489-492. Print.

Kaynor, Campbell, Mei Xin, John Wakefield, James Barsoum, and Xiao-Qiang Qin. "Direct Evidence That IFN-b Functions as a Tumor-Suppressor Protein." *Journal of Interferon and Cytokine Research* 22 (2002): 1089-098. Print.

Keane, Maccon M., Seth A. Ettenberg, Marion M. Nau, Edward K. Russell, and Stan Lipkowitz. "Chemotherapy Augments TRAIL-induced Apoptosis in Breast Cell Lines." *Cancer Research* 59 (1999): 734-41. Print.

Kumar-Sinha, Chandan, Sooryanarayana Varambally, Arun Sreekumar, and Arul M. Chinnaiyan. "Molecular Cross-talk between the TRAIL and Interferon Signaling Pathways." *The Journal of Biological Chemistry* 277.1 (2002): 575-85. Print.

Lagadec, C., E. Adriaenssens, R. A. Toillon, V. Chopin, R. Romon, F. V. Coppenolle, H. Hondermarck, and X. L. Bourhis. "Tamoxifen and TRAIL Synergistically Induce Apoptosis in Breast Cancer Cells." *Oncogene* 27 (2008): 1472-477. Print.

Lawrence, David, Zahra Shahrokh, Scot Marsters, Kirsten Achilles, Danny Shih, Barbara Mounho, Kenneth Hillan, Klara Totpal, Laura DeForge, Peter Schow, Jeffrey Hooley, Steve Sherwood, Roger Pai, Susan Leung, Lolo Khan, Brian Gliniak, Jeanine Bussiere, Craig A. Smith, Stephen S. Strom, Sean Kelley, Judith A. Fox,

Deborah Thomas, and Avi Ashkenazi. "Differential Hepatocyte Toxicity of Recombinant Apo2L/TRAIL Versions." *Nature Medicine* 7.4 (2001): 383-85. Print.

Lengyel, Peter. "Biochemistry of Interferons and Their Actions." *Ann. Rev. Biochem.* 51 (1982): 251-82. Print.

Loebinger, Michael R., Ayad Eddaoudi, Derek Davies, and Sam M. Janes. "Mesenchymal Stem Cell Delivery of TRAIL Can Eliminate Metastatic Cancer." *Cancer Research* 69.10 (2009): 4134-142. Print.

Osborne, C. K. "Tamoxifen in the Treatment of Breast Cancer." Ed. Alastair JJ Wood. *Drug Therapy* 339.22 (1998): 1609-618. Print.

Ozoren, Nesrin, Kunhong Kim, Timothy F. Burns, David T. Dicker, A. D. Moscioni, and Wafik S. El-Deiry. "The Caspase 9 Inhibitor Z-LEHD-FMK Protects Human Liver Cells While Permitting Death of Cancer Cells Exposed to Tumor Necrosis Factor-related Apoptosis-inducing Ligand." *Cancer Research* 60 (2000): 6259-265. Print.

Pan, Guohua, Jian Ni, Ying-Fei Wei, Guo-Liang Yu, Reiner Gentz, and Vishva M. Dixit. "An Antagonist Decoy Receptor and a Death Domain-Containing Receptor for TRAIL." *Science* 277 (1997): 815-18. Print.

Pan, Guohua, Karen O'Rourke, Arul M. Chinnaiyan, Reiner Gentz, Reinhard Ebner, Jian Ni, and Vishva M. Dixit. "The Receptor for the Cytotoxic Ligand TRAIL." *Science* 276 (1997): 111-13. Print.

Pitti, Robert M., Scot A. Marsters, Siegfried Ruppert, Christopher J. Donahuel, Alison Moore, and Avi Ashkenazi. "Induction of Apoptosis by Apo-2 Ligand, a New

Member of the Tumor Necrosis Factor Cytokine Family." *The Journal of Biological Chemistry* 271.22 (1996): 12687-2690. Print.

Pouillart, P., T. Palangie, M. Jouve, E. Garcia-Giralt, W. H. Fridman, H. Magdelenat, E. Falcoff, and A. Billiau. "Administration of Fibroblast Interferon to Patients with Advanced Breast Cancer: Possible Effects on Skin Metastasis and on Hormone Receptors." *European Journal of Cancer & Clinical Oncology* 18.10 (1982): 929-35. Print.

Pulkkinen, L., T. Alitalo, T. Krusius, and L. Peltonen. "Expression of Decorin in Human Tissues and Cell Lines and Defined Chromosomal Assignment of the Gene Locus (DCN)." *Cytogenetics and Cell Genetics* 60 (1992): 107-11. Print.

Qin, Xiao-Qiang, Carla Beckham, Jennifer L. Brown, Matvey Lukashev, and James Barsoum. "Human and Mouse IFN- Gene Therapy Exhibits Different Anti-tumor Mechanisms in Mouse Models." *Molecular Therapy* 4.4 (2001): 356-64. Print.

Rahman, M., S. R. Davis, J. G. Pumphrey, J. Bao, M. M. Nau, P. S. Meltzer, and S. Lipkowitz. "TRAIL Induces Apoptosis in Triple-negative Breast Cancer Cells with a Mesenchymal Phenotype." *Breast Cancer Res Treat* 113.2 (2009): 217-30. Print.

Ricci, M. Stacey, Zhaoyu Jin, Michael Dews, Duonan Yu, Andrei Thomas-Tikhonenko, David T. Dicker, and Wafik S. El-Deiry. "Direct Repression of Flip Expression by C-myc Is a Major Determinant of TRAIL Sensitivity." *Molecular and Cellular Biology* (2004): 8541-555. Print.

Santra, Manoranjan, David M. Mann, Edward W. Mercer, Thomas Skorski, Bruno Calabretta, and Renato V. Iozzo. "Ectopic Expression of Decorin Protein Core

Causes a Generalized Growth Suppression in Neoplastic Cells of Various Histogenetic Origin and Requires Endogenous P21, an Inhibitor of Cyclin-dependent Kinases." *The Journal of Clinical Investigation* 100.1 (1997): 149-57. Print.

Santra, Manoranjan, Tomasz Skorski, Bruno Calabretta, Edmund C. Lattime, and Renato V. Iozzo. "De Novo Decorin Gene Expression Suppresses the Malignant Phenotype in Human Colon Cancer Cells." *Proceedings of the National Academy of Sciences of the United States of America* 92 (1995): 7016-020. Print.

Santra, Manoranjan, Inge Eichstetter, and Renato V. Iozzo. "An Anti-oncogenic Role for Decorin. Down-regulation of ErbB2 Leads to Growth Suppression and Cytodifferentiation of Mammary Carcinoma Cells." *The Journal of Biological Chemistry* 275.45 (2000): 35153-5161. Print.

Shankar, Sharmila, Thiyam R. Singh, Xufeng Chen, Hitesh Thakkar, Jason Firnin, and Rakesh K. Srivastava. "The Sequential Treatment with Ionizing Radiation Followed by TRAIL/Apo-2L Reduces Tumor Growth and Induces Apoptosis of Breast Tumor Xenografts in Nude Mice." *International Journal of Oncology* 24 (2004): 1133-140. Print.

Sheridan, James P., Scot A. Marsters, Robert M. Pitti, Austin Gurney, Maya Skubatch, Daryl Baldwin, Lakshmi Ramakrishnan, Christa L. Gray, Kevin Baker, William I. Wood, Audrey D. Goddard, Paul Godowski, and Avi Ashkenazi. "Control of TRAIL-Induced Apoptosis by a Family of Signaling and Decoy Receptors." *Science* 277 (1997): 818-21. Print.

Singh, Thiyam R., Sharmila Shankar, Xufen Chen, Mohammed Asim, and Rakesh Srivastava. "Synergistic Interactions of Chemotherapeutic Drugs and Tumor Necrosis Factorrelated Apoptosis-inducing Ligand/Apo-2 Ligand on Apoptosis and on Regression of Breast Carcinoma in Vivo." *Cancer Research* 63 (2003): 5390-400. Print.

Studeny, Matus, Frank C. Marini, Richard E. Champlin, Claudia Zompetta, Isaiah J. Fidler, and Michael Andereeff. "Bone Marrow-derived Mesenchymal Stem Cells as Vehicles for Interferon-b Delivery into Tumors." *Cancer Research* 62 (2002): 3603-608. Print.

Studeny, Matus, Frank C. Marini, Jennifer L. Dembinski, Claudia Zompetta, Maria Cabreira-Hansen, Benjamin N. Bekele, Richard E. Champlin, and Michael Andreeff. "Mesenchymal Stem Cells: Potential Precursors for Tumor Stroma and Targeted-Delivery Vehicles for Anticancer Agents." *Journal of the National Cancer Institute* 96.21 (2004): 1593-603. Print.

Suliman, Ayoub, Arh Lam, Rakesh Datta, and Rakesh K. Srivastava. "Intracellular Mechanisms of TRAIL: Apoptosis through Mitochondrial-dependent and -independent Pathways." *Oncogene* 20 (2001): 2122-133. Print.

Thai, Le Minh, Agatha Labrinidis, Shelley Hay, Vasilios Liapis, Steve Bouralexis, Katie Welldon, Brendon J. Coventry, David M. Findlay, and Andreas Evdokiou. "Apo2l/Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Prevents Breast Cancer-Induced Bone Destruction in a Mouse Model." *Cancer Research* 66.10 (2006): 5363-370. Print.

Walczak, Henning, Mariapia A. Degli-Esposti, Richard S. Johnson, Pam J. Smolak, Jennifer Y. Waugh, Norman Boiani, Martin S. Timour, Mary J. Gerhart, Kenneth A. Schooley, Craig A. Smith, Raymond G. Goodwin, and Charles T. Rauch. "TRAIL-R2: A Novel Apoptosis-mediating Receptor for TRAIL." *The EMBO Journal* 16.17 (1997): 5386-397. Print.

Walczak, Henning, Robert E. Miller, Kiley Ariail, Brian Gliniak, Thomas S. Griffith, Marek Kubin, Wilson Chin, Jon Jones, Anne Woodward, Tiep Le, Craig Smith, Pam Smolak, Raymond G. Goodwin, Charles T. Rauch, JoAnn CL Schuh, and David H. Lynch. "Tumoricidal Activity of Tumor Necrosis Factor-related Apoptosis-inducing Ligand in Vivo." *Nature Medicine* 5.2 (1999): 157-63. Print.

Wiley, Steven R., Ken Schooley, Pamela J. Smolak, Wenie S. Din, Chang-Pin Huang, Jillian K. Nicholl, Grant R. Sutherland, Terri D. Smith, and Raymond G. Goodwin. "Identification and Characterization of a New Member of the TNF Family That Induces Apoptosis." *Immunity* 3 (1995): 673-82. Print.

Yamaguchi, Y., and E. Ruoslahti. "Expression of Human Proteoglycan in Chinese Hamster Ovary Cells Inhibits Cell Proliferation." *Nature* 336.6196 (1988): 244-46. Print.

Yamaguchi, Yu, David M. Mann, and Erkki Ruoslahti. "Negative Regulation of Transforming Growth Factor-b by the Proteoglycan Decorin." *Nature* 346 (1990): 281-84. Print.

Yang, Bojie, Xing Wu, Ying Mao, Weiming Bao, Liang Gao, Ping Zhou, Rong Xie, Liangfu Zhou, and Jianhong Zhu. "Dual-Targeted Antitumor Effects Against Brainstem Glioma by Intravenous Delivery of Tumor Necrosis Factor-Related,

Apoptosis-Inducing, Ligand-Engineered Human Mesenchymal Stem Cells." *Neurosurgery* 65 (2009): 610-24. Print.

Yoshida, Tatsushi, Yaqin Zhang, Leslie A. Rivera Rosado, and Baolin Zhang. "Repeated Treatment with Subtoxic Doses of TRAIL Induces Resistance to Apoptosis through Its Death Receptors in MDA-MB-231Breast Cancer Cells." *Mol Cancer Res* 7.11 (2009): 1835-844. Print.

Zhang, Yaqin, and Baolin Zhang. "TRAIL Resistance of Breast Cancer Cells Is Associated with Constitutive Endocytosis of Death Receptors of 4 and 5." *Molecular Cancer Research* 6.12 (2008): 1861-871. Print.

Zhang, Yaqin, Tatsushi Yoshida, and Baolin Zhang. "TRAIL Induces Endocytosis of Its Death Receptors in MDA-MB-231 Breast Cancer Cells." *Cancer Biology & Therapy* 8.10 (2009): 917-22. Print.

Appendix A – DNA Sequence of Factors

hIFNb21 SP6	CCCTCTAGCAGTGGGAATTTCTTGTGTACCGAGCATCGGATCC ATCTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGATGGAT CCTCAGTTTCGGAGGTAACCTGTAAGTCTGTTAATGAAGTAAA AGTTCCTTAGGATTTCCACTCTGACTATGGTCCAGGCACAGTG ACTGTACTCCTTGGCCTTCAGGTAATGCAGAATCCTCCCATAA TATCTTTTCAGGTGCAGACTGCTCATGAGTTTTCCCCTGGTGAA ATCTTCTTTCTCCAGTTTTTCTTCCAGGACTGTCTTCAGATGGT TTATCTGATGATAGACATTAGCCAGGAGGTTCTCAACAATAGT CTCATTCCAGCCAGTGCTAGATGAATCTTGTCTGAAAATAGCA AAGATGTTCTGGAGCATCTCATAGATGGTCAATGCGGCGTCCT CCTTCTGGAACTGCTGCAGCTGCTTAATCTCCTCAGGGATGTC AAAGTTCATCCTGTCCTTGAGGCAATATCCAAGCCTCCCATTC AATTGCCACAGGAGCTTCTGACACTGAAAATGGCTGCTTCTTT GTAGGAATCCAAGCAAGTTGTAGCTCATGGAAAGAGCTGTAG TGGAGAAGCACAACAGGAGAGCAATAAGGAGGAGACACTTGT TGGTCATGGTGGATCCATCCATCACACTGGCGGCCGCTCGAGC ATGCATCTAGAGGGGCCCATTTCGCCCTATTCGAAGTCGTATT ACGAGTCACTGG
hIFNb21 M13For	GGGTGGATCGGTATTCTCAATAGGGCGAATATGGGCCCTCTAG ATGCATGCTCGAGCGGCCGCCAGTGTGATGGATGGATCCACC ATGACCAACAAGTGTCTCCTCCAAATTGCTCTCCTGTTGTGCTT CTCCACTACAGCTCTTTCCATGAGCTACAACTTGCTTGGATTCC TACAAAGAAGCAGCAATTTTCAGTGTCAGAAGCTCCTGTGGCA ATTGAATGGGAGGCTTGAATATTGCCTCAAGGACAGGATGAA CTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAG AAGGAGGACGCCGCATTGACCATCTATGAGATGCTCCAGAAC ATCTTTGCTATTTTCAGACAAGATTCATCTAGCACTGGCTGGA ATGAGACTATTGTTGAGAACCTCCTGGCTAATGTCTATCATCA GATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAA AGAAGATTTCACCAGGGGAAAACTCATGAGCAGTCTGCACCT GAAAAGATATTATGGGAGGATTCTGCATTACCTGAAGGCCAA GGAGTACAGTCACTGTGCCTGGACCATAGTCAGAGTGGAAAT CCTAAGGAACTTTAACTTCATTAACAGACTTACAGGTTACCTC CGAAACTGAGGATCCATCTGCAGAATCCAGCACACTGGCGGG CGTTACTAGTTGGATCCGAGCTCGGNACCCAAGCCTGAATGCA TAGCCTGGAGTATTCTATAGGTG

hIFNb24 M13Rev	GTGGGGCGCAAGTCATTTAGGTGACACTATAGAATACTCAAG CTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACG GCCGCCAGTGTGCTGGAATTCTGCAGATGGATCCTCAGTTTCG GAGGTAACCTGTAAGTCTGTTAATGAAGTAAAAGTTCCTTAGG ATTTCCACTCTGACTATGGTCCAGGCACAGTGACTGTACTCCT TGGCCTTCAGGTAATGCAGAATCCTCCCATAATATCTTTTCAG GTGCAGACTGCTCATGAGTTTTCCCCTGGTGAAATCTTCTTTCT CCAGTTTTTCTTCCAGGACTGTCTTCAGATGGTTTATCTGATGA TAGACATTAGCCAGGAGGTTCTCAACAATAGTCTCATTCCAGC CAGTGCTAGATGAATCTTGTCTGAAAATAGCAAAGATGTTCTG GAGCATCTCATAGATGGTCAATGCGGCGTCCTCCTTCTGGAAC TGCTGCAGCTGCTTAATCTCCTCAGGGATGTCAAAGTTCATCC TGTCCTTGAGGCAATATTCAAGCCTCCCATTCAATTGCCACAG GAGCTTCTGACACTGAAAATTGCTGCTTCTTTGTAGGAATCCA AGCAAGTTGTAGCTCATGGAAAGAGCTGTAGTGGAGAAGCAC AACAGGAGAGCAATTTGGAGGAGACACTTGTTGGTCATGGGT GGATCCATCCATCAC
hIFNb24 M13For	TTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATG GATGGATCCACCATGACCAACAAGTGTCTCCTCCAAATTGCTC TCCTGTTGTGCTTCTCCACTACAGCTCTTTCCATGAGCTACAAC TTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGTCAGA AGCTCCTGTGGCAATTGAATGGGAGGCTTGAATATTGCCTCAA GGACAGGATGAACTTTGACATCCCTGAGGAGATTAAGCAGCT GCAGCAGTTCCAGAAGGAGGACGCCGCATTGACCATCTATGA GATGCTCCAGAACATCTTTGCTATTTTCAGACAAGATTCATCT AGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTA ATGTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGA AAAACTGGAGAAAGAAGATTTCACCAGGGGAAAACTCATGAG CAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGCATTAC CTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACCATAGTC AGAGTGGAAATCCTAAGGAACTTTTACTTCATTAACAGACTTA CAGGTTACCTCCGAAACTGAGGATCATCTGCAGAATCCAGCAC ACTGGCGGCCGTTACTAGTGGATCNAGCTCGGTACCAGCCTGA TGCATAGCCTGAGTATTCTATAGTGTCACCTAATAGCTGGGCG TAATATTGNCATAGCTGTNTCGGG

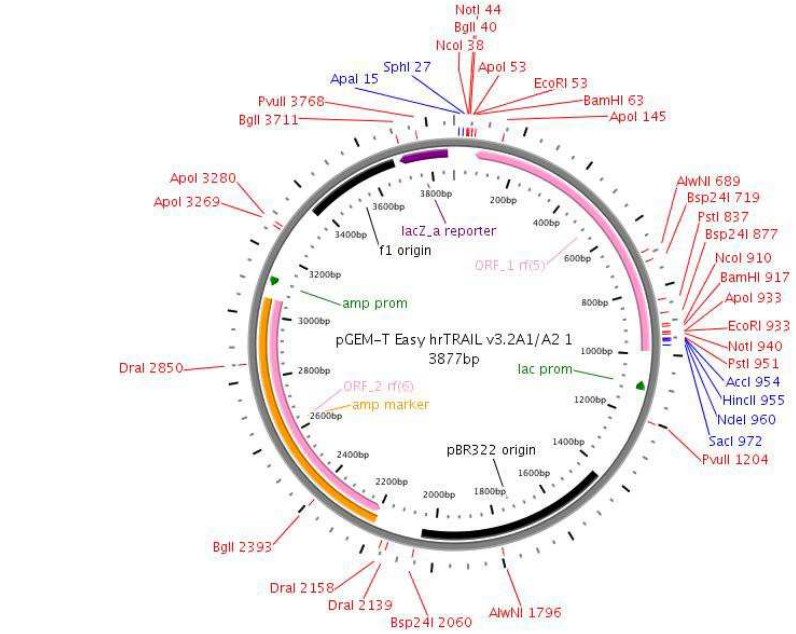
hDecorin 1 SP6	GTCATTGTTGTGGTGGCATGAAAACTACAGAATACCTATTAAT TACCAGGCCGCCGGATGTGCTGGAATTCTGCAGATGGATCCAC CATGAAGGCCACTATCATCCTCCTTCTGCTTGCACAAGTTTCCT GGGCTGGACCGTTTCAACAGAGAGGCTTATTTGACTTTATGCT AGAAGATGAGGCTTCTGGGATAGGCCCAGAAGTTCCTGATGA CCGCGACTTCGAGCCCTCCCTAGGCCCAGTGTGCCCCTTCCGC TGTCAATGCCATCTTCGAGTGGTCCAGTGTTCTGATTTGGGTCT GGACAAAGTGCCAAAGGATCTTCCCCCTGACACAACTCTGCTA GACCTGCAAAACAACAAAATAACCGAAATCAAAGATGGAGAC TTTAAGAACCTGAAGAACCTTCACGCATTGATTCTTGTCAACA ATAAAATTAGCAAAGTTAGTCCTGGAGCATTTACACCTTTGGT GAAGTTGGAACGACTTTATCTGTCCAAGAATCAGCTGAAGGA ATTGCCAGAAAAAATGCCCAAAACTCTTCAGGAGCTGCGTGC CCATGAGAATGAGATCACCAAAGTGCGAAAAGTTACTTTCCAT GGACTGAAACAGATGATTGTCATAGAACTGGGCACCCATTCG CTGAAGAGCTCAGGAATTGAAATGGGGCCTTCCAGGGAATGA GCAAGCTCTTCTACATTCGCATTGCTGATTCCATTATCACCAGC ATGCCC
hDecorin 1 M13For	GTGGATCTTTATTCTCAATAGGGGCGAATTGGGCCCTCTAGAT GCATGCTCGAGCGGCCGCCAGTGTGATGGATGATCCTTACTTA TAGTTTCCGAGTTGAATGGCAGAGCGCACGTAGACACATCTGA AGGTGGATGGCTGTATCTCCCAGTACTGGACCGGGTTGCTGAA AAGACTCACACCCGAATAAGAAGCCTTTTTGGTGTTGTGTCCA GGTGGGCAGAAGTCACTTGATCCAACTACAGAGATATTGTTGT TATGAAGGTAGACAACCTGGATGTACTTATGCTCTGCCAGCCC ACCAGGTACTCTGGTAAGCTTGTTGTTGTCCAAGTGAAGCTCC CTCAGATGAGGCGTGTTGGCCAGAGAGCCATTGTCAACAGCA GAGATGCTGTTGAAACTCAATCCCAACTTAGCCAAATTATTCA GTCCTTTCAGGCTAGCTGCATCAACTCTGCTGATTTTGTTGCCA TCAAGATGTAATTCCGTAAGGGAAGGAGGAAGACCTTGAGGA ATGCTGGTGATATTGGTATCAGCAATGCGGATGTAGGAGAGCT TCTTCATTCCCTGGAAAGCCCCATTTTCAATTCCTGAGCTCTTC AGCGGATTGGTGCCCAGTTCTATGACAATCATCTGGTTCAGTC CATTGAAAGTAACTTTTCGCACCTTGGGTGATCTCATTCTCATT GGGCACGCAGCTCCTGAAGAGTTTTTGGGCATTTTTTCTTGGC AATCCCTCAGCTGATTCTTGGACCGATTANGTCGTCCCACCTC

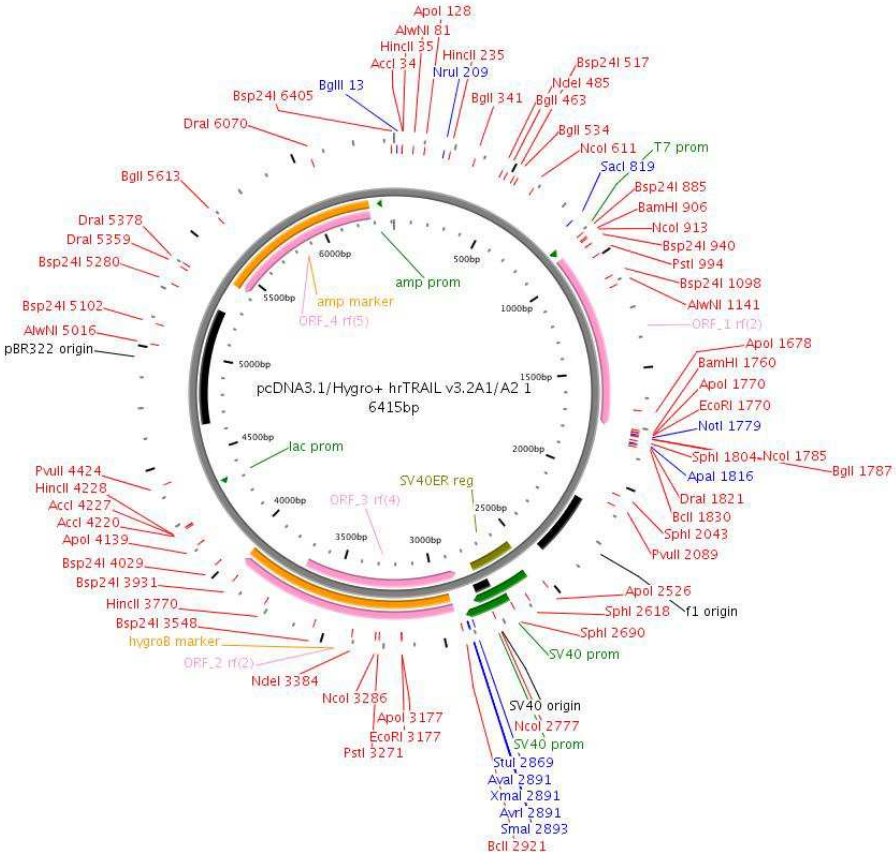
hDecorin 2 M13Rev	GCCAATTCTATTTAGGTGACACTATAGAATACTCAAGCTATGC ATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCC AGTGTGCTGGAATTCTGCAGATGGATCCACCATGAAGGCCACT ATCATCCTCCTTCTGCTTGCACAAGTTTCCTGGGCTGGACCGTT TCAACAGAGAGGCTTATTTGACTTTATGCTAGAAGATGAGGCT TCTGGGATAGGCCCAGAAGTTCCTGATGACCGCGACTTCGAGC CCTCCCTAGGCCCAGTGTGCCCCTTCCGCTGTCAATGCCATCTT CGAGTGGTCCAGTGTTCTGATTTGGGTCTGGACAAAGTGCCAA AGGATCTTCCCCCTGACACAACTCTGCTAGACCTGCAAAACAA CAAAATAACCGAAATCAAAGATGGAGACTTTAAGAACCTGAA GAACCTTCACGCATTGATTCTTGTCAACAATAAAATTAGCAAA GTTAGTCCTGGAGCATTTACACCTTTGGTGAAGTTGGAACGAC TTTATCTGTCCAAGAATCAGCTGAAGGAATTGCCAGAAAAAAT GCCCAAAACTCTTCAGGAGCTGCGTGCCCATGAGAATGAGAT CACCAAAGTGCGAAAAGTTACTTTCAATGGACTGAACCAGAT GATTGTCATAGAACTGGGCACCANTCCGCTGAAGAGCTCAGG AATTGAAATTGGGGCCTTCCAGGGGAATGAAGAAGCTCCTCCT ACATTTCGCATTTGCCTGA
hDecorin 2 M13For	ATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGAT GGATGGATCCTTACTTATAGTTTCCGAGTTGAATGGCAGAGCG CACGTAGACACATCTGAAGGTGGATGGCTGTATCTCCCAGTAC TGGACCGGGTTGCTGAAAAGACTCACACCCGAATAAGAAGCC TTTTTGGTGTTGTGTCCAGGTGGGCAGAAGTCACTTGATCCAA CTACAGAGATATTGTTGTTATGAAGGTAGACAACCTGGATGTA CTTATGCTCTGCCAGCCCACCAGGTACTCTGGTAAGCTTGTTG TTGTCCAAGTGAAGCTCCCTCAGATGAGGCGTGTTGGCCAGAG AGCCATTGTCAACAGCAGAGATGCTGTTGAAACTCAATCCCAA CTTAGCCAAATTATTCAGTCCTTTCAGGCTAGCTGCATCAACT CTGCTGATTTTGTTGCCATCAAGATGTAATTCCGTAAGGGAAG GAGGAAGACCTTGAGGAATGCTGGTGATATTGGTATCAGCAA TGCGGATGTAGGAGAGCTTCTTCATTCCCTGGAAAGCCCCATT TTCAATTCCTGAGCTCTTCAGCGGATGGGTGCCCAGTTCTATG ACAATCATCTGGTTCAGTCCATTGAAAGTAACTTTTCGCACCT TGGGTGATCTCATTCTCATTGGGCACGCAGCCTCCTGAAGAGT TTTTGGGCATTTTTTTCTTGGCCANTTCCNTTCAGCCTGATTTC TTGG

hTRAIL 3.2 A1-A2 combi 1 For	TAATTCAGCCACTATAGGGCGAATTGGGCCCGACGTCGCATGC TCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGGATCCTAG CCAACTAAAAAGGCCCCGAAAAAACTGGCTTCATGGTCCATG TCTATCAAGTGCTCATTTGTTACAGAAACAAAAATTCTGTCAT TTTCCTTAAGCTCAAATATTCCCCCTTGATAGATGGAATAGAG TCCATATTCTGCATCTTTAGACCAACAACTATTTCTAGCACTTT TCATCAACAATATAGGGTCAGGATAACTTGTGTATTTGTAAAT ATATTGGACCATTTGTTTGTCGTTCTTTGTGTTTTCTTTTATTTC CTCCTGAAATCGAAAGTATGTTTGGGAATAGATGTAGTAAAAC CCTTTTTCATGGATGACCAGTTCACCATTCCTCAAGTGCAAGTT GCTCAGGAATGAATGCCCACTCCTTGATGATTCCCAGGAGTTT ATTTTGCGGCCCAGAGCCTTTTCATTCTTGGAGTTTGGAGAAG ACAATGTGTTGCTTCTTCCTCTGGTCCCAGTTATGTGAGCTGCT ACTCTCTGAGGACCTCTTTCTCTCACTAGGGGAGAAATATTTT GTTGCTTTTCTTGAACTGTAGAAATGGTTTCCTCAGAGGTTCTC AAAATCATCTTTCTAACGAGCTGACGGAGTTGCNCTTGACTTG CCAGCAGGGGCTGTCATACTCTCTTCGTCATTGGGGTCCCATT T
hTRAIL 3.2 A1-A2 combi 1 rev	TATTTAGGTGACACTATAGAATACTCAAGCTATGCATCCAACG CGTGGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCG AATTCACTAGTGATTGGATCCACCATGGCTATGATGGAGGTCC AGGGGGGACCCAGCCTGGGACAGACCTGCGTGCTGATCGTGA TCTTCACAGTGCTCCTGCAGTCTCTCTGTGTGGCTGTAACTTAC GTGTACTTTACCAACGAGCTGAAGCAGATGCAGGACAAGTAC TCCAAAAGTGGCATTGCTTGTTTCTTAAAAGAAGATGACAGTT ATTGGGACCCCAATGACGAAGAGAGTATGAACAGCCCCTGCT GGCAAGTCAAGTGGCAACTCCGTCAGCTCGTTAGAAAGATGA TTTTGAGAACCTCTGAGGAAACCATTTCTACAGTTCAAGAAAA GCAACAAAATATTTCTCCCCTAGTGAGAGAAAGAGGTCCTCA GAGAGTAGCAGCTCACATAACTGGGACCAGAGGAAGAAGCAA CACATTGTCTTCTCCAAACTCCCAGAATGAAAAGGCTCTGGGC CGCAAAATAAACTCCTGGGAATCATCAAGGAGTGGGCATTCA TTCCTGAGCCACTTGCACTTGAGGAATGGTGAACTGGTCATCC ATGAAAAAGGGTTTACTACATCTATTCCCAACATACTTCCGAT NCAGGAGGAAATAAAGAAACCCCAAGAACGACAAACAAATG GTCCATATATTTACAATTACCCAGGG

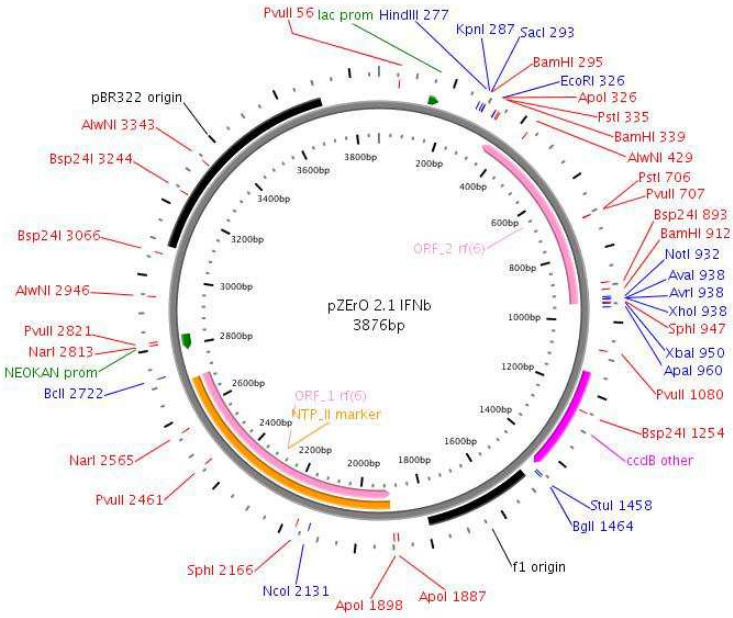
hTRAIL 3.2 A1-A2 combi 4 For	CTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCC ATGGCGGCCGCGGGAATTCGATTGGATCCTAGCCAACTAAAA AGGCCCCGAAAAAACTGGCTTCATGGTCCATGTCTATCAAGTG CTCATTTGTTACAGAAACAAAAATTCTGTCATTTTCCTTAAGCT CAAATATTCCCCCTTGATAGATGGAATAGAGTCCATATTCTGC ATCTTTAGACCAACAACTATTTCTAGCACTTTTCATCAACAAT ATAGGGTCAGGATAACTTGTGTATTTGTAAATATATTGGACCA TTTGTTTGTCGTTCTTTGTGTTTTCTTTTATTTCCTCCTGAAATC GAAAGTATGTTTGGGAATAGATGTAGTAAAACCCTTTTTCATG GATGACCAGTTCACCATTCCTCAAGTGCAAGTTGCTCAGGAAT GAATGCCCACTCCTTGATGATTCCCAGGAGTTTATATTGCGGC CCAGAGCCTTTTCATTCTTGGAGTTTGGAGAAGACAATGTGTT GCTTCTTCCTCTGGTCCCAGTTATGTGAGCTGCTACTCTCTGAG GACCTCTTTCTCTCACTAGGGGAGAAATATTTTGTTGCTTTTCT AGAACTGTAGAAATGGTTTCCTCAGAGGTTCTCAAAATCATCT ATCTAACGAGCTGACGGAGTTGCCACTAGACTTGCCAGCAGG GGCTGTTCATACTCTCATTCGTCATNGGGGGTCCCAATAACTG TCATCTACATATTAAGAAACAAGCCAATGCCACATTTG
hTRAIL 3.2 A1-A2 combi 4 rev	TATTGTAGGTTGACACTATAGGCATACTCAAGCTATGCATGCA ACGCGTGGGGAGCCTCTCCCATATGGGTCGACCTGCAGGCGG CCGCGAATGTCACTAGTGATTGGATCCACCATGGCTATGATGG AGGTCCAGGGGGGACCCAGCCTGGGACAGACCTGCGTGCTGA TCGTGATCTTCACAGTGCTCCTGCAGTCTCTCTGTGTGGCTGTA ACTTACGTGTACTTTACCAACGAGCTGAAGCAGATGCAGGAC AAGTACTCCAAAAGTGGCATTGCTTGTTTCTTAAAAGAAGATG ACAGTTATTGGGACCCCAATGACGAAGAGAGTATGAACAGCC CCTGCTGGCAAGTCAAGTGGCAACTCCGTCAGCTCGTTAGAAA GATGATTTTGAGAACCTCTGAGGAAACCATTTCTACAGTTCAA GAAAAGCAACAAAATATTTCTCCCCTAGTGAGAGAAAGAGGT CCTCAGAGAGTAGCAGCTCACATAACTGGGACCAGAGGAAGA AGCAACACATAGTCTTCTCCAAACTCCAAGAATGAAAAGGCT CTGGGCCGCAAAATAAACTCCTGGGAATCATCAAGGAGTGGG CATTCATACCTGAGCAACTTGCACTTGAGGAATGGTGAACTGG TCATCCATGAAAAAAGGGTTTTACTACATCTATTCCCCAAACA TACTTTTCGAATTTCAGGGAAGGAAATTAAAAAGAAAAACCC CCAANGAACCC

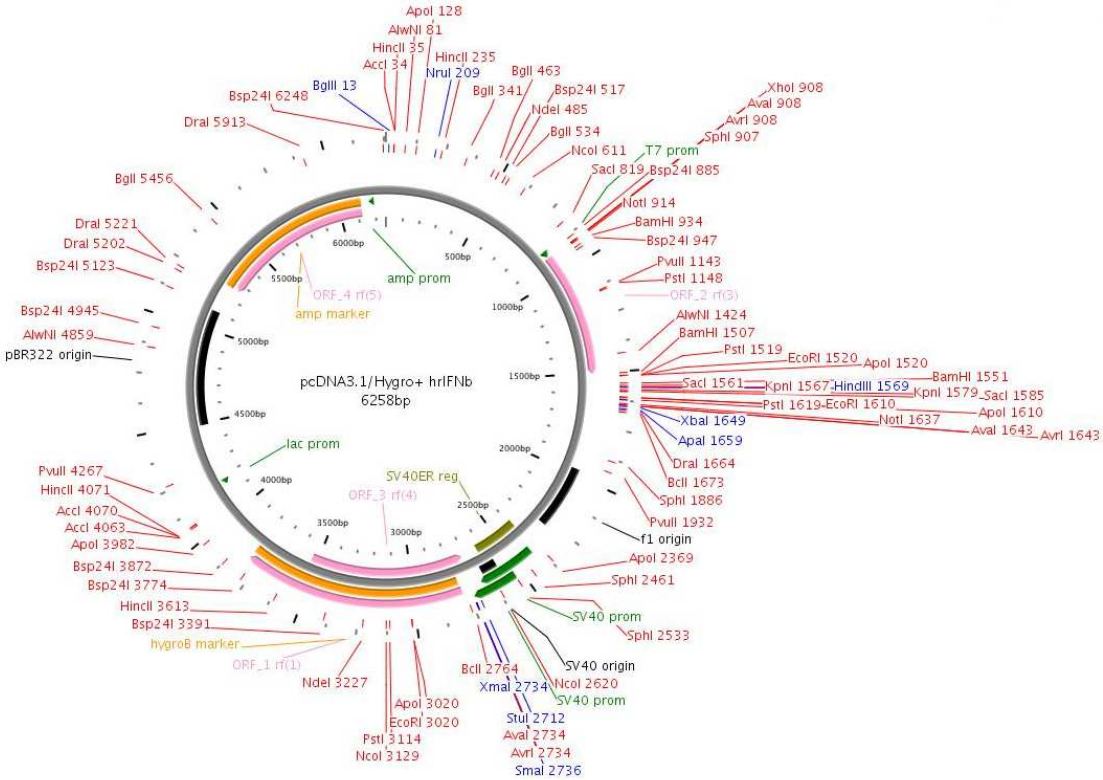
Appendix B – hrTRAIL constructs





Appendix C- hrIFNb Constructs





Appendix D- hrDecorin Constructs