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# The Transcriptional Regulation of HLA-E by Interferon-Gamma in Tumor Cells

Quintesia Grant
*Virginia Commonwealth University*

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# TRANSCRIPTIONAL REGULATION OF HLA-E BY INTERFERON-GAMMA IN TUMOR CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University


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# DEDICATION

To Sheng Zu Zhu and Claudia Carroll for always believing.

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| | |
|---|---|
| α | Alpha |
| ab | Antibody |
| β | Beta |
| β-Gal | Beta-Galactosidase |
| $^{14}C$ | Carbon 14 |
| CAT | Chloramphenicol acetyltransferase |
| CBP | CREB binding protein |
| CEBP | CCAAT-Enhancer Binding Protein |
| CMV | Cytomegalovirus |
| cpm | Counts per minute |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Deoxyribonucleic Acid |
| DTT | Dithioerythritol |
| ECL | Enhanced Chemiluminescence |
| FBS | Fetal Bovine Serum |
| FOG | Friend of GATA |
| γ | Gamma |
| GAS | Gamma Activation Sequence |
| HCl | Hydrogen Chloride |

| | |
|---|---|
| HLA | Human Leukocyte Antigen |
| HRP | Horseradish peroxidase |
| HMG | High Mobility Group |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IRF | Interferon Regulatory Factor |
| IRR | Interferon Response Region |
| IRR-AC | Interferon Response Region-Activation Complex |
| ISGF | Interferon Stimulated Gene Factor |
| ISRE | Interferon Stimulated Response Region |
| ITIM | Immune receptor Tyrosine based Inhibitory Motif |
| JAK | Janus Kinase |
| kDa | Kilodalton |
| KIR | Killer Inhibitory Receptor |
| kg | Kilogram |
| M | Molar |
| MHC | Major Histocompatibility Complex |
| µM | Micromolar |
| mM | Millimolar |
| mg | Milligram |
| ul | Microliter |
| µg/ml | Microgram per Milliliter |
| ml | Milliliter |

| | |
|---|---|
| mRNA | Messenger Ribonucleic Acid |
| $Na_2CO_3$ | Sodium Carbonate |
| NaCl | Sodium Chloride |
| NK cell | Natural Killer Cell |
| ONPG | o-Nitrophenyl-β-D galactopyranoside |
| pM | Picomolar |
| PMSF | Phenylmethylsulfonyl Fluoride |
| PVDF | Polyvinylidene Fluoride |
| RNA | Ribonucleic Acid |
| rpm | Revolutions per Minute |
| RPMI | Roswell Park Memorial Institute |
| SDS-PAGE | Sodium dodecylsulfate-polyacrylamide gel electrophoresis |
| STAT | Signal Transducer of Transcription |
| TCR | T-Cell Receptor |
| UIRR | Upstream Interferon Response Region |
| v/v | Volume/Volume |
| w/v | Weight/Volume |

# ABSTRACT

Transcriptional Regulation of HLA-E by Interferon Gamma in Tumor Cells

A dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Virginia Commonwealth University, 2010

Advisor: Gordon Ginder, M.D., Professor, Departments of Internal Medicine and Microbiology/Immunology

The human Class Ib gene, HLA-E inhibits both Natural Killer Cells and a subset of CD8+ cytotoxic T lymphocytes by engaging the CD94/NKG2A inhibitory receptor. IFN-γ induces the expression of HLA-E as well as Class Ia molecules, which are required for the killing of target cells. Since HLA-E has negative effects on immune killing of target cells, we have sought to identify locus specific mechanisms of IFN-γ induction in order to identify molecular targets for selective activation of Class Ia genes, but not HLA-E. We have previously identified a unique upstream IFN-γ response region in the HLA-E promoter and showed that GATA-1 is required for its function in the K562 leukemic cell line. We have now examined the effect of GATA family members on IFN-γ induction of HLA-E in other cell types. HLA-E CAT reporter gene assays

demonstrate that tumor cells that express GATA factors as determined by western blot and quantitative PCR, mediate a 2.4 to 4.0 fold enhanced response to IFN-γ stimulation. Functional constructs containing mutations of the core nucleotides in the GATA binding site had a 4.8 fold decreased response to IFN-γ in A2780 cells and a 8.5 to 14.0 fold decreased response to IFN-γ in SKOV3 cells. Knockdown of GATA-6 using siRNA resulted in a 40% decrease in HLA-E induction in Seg1 cells and a 30% decrease in HLA-E induction in HCT116 cells. Tetracycline regulated shRNA knockdown of GATA-6 expression in the SKOV3 cell line revealed a 3 fold decrease in the IFN-γ response of HLA-E reporter driven constructs. Additionally we observed a decreased IFN-γ response in SKOV3 cells transfected with siRNA specific for CBP and IRF-9. We conclude that GATA factors play a tissue specific role in regulation of IFN-γ mediated HLA-E expression and that IRF-9 may be a target for the differential manipulation of classical MHC and HLA-E.

# Chapter 1: Introduction

## Major Histocompatibility Complex

The Major Histocompatibility Complex is a generic term given to a number of genes, located on chromosome six, that determine the fate of grafted tissues. The highly polymorphic nature of the classical MHC loci ensures diversity in MHC gene expression of the population as a whole (Parham, Lawlor, Lomen, & Ennis, 1989). Most of the genes in the MHC family are involved in immunity, including the MHC class I loci, which can be divided into classical and nonclassical subdivisions(Gobin, Keijsers, van Zutphen, & van den Elsen, 1998; Howcroft & Singer, 2003; van den Elsen, Gobin, van Eggermond, & Peijnenburg, 1998). The function of Class I molecules is to present foreign peptide antigens to T cells. Both the classical and nonclassical MHC genes encode integral membrane proteins called class I heavy chains, which are comprised of three globular domains (α1, α2, and α3), a transmembrane region, and a cytoplasmic domain (Parham et al., 1989; Williams, 2001). The α1 and α2 domains form the peptide binding cleft of MHC. This region interacts with peptides processed by the cytoplasmic proteasome complex that are eight to ten amino acids in length. Interestingly, the peptides that bind to a particular allelic form of MHC have been shown to exhibit common features, such as a hydrophobic residue at position two or a positively charged residue at position nine (Cunningham, 1977).

All MHC molecules must associate with beta-2 microglobulin in order to be expressed on the cell surface (Peterson, Rask, & Ostberg, 1977). The interaction between the two molecules occurs via the α3 domain as does the interaction between MHC and the CD8

molecule present on cytotoxic T lymphocytes. The members of the classical or Class Ia are HLA-A, HLA-B, and HLA-C in humans and H-2K, H-2D, and H-2L in mice. As stated previously, Class Ia genes are diverse and highly polymorphic. For example there are several hundred HLA-A, B, and C alleles (Williams, 2001). Furthermore, it has been observed that alleles differ in the ability to bind peptides. It is important to note that if a peptide cannot be bound by any of the Class Ia alleles, it will render that peptide non-antigenic as there will be no corresponding T-cell that can recognize it. Class Ia molecules are present on the surface of most nucleated cells.

In contrast, Class Ib MHC, whose members are HLA-E, HLA-F, and HLA-G in humans and Qa-1 in mice, show limited polymorphism. For example there are only six HLA-E alleles, nineteen HLA-F alleles, and fifteen HLA-G alleles that have been discovered to date (Ishitani, Sageshima, & Hatake, 2006; Kamishikiryo & Maenaka, 2009; Pyo et al., 2006). Since bound peptide is a requirement for expression of Class I molecules on the cells surface it is likely this low expression is due to a limited amount of peptides that can be recognized and presented by these molecules. Additionally, both HLA-F and HLA-G exhibit limited tissue distribution, while HLA-E is ubiquitously expressed (Gobin & van den Elsen, 2000). Interestingly, all three class Ib molecules have been identified at the maternal-fetal interface, suggesting that these molecules may have evolved as a method of immunotolerance (Copeman et al., 2000; Gobin & van den Elsen, 2000; Ishitani et al., 2006).

**HLA-E**

The Class Ib molecule, HLA-E functions as a sentinel molecule for Class Ia expression. It has been shown to bind to the nonameric leader peptides of Class I molecules. After synthesis, the Class I preprotein is cleaved by signal peptidase before being further processed by signal peptide peptidase and transported into the endoplasmic reticulum lumen via the TAP transporter (Borrego, Ulbrecht, Weiss, Coligan, & Brooks, 1998; V. M. Braud, Allan, Wilson, & McMichael, 1998). Here a newly formed HLA-E molecule, in conjunction with a beta-microglobulin molecule binds to the leader peptide. Furthermore, HLA-E has been shown to bind strongly to the HLA-A2 sequence, VMAPRTLV, with the key residues required for peptide binding being located at position 2 and position 9 of the leader peptide (V. Braud, Jones, & McMichael, 1997). Although the leader peptides of Class Ia molecules are most often presented by HLA-E molecules, it has recently been discovered that HLA-E can bind gene products from cytomegalovirus, Mycobacterium tuberculosis, and Epstein Barr virus (V. M. Braud, Tomasec, & Wilkinson, 2002; Heinzel et al., 2002; Pietra et al., 2003; Ulbrecht et al., 2000). Additionally, HLA-E can bind to mimic HLA Class I leader peptides produced by CMV, resulting in immune evasion (Llano, Guma, Ortega, Angulo, & Lopez-Botet, 2003; Ulbrecht et al., 2000).

HLA-E binds avidly to the C-lectin type receptor NKG2A/CD94, which is also found on certain types of cytotoxic T-cells (Lee et al., 1998) . Furthermore, a knockout mouse model of the murine homologue of HLA-E, Qa-1, has been shown to play a key role in the development of CD8+ T suppressor cells (Wang, Ramaswamy, Hu, & Cantor, 2001). Recent evidence also shows that in some instances HLA-E has the ability to stimulate cytotoxic activity via the interaction with the T-Cell Receptor on CD8+ T cells

(Romagnani et al., 2002). Although HLA-E has the ability to behave as a positive regulator of the immune system in specific circumstances, its primary action is to inhibit the cytotoxic activity of both natural killer cells and CTLs, thereby mediating an opposite effect on the immune system in comparison to Class Ia molecules.

**Interaction of MHC Class I and Natural Killer Cells**

MHC molecules interact with cells from both the innate and adaptive immunity. Class Ia molecules are critical for CD8+ Cytotoxic T cell development, as well as the recognition and killing of target cells. Additionally, Class Ia molecules have been shown to interact with Natural Killer Cells via their Killer Inhibitory Receptors (KIRs) which results in blockade of Natural Killer Cell activation and cytolytic function (Lanier, 1998; Moretta & Moretta, 2004). NK cells are an integral component of innate immunity as their activity not dependent on prior sensitization to antigen or clonal expansion. This inhibition by Class I molecules provides protection to normal cells from NK activity, while at the same time sensitizing the NK cells to compromised or decreased Class I expression. Recent studies show that the activity of NK cells may be contigent on prior interaction with self-MHC before the cells are "licensed to kill" (Yokoyama & Kim, 2006). Specifically, authors postulated that NK cells would have a reduced capacity to recognize and kill non-self virally infected or transformed tissues prior to being educated on what designated a tissue as "self."

Interaction between HLA-E and NK cells has also been described. In fact HLA-E is the principal ligand for the inhibitory CD94/NKG2A receptor (Lee et al., 1998; Vales-Gomez, Reyburn, Erskine, Lopez-Botet, & Strominger, 1999). HLA-E binds and presents the

leader peptide of other class I molecules such as HLA-A, B, C and G (Pietra, Romagnani, Moretta, & Mingari, 2009). As such its presence is indicative of global Class I expression. The inhibitory action of both CD94/NKG2A and Killer Inhibitory Receptors is mediated by immunotyrosine-based inhibitory motifs (ITIMS) located in the cytoplasmic tails of the molecules. It is also important to note that HLA-E has the ability to engage the activating receptor CD94/NKG2C (V. M. Braud et al., 1998; Vales-Gomez et al., 1999). However it seems that the molecule is preferably bound by NKG2A as it has a higher affinity for this receptor.

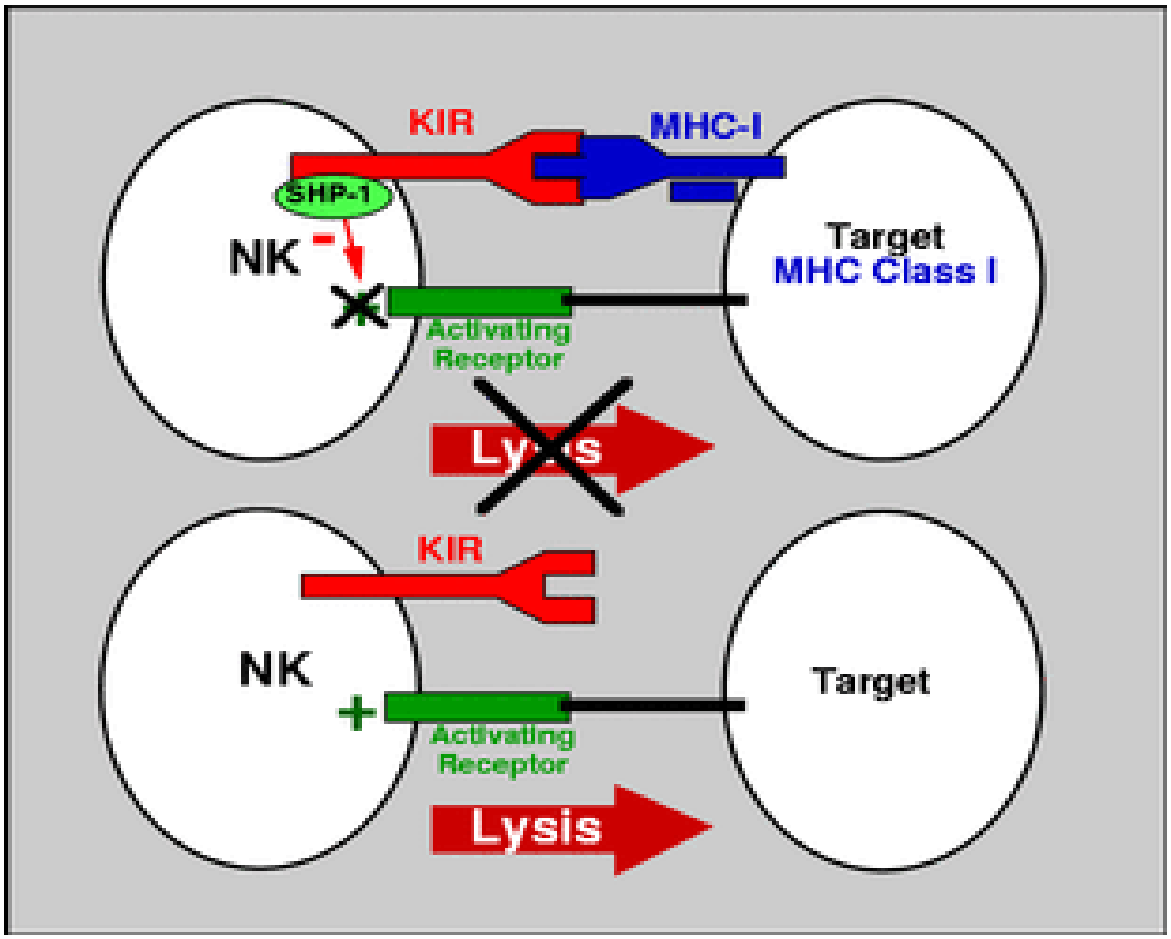



**Figure 1. Effect of MHC on NK cell function.** Interaction of MHC class I molecules with the KIR receptors results in the inhibition of NK cell lysis of target cells. Loss of MHC class promotes cytolysis. (arthritis-research.com/.../figures/ar1034-1.gif)

**Interferon Gamma**

Interferon gamma is a 48 kilodalton homodimeric glycoprotein that was first discovered by EF Wheelock in 1965 (Boehm, Klamp, Groot, & Howard, 1997). He observed that a substance in the supernatant of phytohemagglutinin stimulated lymphocytes was able to inhibit the cytopathic effects of the Sindbis virus (Wheelock, 1965). Later this molecule was labeled as the type II interferon with special

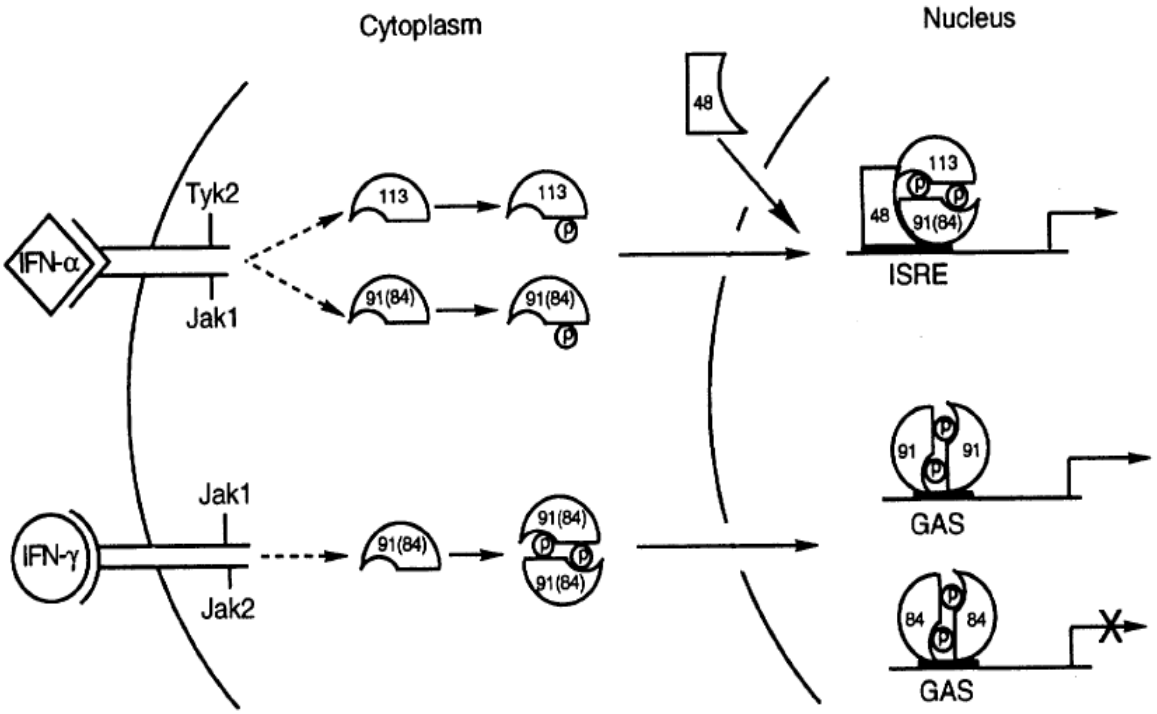



**Fig. 2.** Diagram of proteins identified in IFN-α– and IFN-γ–dependent signal transduction and gene activation. The Jak kinases are phosphorylated on tyrosine in response to ligand, but the sites and the requirement for such modification are not yet established. The circled P's on the STAT proteins are tyrosine phosphates and the indentations symbolize SH2 domains.

**Figure 2. Type I and Type II Interferon Signaling**. Adapted from Stark et al. Jak-STAT Pathways and Transcriptional Activation in Response to IFNs and Other Extracellular Signaling Proteins. Science. 1994; 264: 1415-1421.

emphasis being placed on its immune function. Interferon gamma is principally produced by cytotoxic T-lymphocytes, CD4+ T helper 1 cells, and Natural Killer cells (Darnell, Kerr, & Stark, 1994). Secretion of the molecule is stimulated by ligation of the T-cell receptor or in response to the cytokine interleukin 12 that is produced by macrophages. Interferon gamma has a number of functions including control of viral infection, increasing expression of MHC genes, enhancing the respiratory burst, apoptosis, and regulation of leukocyte adhesion to the endothelium (Boehm et al., 1997). Interferon gamma has also been implicated in the differentiation of naïve T helper cells into Th1 cells which are critical for cellular immunity.

The importance of IFN gamma is demonstrated by knockout studies of the cytokine and or its receptor in mice. Unchallenged mice show phenotypic changes specifically in lymphoid cells. Once challenged the mice show a particular deficiency in responding to intracellular pathogens, specifically Mycobacterium, Listeria monocytogenes, and Leishmania major (Lu et al., 1998). These mice are also more susceptible to infection with vaccinia virus (Xu, Johnson, Liggitt, & Bevan, 2004). When challenged with Mycobacterium, interferon gamma deficient mice are capable of developing granulomas but are unable to produce the reactive oxygen species which are necessary to clear the pathogen (Flynn et al., 1993). Additionally mice that are null for the interferon gamma receptor died within nine weeks after infection with the BCG strain of Mycobacterium bovis, whereas infection in wild type mice was not fatal (Erb,K.J. 1999). Interferon gamma null mice are also highly susceptible to Listeria monocytogenes (DiTirro et al., 1998; Lu et al., 1998). Further characterization of these mice showed that cells were unable to induce B-cell class switching to IgG2a, produced lower amounts of interferon

gamma in response to antigenic challenge and was unable to activate JAK-Stat signaling in response to interferon gamma. These studies demonstrate the importance of interferon gamma for enhancing the activity of macrophages.

Interferon gamma signaling is activated when the cytokine binds to its heterodimeric receptor IFNGR. This interaction results in a conformational change that culminates in the transphosporylation by the associated Janus kinases, JAK 1 and JAK 2 (Darnell et al., 1994). Specifically, JAK1 is associated with IFNGR1 and JAK2 is associated with IFNGR2. The phosporylated residues then serve as docking sites for STAT1 alpha molecules via the SH2 domain. The subsequent phosphorylation of the STAT 1 molecules on tyrosine 701 leads to dissociation from the receptor and homodimerization (Boehm et al., 1997). Specifically, the molecules associate via the SH2 domain of one STAT1 alpha molecule and the phosphorylated tyrosine residue of the other. The newly formed homodimer translocates into the nucleus and then binds to the gamma activation site of interferon stimulated target genes.

Stat 1 alpha is the principle molecule involved in interferon gamma signal transduction. This fact is illustrated by STAT1 knockout mice which exhibit similar deficiencies as interferon gamma or interferon-gamma receptor null mice (Levy & Darnell, 2002). STAT 1α is a 91 kD protein, which like the other members of the STAT family, consists of six conserved domains: n-terminal domain, coiled-coiled domain, DNA-binding domain, linker domain, Src-homology (SH2) domain, and transcriptional activation domain (Brierley & Fish, 2005). The amino terminal domain is critical for stabilizing homodimer or heterodimer formation. The coiled-coiled domain is critical for protein interactions, such as that of STAT 1α and STAT2 with interferon regulatory factor nine (IRF-9). The

Src-homology domain (SH2) is important for dimerization and receptor binding. The transcriptional activation domain, mediates interactions with nuclear coactivators and histone acetyltransferases (Brierley & Fish, 2005).

Additionally, the transcriptional activation domain contains two residues that are critical for STAT function. There is a conserved tyrosine residue that is absolutely required for STAT activation as its phosphorylation permits interaction with the SH2 domain of other STAT molecules as well as a conserved serine residue whose phosphorylation is necessary for full transcriptional activity (Levy & Darnell, 2002; Shuai, Stark, Kerr, & Darnell, 1993). In fact loss of serine phosphorylation as a result of pharmacologic inhibition of Protein Kinase C-delta, causes a decrease in the association between STAT molecules and p300. STAT1 is able to interact with p300 via the transcriptional activation domain and the n-terminal domain (J. J. Zhang et al., 1996). Interestingly, the ability of STAT1 alpha to transactivate genes is inhibited after incubation with the adenovirus E1a protein and after pharmacologic inhibition of histone deacetylase activity. Furthermore, Stat 1β is a 84 kD protein that is derived from the same gene as Stat 1α. Differential splicing results in a shorter c-terminus than the Stat1a molecule and as a result, Stat1b has the ability to bind DNA but is unable to transactivate genes (Brierley & Fish, 2005).

STAT1 homodimers bind the Gamma Activation Site (GAS), a nine base pair palindromic sequence (TTNCNNAA) first described in regards to the transcriptional activation of the guanylate binding protein (GBP) gene (Decker, Lew, Mirkovitch, & Darnell, 1991). The interaction of STAT molecules with its consensus region has been shown to be increased by the cooperative binding of adjacent homodimers. In fact, two

STAT1 homodimers bind to the Interferon Response Region (IRR) of the HLA-E promoter (Gustafason K 1996). Mutagenesis studies demonstrate that lysine 366, aspartate 421, and arginine 460 are crucial for STAT1 binding in response to Interferon-gamma (Yang, Henriksen, Schaefer, Zakharova, & Darnell, 2002).

In addition to the Gamma Activation Site, STAT1 has also been shown to interact with the Interferon Stimulated Response Element (ISRE). The consensus sequence AGTTTCNNTTTCC/A is bound by Interferon Stimulated Gene factor 3 (ISGF-3), which consists of STAT1, STAT2, and Interferon Regulatory Factor 9 (Bluyssen et al., 1995; Waring, Radford, Burns, & Ginder, 1995). Studies demonstrate that IRF-9 recognizes and binds to the ISRE while STAT1 interacts with flanking sequences (Wesoly, Szweykowska-Kulinska, & Bluyssen, 2007). It is important to note that ISGF3 formation is a consequence of type I interferon signaling. In the case of most HLA Class Ia genes, IFN-γ induces the Interferon Response Factor-1 (IRF-1) transcription through its gamma activation sequence (GAS) sequence, thereby allowing the IRF-1 protein to stimulate HLA-B or HLA-C transcription through binding to the Interferon Stimulated Response Element (ISRE). Additionally, some studies show that a STAT1 homodimer in conjunction with IRF-9 is capable in binding to the ISRE in response to IFN-γ.

**Interferon Response Region and Upstream Interferon Response Region of the HLA-E promoter**

The promoter of HLA-E differs significantly from that of Class Ia genes. Class Ia molecules have increased expression as a result of both Interferon-alpha, beta as well as interferon-gamma via the interferon stimulated response element. HLA-E

transcription is also induced by interferon gamma, although the HLA-E promoter does not contain a functional ISRE. Kuluski and colleagues reported the insertion of Alu elements and Charlie fragments within the enhancer and proximal promoter regions of HLA-E and postulated that these insertions contributed to the deletion of the enhancer A region and the ISRE that is present in Class Ia promoters (Kulski et al., 1998; Kulski, Shigenari, Shiina, & Inoko, 2010). Indeed it has been shown via electrophoretic mobility shift assays (EMSA) and reporter gene assays that the putative enhancer A and ISRE regions of HLA-E do not bind IRF-1 or NFkB and that these factors are incapable of transactivating the HLA-E gene.

Furthermore it has been demonstrated that the response to interferon gamma is mediated by a unique response element in the HLA-E promoter, termed the Interferon Response Region (IRR). The region extends from -193 to -146 and is composed of an extended, imperfect repeat consisting of two distinct half sites. The 5' half is homologous to a gamma activation site (GAS), while the 3' half is similar to the ISRE. Gel mobility shift assays using probes containing a duplication of the 5' half or the 3' half of the IRR bound IFN-γ induced complexes that exhibited the same mobility as the IFN-γ activation complex that bound the intact IRR. The observation of a symmetric methylation interference pattern in conjunction with the binding of STAT1 alpha to the IRR on EMSA led to the conclusion that a STAT1 alpha homodimer bound the IRR and helped to mediate the transcriptional response to Interferon gamma (Gustafason, K 1996).

Further experiments suggested that additional factors could be a part of the activating protein complex that binds the interferon response region in addition to STAT 1α.

Treatment of U937 nuclear extracts with the minor groove binding drugs Netropsin and Dystamicin A inhibited the formation of IRR-AC and IC substitution of the AT rich portion of the IRR further demonstrated that minor groove contacts were required for full induction of HLA-E by IFN- $\gamma$. Specifically, a minor groove binding protein, HMGA1, has been implicated as part of the complex. Purification of the protein complex led to the verification of the STAT1 alpha molecule in the IRR-AC and suggested that the polypyrimidine tract binding protein, PSF, may be a part of the complex (unpublished results).

Characterization of HLA-E induction in K562 leukemia cells led to the discovery of the Upstream Interferon Response Region (UIRR). This element is located 38 base pairs immediately upstream of the IRR and is dependent on the IRR for its function. Analysis of this region of the promoter with MATInspector software demonstrated a nonconsensus GATA transcription factor binding sequence. Subsequently, it was shown that when the UIRR is bound by GATA-1 in K562 cells, a five-fold enhanced transcriptional response to IFN-γ can be achieved. (Barrett,DM 2004). However it is important to note that this enhanced response was not observed in the U937 cell line. Therefore other cell lines were examined for their ability to promote UIRR function. We observed that those cell lines that expressed a GATA transcription family member also demonstrated an enhanced transcriptional response to IFN-γ via the UIRR. These observations led to the hypothesis that there is a cell-type restricted upstream enhancer element present in the HLA-E promoter that can be bound by various GATA transcription family members.

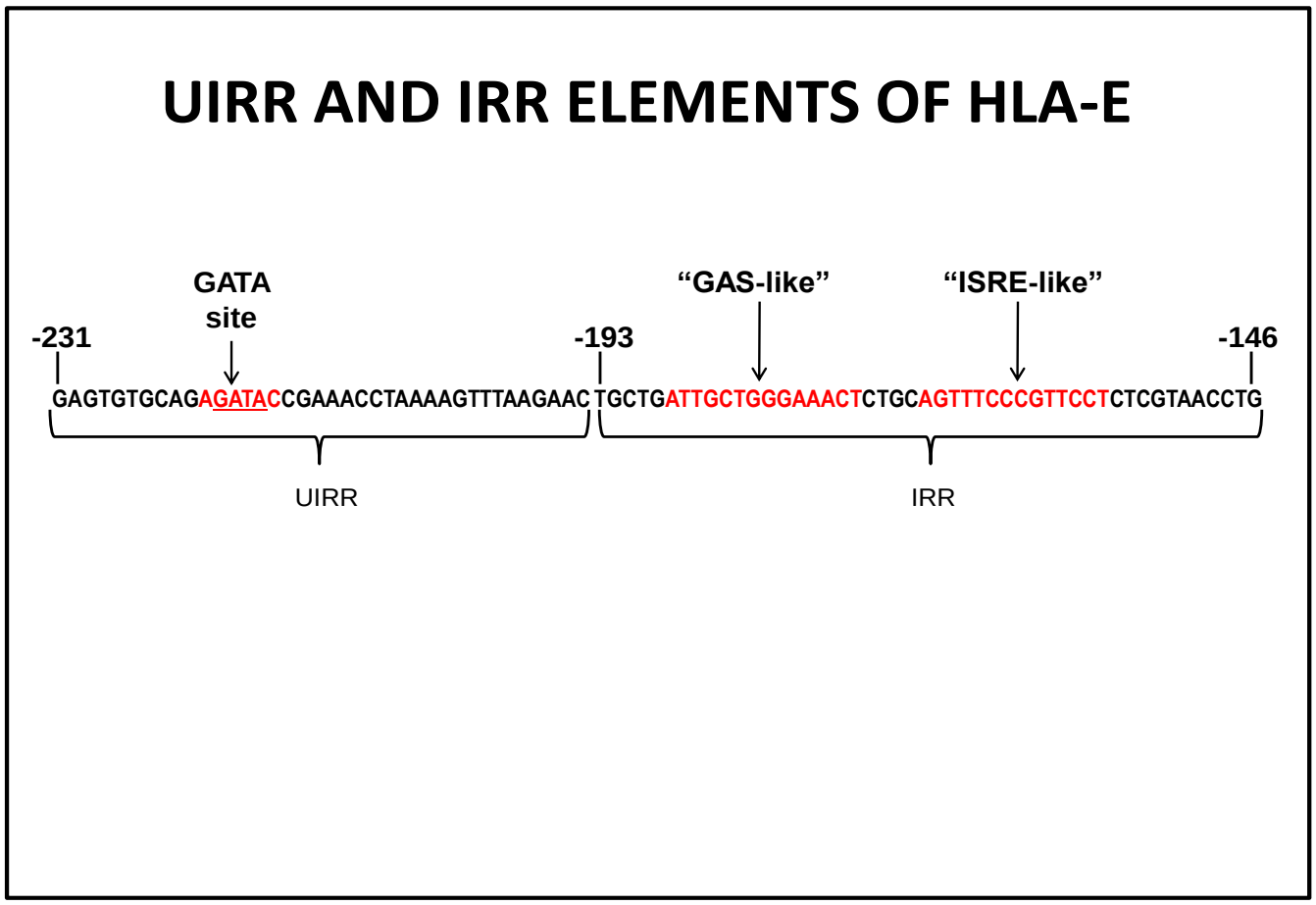



**Figure 3. Diagram of UIRR and IRR elements in the HLA-E promoter.** Highlighted region in the UIRR denotes the GATA binding element. Highlighted portions of the IRR region denotes the 5' and 3' half sites which are similar to the Gamma Activation Site and Interferon Stimulated Response Element.

**GATA Transcription Factors**

The development of differentiated cell types from multipotent progenitor cells is mediated by the binding of transcription factors to cis regulatory elements in their target genes or by the repressing the expression of genes which promote alternate cell fates. GATA transcription factors play an essential role in the differentiation and development of cells. The first GATA transcription family member to be discovered and investigated was GATA binding protein 1 (GATA-1). It was discovered due to its ability to bind to the 3' region of the human β globin gene (Lowry 1999). Subsequently five more transcription family members were discovered. The GATA transcription family members can be divided into two subgroups based upon their tissue distribution and subsequent roles in cell differentiation. GATA-1, GATA-2, and GATA-3 are characterized by their role in hematopoetic cell fates. GATA-4, GATA-5, and GATA-6 are linked to the development of mesoendodermal tissues such as heart, lung, pancreas, gonads, and intestines. GATA binding proteins have also been identified in other species such as C.elegans (ELT-1), Aspergillus (areA), Neurospora (nit-2), S. cerevisiae (DAL 80), S.pombe (GAT-2), and Drosophila (pannier and serpent) (Dorfman 1992, Lowry 1999).

. The GATA family of transcription factors were named based on their ability to bind to the DNA consensus sequence WGATAR or (A/G)GATA(A/G (Patient & McGhee, 2002) Although the family members share common structural features such as an N-terminal transactivation domain and two C-terminal zinc fingers DNA binding domains, they exhibit less homology at the amino acid level (Lowry & Atchley, 2000). For example GATA-2 and GATA-3 are 55% homologous, GATA-3 and GATA-4 are 20% homologous (Maeda, Kubo, Nishi, & Futai, 1996). All family members exhibit an 80% homology at

the zinc finger regions which consist of CX2-CX17-CX2-C (Molkentin, 2000; Simon, 1995). The C-terminal zinc finger is the primary DNA binding element of the GATA protein, whereas the N-terminal zinc finger participates in protein-protein interaction and stabilization of C-terminal binding. Pedone et al also demonstrated that the N-terminal zinc finger has the ability to bind to DNA in the case of GATA-2 and GATA-3 but not the other family members. GATA-2 and GATA-3 have also been shown to recognize the GATC sequence equally as well as the GATA sequence (Ko 1993).

GATA-1 was identified based upon its ability to bind to the human β globin locus control region. It was previously known as Eryf-1, NF-E1, NF-1, and GF-1. In addition to its role as an erythroid cell regulator, GATA-1 also plays a role in Sertoli cell and eosinophil development (Hirasawa 2002). GATA-1 null mice die in utero at approximately embryonic day 10 from severe anemia and exhibit arrest of erythroid maturation. There are several instances where GATA-1 mutations have been linked to human disease. The R216Q missence mutation in the N-terminal zinc finger causes X-linked thrombocytopenia with thalassemia (Lowry 2006). Mutations within the transactivation domain of GATA-1 have been linked to the development of transient myeloproliferative disorder (TMD) and acute megakaryoblastic leukemia.

GATA-2 is expressed in erythroid cells, fibroblasts, endothelial cells, embryonic brain, and adult kidney (Dorfman 1992). Furthermore the expression of GATA-2 is required for the survival of pluripotent hematopoetic stem cells. Interestingly, GATA-1 and GATA-2 are reciprocally expressed during erythropoesis with GATA-2 levels declining as GATA-1 levels increase (Shivdasani 1997). Also, both GATA-2 and GATA-3 are required for megakaryopoesis (Chang 2002). Recently, an interplay between C/EBP α and GATA-2

expression during basophil development has been demonstrated. Arinobu et al conducted experiments where they introduced GATA-2, C/EBPα or both into common lymphoid progenitor cells and determined that expression of GATA-2 or C/EBPα alone resulted in development of mast cells or granulocytes and monocytes, respectively. However, overexpression of GATA-2 followed by C/EBPα resulted in pure basophil colonies, whereas C/EBP α followed by GATA-2 promote a mixed myeloid population (Arinobu 2009). Like GATA-1 null mice, GATA-2 knockout mice die at embryonic day 10.5 from severe anemia and exhibit reduced numbers of erythroid cells.

GATA-3 is an important transcription in the regulation of target genes. Interestingly, GATA-3 plays a role in the expression of the NKG2A ligand, which is the receptor for HLA-E (Marusina, Kim, Lieto, Borrego, & Coligan, 2005). GATA-3 is expressed in T cells and plays a critical role in Th2 development (Ho, Tai, & Pai, 2009; Okazaki, Maeda, Chiba, Doi, & Imai, 2009). Once again there is an antagonistic or concentration dependent mechanism at work between a GATA transcription family member and another transcription factor. In this case Tbet and GATA-3 have opposing roles in the differentiation of T cells. T-bet induces the Th1 genetic program while GATA-3 induces the Th2 phenotype. Specific deletion of GATA-3 in CD4+ T cells causes cells to develop in Th1 cells ((Ho et al., 2009). GATA-3 has recently been shown to play a role in embryonic mammary tissue morphogenesis and maintenance of the differentiated state in adult luminal epithelial cells. Expression of GATA-3 is detected in estrogen receptor positive breast cancers and several groups have shown that GATA-3 is a positive regulator of estrogen receptor alpha (ERα) (Mehra 2005, Garcia-Closas 2007, Voduc 2008, Eeckhoute 2007). Furthermore loss of GATA-3 expression has been

shown to promote epithelial-mesenchymal transition (EMT), where an epithelial tumor devolves into a more invasive fibroblast like tumor (Yan 2010 and Chou 2010). Pandolfi et al (1995) reported that GATA-3 null embryos die between E11 and E12. These mice show an aberration in fetal liver hematopoesis and deformities in the brain and spinal cord. Furthermore, mutated GATA-3 in humans has been linked to HDR syndrome, which consists of a constellation of symptoms which includes hypoparathyroidism, sensorineural deafness, and renal dysplasia (Van Esch 2000).

GATA-4 is expressed in the heart, gut, gonads, liver, visceral endoderm, and parietal endoderm (Molkentin 2000). GATA-4 is one of the earliest transcription factors expressed in cardiac cells (Pikkarainen 2004) and continues to be expressed in the adult. GATA-4 null mice die between embryonic days 8.0 and 9.0 due to cardiac defects (Molkentin 2000). GATA-4 deficient mice exhibit an increase in GATA-6 expression, suggesting that it is a negative regulator of GATA-6 (Morrisey 1998). Mutations of the GATA-4 protein can be associated with disease in humans. GATA-4 haploinsufficiency has been linked to atrial septal defects and congenital heart disease (Pehlivan 1999 and Garg 2003).GATA-4 expression has been detected in the granulosa and theca cell tumors of the ovary and high levels of GATA-4 has been shown to correlate with risk of disease recurrence ( Laitinen 2000 and Kyronlanti 2008). Capo-chichi et al report that ovarian carcinomas that arise from epithelial cells lose expression of both GATA-4 and GATA-6 due to promoter hypermethylation, which leads to deficient expression of molecules that are important for cellular differentiation, such as collagen IV, Dab-2, and laminin (2003).

GATA-5 is expressed in the embryonic heart, lungs, urogenital ridge, bladder, and gut epithelium. However during adulthood expression in the heart is lost (Pikkarainen 2004). GATA-5 null mice, which are viable, show no cardiac defects but do exhibit genitourinary malformation (Viger 2008). Additionally, loss of GATA-5 expression via promoter hypermethylation has been implicated in pancreatic cancer development and gastric carcinoma (Fu 2007).

GATA-6 is expressed during development in the visceral endoderm, heart, lungs, urogenital ridge, vascular smooth muscle cells, and in the gastrointestinal tract (Molkentin 2000). Its expression in retained during adulthood in these tissues although its expression is decreased in the liver and lungs. GATA-6 deficient mice die at embryonic day 6.5 to 7.5 due to problems with lung development (Morrisey 1998). Interestingly, GATA-6 null mice also exhibit a down regulation in GATA-4 gene expression (Morrisey 1998). It has been demonstrated that GATA-6 has the ability to bind to a wider range of DNA sequences than the other GATA transcription family members. Sakai et al showed via polymerase chain random site selection that GATA-6 has the ability to bind to the GATT and GATC sequences in addition to the GATA sequence, as long as an adenine is located at both ends of the sequence, thus AGAT(A/T/C)A (1998). GATA-6 expression has been detected in malignant mesothelioma and metastatic pulmonary adenocarcinoma where it was shown that tumors that expressed GATA-6 resulted in a better prognosis than those with no GATA-6 expression (Lindholm 2009).

**Interferon Regulatory Factors**

The interferon regulatory factor family consists of nine transcription factors: IRF-1, IRF-2, IRF-3, IRF-4/PIP/LSIRF/ICSAT, IRF-5, IRF-6, IRF-7, IRF-8/ICSBP and IRF-9/ISGF3γ (Savistsky D 2010). The IRF family members were first described due to their interaction with interferon inducible genes. In fact, the founding member of the IRF family, IRF-1, was identified based upon its ability to bind to the IFN-β gene promoter and cause transcriptional activation (Reis,L.F. 1992; Taniguchi,T. 2001). Analysis for transcription factors with sequence similarity to IRF-1 led to the discovery of IRF-2, which acts as a repressor of interferon α/β. The expression of IRF-1, IRF-2, IRF-7, and IRF-9 is induced by viral infection or interferon stimulation. The core DNA sequence recognized by IRF family was initially defined as 5'-GAAA-3'. Crystal structure analysis between IRF-2 DNA binding domain and tandem repeats of GAAA sequence further defined the IRF recognition sequence as 5'-AANNGAAA-3' (Fujii,Y. 1999). This sequence is strikingly similar to the ISRE (5'-A/GNGAAANNGAAACT-3'), which explains why IRF factors can interact with the ISRE element in Classical MHC promoters in response to IFN-α/β (IRF-9) or IFN-γ (IRF-1). All IRF family members contain a well conserved N-terminal DNA binding domain that consists of five tryptophan rich repeats, while the C-terminal region mediates protein-protein interaction (Taniguchi,T. 2001).

**Clinical Significance**

Cancer immunotherapy attempts to use the specificity of the immune system to help with the treatment of malignancy. Most human cancers are currently incurable unless they are discovered and surgically removed at an early stage. The use of immunotherapy approaches could potentially serve to help control the spread of

cancerous cells and to facilitate the destruction of transformed cells that are resistant to chemotherapy. Current biological therapies include passive antibody transfer, tumor-specific vaccines, and adoptive immunotherapy (Blattman & Greenberg, 2004). While these treatment methods have proven to be successful clinically, there has been limited success with cell-mediated immunotherapy. An example of successful treatment using cell-mediated therapy is illustrated by the use of allogeneic bone marrow transplantation to treat chronic myeloid leukemia. This method takes advantage of the graft-versus-tumor response in order to eliminate cancer cells (Dermime et al., 1997). Other attempts to use immunotherapy without taking advantage of allo-reactive mechanisms have proven more challenging primarily as a result of tumor immune evasion mechanisms.

Transformed cells use a variety of methods to hamper the immune system including physical exclusion of immune cells, disruption of the function of natural killer and NK-T cells, and down-regulation of MHC Class Ia expression. Defective class Ia expression is a common occurrence in human tumors such as breast cancer, prostate cancer, and melanoma (Gasparollo et al., 2001; Maleno, Lopez-Nevot, Cabrera, Salinero, & Garrido, 2002; Maleno et al., 2004; Palmisano et al., 2001). The deficient expression often involves a specific locus or allele and studies have shown that even minute changes in HLA-A2 expression have a negative impact on the tumoricidal activity of CTLs (Gasparollo et al., 2001). Furthermore some transformed cells use molecular mimicry to escape immune recognition by creating peptides that are similar to the leader peptides of HLA Class Ia molecules, which are presented by the HLA-E molecule and inhibit the activity of natural killer cells. In these instances, the lack of HLA-E

expression results in a negative impact on both cytotoxic T-lymphocytes as well as natural killer cells. Since HLA-E serves a ligand for the NKG2A/CD94 receptor that is present on many natural killer cells, NK-T cells, and a subset of CD8+ T cells, the ability to regulate this gene has the potential to have a major impact on adoptive immunotherapy treatment strategies.

# Chapter 2: Characterization of the Upstream Interferon Response Region in Epithelial Tumor Cells

## Introduction

Interferon gamma is a soluble cytokine that is secreted by a variety of cells including CD4+ T cells and NK cells. It has the ability to activate macrophages and promotes intracellular anti-viral activity by increasing the expression of both Class Ia and Ib molecules. The mechanism of action of this cytokine has been shown to be mediated primarily through tyrosine phosphorylation of STAT1α by JAK kinases upon binding to the interferon gamma receptor. Phosphorylated STAT 1α then forms a homodimer, which translocates to the nucleus and binds to the gamma activation site (GAS) of various target genes. In the case of the HLA Class Ia genes, IFN-γ induces the transcription of the interferon response factor-1 (IRF-1), which binds to the interferon stimulated response element (ISRE) in the promoters of HLA-B or HLA-C, thereby inducing transcription.

Similarly, the transcription of the HLA Class Ib gene, HLA-E, is also stimulated by IFN-γ despite the lack of a functional ISRE in its promoter. Previous studies in our laboratory show that HLA-E is induced via a variant STAT1α binding element, named the Interferon Response Region (Gustafason et al 1996). The Upstream Interferon Response Region (UIRR) is immediately adjacent to the IRR. We previously demonstrated that the UIRR is bound by GATA1 in K562 stimulated cells and that the activity of the UIRR confers a 5-fold enhanced response to IFN-γ above that of the IRR (Barrett, Gustafson, Wang, Wang, & Ginder, 2004). GATA1 interacts with the HLA-E

promoter in vivo, while GATA1 overexpression in the U937 cell line resulted in a 5-fold increase in HLA-E induction. Since the expression of GATA transcription factors is cell-type restricted, we investigated whether family members other than GATA1 could support UIRR enhancer function.

## Material and Methods

### Cell Culture

The promonocytic cell line U937, erythroid leukemia K562, ovarian carcinoma OvCar 8, ovarian carcinoma A2780, esophageal carcinoma Seg1, colon carcinoma HCT 116 were maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen, Carlsbad, CA) which was supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 ug/ml streptomycin (Invitrogen, Carlsbad, CA). The melanoma cell line, MeWo, was maintained in MEM α medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 ug/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were induced with interferon-gamma (RnD systems) at 200 units/ml or 300 units/ml for times indicated. Doxycycline (Sigma-Aldrich, Saint Louis, MI) was used at 2 ug/ml and added prior to interferon-gamma induction.

### Quantitative reverse transcriptase PCR

Cytoplasmic RNA was isolated using the Trizol reagent system (Invitrogen, Carlsbad, California). 1 ml or 0.5 ml of Trizol reagent was added to approximately 1 x$10^6$ (six well dishes) or 3 x $10^5$ (twelve well dishes), respectively, for ten minutes. The cells were collected an added to a 1.5 ml microcentrifuge tube prior to the addition of 200

microliters of chloroform. Cell lysate suspensions were shaken for thirty seconds and allowed to settle for three minutes followed by centrifugation at 12000 x g at 4˚C. The upper phase was removed to a fresh tube and 550 microliters of isopropanol was added before a ten minute incubation at room temperature. The samples were then spun at 12000 x g at 4˚C for fifteen minutes. The supernatant was removed and the RNA pellet was washed with 600 microliters of 70% ethanol/30% DEPC treated water. Samples were spun at 7500 x g at 4˚C for five minutes. The supernatant was removed and pellets were allowed to air dry for ten minutes. The pellet was resuspended in 100 microliters of 100% DEPC treated water. RNA was quantified by spectrophotometry. Each RNA sample was then DNAse treated to remove any contaminated genomic DNA. Specifically, 0.5 ul of Dnase I, 0.5 ul of Rnase Inhibin, plus water added to a total volume of 20ul was added to 2 ug of RNA. The samples were incubated at 37˚C for thirty minutes, and 75˚C for ten minutes to cause inactivation of the enzymes. Next, cDNA was synthesized using the i-Script cDNA synthesis kit by Biorad. 500 ng of DNAse I treated RNA was used for cDNA synthesis. The reaction mix consisted of 2 ul of 5x iScript reaction mix, 0.5 ul of iScript reverse transcriptase, and nuclease free water to ten microliters. The samples were then incubated as follows: 5 minutes at 25˚C, 30 minutes at 42˚C, 5 minutes at 85˚C, and 10 minutes at 4˚C. Each qPCR reaction was performed using 20ng of cDNA. 12.5 ul of 2x Sybr Green Mix (Roche) and 7.5 ul of 1 nM primer mix containing forward and reverse primers (IDT) were used in each sample.

The primer sequences were as follows:

| Primers | Forward | Reverse |
|---|---|---|
| HLA-E | TTGCAAGGGCCTCTGAATCTGTCT | AGGAACACAGGTCAGTGTGAGGAA |
| STAT 1α | GTGCATCATGGGCTTCATCAGCAA | TAGGGTTCAACCGCATGGAAGTCA |
| Cyclophilin A | AGACAAGGTCCCAAAAGCAGA | TGTGAAGTCACCCTGACACAT |
| CAT | ATCCCTGGGTGAGTTTCACCAGTT | GGAAGCCATCACAAACGGCATGAT |
| GFP | TGACCCTGAAGTTCAGCACCA | TGTAGTTGCCGTCGTCCTTGAAGA |
| GATA-1 | AGACTTTGAAGACAGAGCGGCTGA | TTGGGAGAGGAATAGGCTGCTGAA |
| GATA-2 | ATTGTCAGACGACAACCACCACCT | AGTGGCCTGTTAACATTGTGCAGC |
| GATA-3 | TGCATCTGGGTAGCTGTAAGGCAT | GCATCAAACAACTGTGGCCAGTGA |
| GATA-4 | TACATCAGCTTCCGGAACCACCAA | AATCCAGCATTGAGCAAAGGGCTC |
| GATA-5 | AATGGCCGGTGATGTATGTCAGGA | AATCCAGGTTTCTGGCATTGCTGG |
| GATA-6 | TCTACAGCAAGATGAACGGCCTCA | GTGTGACAGTTGGCACAGGACAAT |
| mGATA4 | TCAAATTCCTGCTCGGACTTGGGA | GTTTGAACAACCCGGAACACCCAT |

**Creation of stable cell lines**

**Knockdown of GATA-6**

Previously the SKOV3TR cell line was created in the laboratory. Specifically, the ovarian carcinoma SKOV3 was transfected with a plasmid that express the tet Repressor protein. The cell line with the highest expression of recombinant protein was named SKOV3TR and chosen to be transfected with the tetracycline inducible shRNA vector, pSuperior.neo, which contained a 19 base pair sequence directed against GATA6. The superior.neo vector contains a hybrid promoter with two binding sites for the Tet Repressor molecules. The expression of GATA-6 shRNA is repressed in the absence of tetracycline or doxycycline. Cells were selected with G418 (Invitrogen, Carlsbad, CA) at 500 ug/ml until the cells began to proliferate at pre-selection levels. The decrease of GATA-6 expression was confirmed using qPCR.

**Overexpression of GATA-4 and GATA-6**

The cell lines MeWo and OvCar 8 were transfected with the expression vector pCDNA/hGATA6, pCDNA/mGATA4, or a combination of both. The pCDNA1/mGATA-4 vector was a much appreciated gift from Dr. Jeff Molkentin. First the plasmids were subcloned from the pCDNA1 vector into the pCDNA4 vector. The hGATA6 and mGATA-4 cDNA fragments were isolated from the pCDNA1 plasmid by digestion with Xho and BamH1.The resulting fragment was gel isolated and then ligated into the pCDA4/TO vector that had also been digested with Xho and BamH1. The resulting pcDNA4/hGATA6 and pcDNA4/mGATA4 plasmids were transfected both separately and together into the MeWo and OvCar 8 cell lines. The cells were selected with Zeocin at 150 ug/ml until the cells began to proliferate at pre-selection rates (approximately four weeks). The cells were then pooled and will be referred to as MeWo mGATA4, MeWo hGATA6, MeWo mGATA4/hGATA6, OvCar mGATA4, OvCar hGATA6, and OvCar mGATA4/hGATA6. Overexpression of the GATA4 and GATA6 proteins were verified with western blot.

**Preparation of CAT reporter gene constructs**

The HLA-E 6.2 genomic clone, which was a gift from Dr. Harry Orr, was used to generate the pECAT clone by sequentially subcloning Hind III-Pst I (-1700 to -174) and PstI-AlwNI (-174 to +2) promoter fragments into the promotorless chloramphenicol acetyltransferase (CAT) reporter gene (Promega, Madison, WI). The 5' deletion mutants pE386 and pE128 were generated by restriction digestion and subsequent re-ligation of the plasmid. Further deletion mutants were generated by polymerase chain

reactions (PCR) using primers corresponding to -331 to -311, -281 to -262, and -231 to -210 of the HLA-E promoter, respectively, along with the addition of a Hind III site at the 5' end of each primer. The primers were used in conjunction with an internal primer corresponding to sequences in the CAT gene and pE386 plasmid as a template. The PCR products were gel isolated, digested with Hind III and Pst I, gel isolated again, then ligated into the pE386 plasmid that had been similarly digested and gel isolated (Gustafson & Ginder, 1996).

The pCAT3/GFP construct was created by digesting a CMV driven GFP expression plasmid with BamH1 and Xho I. The resulting fragment was gel isolated and ligated into a pCAT3 plasmid that had been digested with Sal I and BamH1 and then gel isolated. The resulting pCAT3/GFP plasmid was used to generate the CAT reporter gene plasmids pE231/GFP, pE231mut/GFP, pE191/GFP and pE128/GFP. Specifically, the UIRR-CAT, IRR-CAT, and Basal-CAT plasmids that were created previously were digested with Kpn I and Nhe I. The resulting fragment was gel purified and ligated into the pCAT3/GFP plasmid that was similarly digested and gel purified. The constructs were sequenced by the dideoxy-chain termination method to verify endpoints and mutated sequences. All restriction enzymes were purchased from New England Biolabs (Beverly, MA).

**Transient Transfection and CAT Reporter Gene Assays**

Cells were plated the day before in 6 well dishes at a density of $3.0 \times 10^5$ using Lipofectamine 2000 (Invitrogen). Briefly, 3 ug of CAT reporter plasmid plus 0.3 ug of beta-galactosidase was diluted in 250 ul of serum free media. In a separate tube, 10 ul

of Lipfectamine was added to 250 ul of serum free media. The mixture was incubated at room temperature prior to addition to the DNA mixture. The diluted DNA plus the diluted Lipofectamine was incubated for thirty minutes. 500 ul was added to each well. The following day the appropriate wells were stimulated with 300 units of IFN-y/ml. Transfected cells were removed from the 6 well dishes using trypsin (Invitrogen). The cells were centrifuged at 8000xg at room temperature for 3 minutes. The media was removed and the remaining cell pellet was washed with 800 ul of cold 1x PBS. The cells were centrifuged at 8000xg at room temperature for 3 minutes. After removal of the supernatant, the cell pellet was resuspended in 100 ul of 0.25M Tris-Cl and placed in -80˚C for one hour. The cells were then lysed using a free-thaw method. Briefly, the cells were placed at 37˚C for five minutes by vortexing. Next the cells were placed in a 100% ethanol ice bath for five minutes. The cells were then placed at 37˚C for five minutes. These steps were repeated twice. Next the cells were centrifuged at 12000xg for five minutes. The supernatant was removed to a fresh tube. 15 ul of sample was combined with 1ul of $^{14}$C, 5 ul of n-Butyryl CoA, and 89 ul of 0.25M Tris-Cl. Each sample was incubated for 90 minutes at 37˚C. The reaction was terminated by the addition of 600 ul of xylenes. The samples were vigorously shaken then centrifuged at 12000xg for three minutes. 500 ul of the supernatant was removed to a fresh tube followed by the addition of 100 ul of 0.25M Tris-Cl. After being vigorously shaken, the samples were centrifuged at 12000xg for three minutes. 400 ul of the supernatant was removed to a fresh tube followed by the addition of 100 ul of 0.25M Tris-Cl. The samples were shaken and then centrifuged as above. 300 ul of the supernatant was added to 6 milliliters of 4a20 scintillation fluid.

CAT activity was normalized to internal beta-galactosidase activity. 15 ul of the cell lysate was mixed with 135 ul of 0.25M Tris-Cl and 150 ul of 2x Assay Buffer (200 mM $Na_2PO_4$, 2mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml O-nitrophenyl-β-D-galactopyranoside (ONPG) pH 7.3) the incubated thirty minutes at 37˚C. Reactions were terminated with 700 ul of 1M $Na_2CO_3$ amd read at 420 nm on a UV spectrophotometer. Units of β-galactosidase activity were determined using a standard curve.

**Transient Transfection and siRNA treatment**

Cells were plated in a 12-well dish at a density of $2x10^4$ cells per well. 40 picomoles of siRNA was diluted in 100 ul of serum free media that was combined with 100 ul of serum free media plus 5 ul of Lipofectamine. The mixture was incubated at room temperature before addition of 200 ul to the appropriate well. After 72 hours of sirRNA treatment, 200 units/ml of IFN-y was added to the appropriate wells. Cells were then incubated for twenty-four hours at 37˚C and 5% $CO_2$ before being harvested.

**Western Blotting**

Whole cell extracts were mixed with 2x SDS sample buffer [62.5 mM Tris pH 6.8, 30% glycerol (v/v), 0.01% bromophenol blue (w/v)] then boiled for five minurtes at 100˚C. Samples were loaded on a 4% stacking/10% resolving SDS Ready Gel from BioRad (Hercules, CA) and run at 88 V at room temperature. The gel was first equilibrated in 100% methanol for twenty seconds and then in transfer buffer [(25mM Tris pH 8.3, 192 mM glycine, 20% methanol (v/v)] for ten minutes. Next the gel was transferred to a PVDF membrane at 100 V and 4˚C for one hour. The membrane was blocked in 5%

nonfat powdered Carnation milk (Nestle, Young America, MN) and 1x PBS-T [40 ml of 10x PBS-T (10 x PBS and 2.0 ml of Tween 20)] for one hour at room temperature with shaking. Next the membrane was incubated in the appropriate primary antibody (2ug/ml) in PBS-T plus 5% nonfat milk overnight at 4˚C with rocking. Unbound primary antibody was removed by washing the blot for ten minutes with ten milliliters of 1x PBS-T followed by 3 five minute washes with 1x PBS-T. The membrane was then incubated for one hour at room temperature with shaking in the appropriate secondary antibody (1:4000) in PBS-T plus 5% nonfat milk. Unbound secondary antibody was removed by one ten minute wash with PBS-T followed by 3 five minute washes. Protein was visualized by enhanced chemiluminesence from BioRad (Hercules, CA).

**Statistical Analysis**

CAT reporter gene assay data was analyzed using Microsoft Excel to determine the standard error of the mean. The standard deviation between samples was first determined and then this value was divided by the square root of the number of biological replicates. qPCR data was analyzed using the $2^{-\Delta CT}$ method (Schmittgen 2008). Cyclophilin A was used as an endogenous control. Relative quantification was determined as the ratio of the target gene to the housekeeping gene. Error was reported as above. Significance was determined using the student's t-test.

## Results

### GATA Factor Screen and Analysis of UIRR Function

Previous investigation of the functional activity of the HLA-E promoter in the context of the chloramphenicol acetyltransferase reporter gene assay demonstrated that the interferon response region of the HLA-E promoter resides between -193 and -146 portion of the promoter. Inclusion of the promoter elements spanning to -386 of the promoter resulted in a decrease in the transcriptional response to interferon gamma treatment compared to inclusion up to -193 base pairs. In order to determine whether or not this effect was cell type specific, CAT reporter constructs were also transfected into the K562 erythroleukemia cell line (see Figure 4). When cells expressing the -386 and -193 CAT plasmids expressed and the cells were stimulated with interferon gamma, there was a five-fold increase in the transcriptional response with the -386 CAT plasmid compared to the -193 CAT plasmid.

To further define the upstream element responsible increased CAT activity, we constructed a series of 5' deletions of the HLA-E promoter. It was determined that there was an Upstream Interferon Response Region (UIRR) that could be localized to the -231 to the -194 portion of the HLA-E promoter. Analysis of potential transcription factor binding sites using the MATInpsector 2.0 software showed that the potential candidates that could be binding to the region were GATA binding transcription factor-1, Creb Binding Protein, and My-T1 neurotransgenic factor. The Myt-1 transcription factor was excluded from further analysis since it is only expressed in neural tissue. Mutagenesis of the potential binding sites for GATA-1 and Creb binding protein indicated that

changing the GATA binding site decreased the IFN-γ transcriptional activation of HLA-E, whereas disrupting the CEBP binding site had no effect. Additionally, analysis of protein expression showed expression of GATA-1 in the K562 cell line, which supports the UIRR function, but not the in the U937 cell line, which does not support UIRR function. This led to the hypothesis that GATA expression was necessary for the UIRR response. Further investigation of the HLA-E transcriptional response in other tumor cell lines seemed to uphold this theory. Specifically, the cell lines that were examined were SKnMC, Tera-2, Hela, MeWo, Panc-1 (Figure 5). It was determined that those cell lines that exhibited UIRR enhancer like function were also expressors of GATA binding transcription factors. We decided to further investigate this phenomenon in cell lines that were epithelial rather than hematopoietic in origin. This was of interest because the expression of GATA transcription factors can be divided into two families. GATAs 1-3 are critical for hematopoietic cell development whereas GATAs 4-6 are necessary for mesoendoderm development. Since we had previously demonstrated that GATA-1 could support UIRR function we decided to focus on the family members in the "mesoendoderm" family. Based upon the known tissue expression of GATA4, GATA-5, and GATA-6, the ovarian carcinoma A2780, the melanoma MeWo, the esophageal carcinoma Seg1, the ovarian carcinoma OvCar8, and HCT 116 were screened for their GATA expression via QPCR (Figures 6-11). Expression was normalized to expression of the housekeeping gene Cyclophilin A. The ovarian carcinoma SKOV3, colon carcinoma HCT 116, and esophageal carcinoma Seg1 were all determined to express GATA6 mRNA.

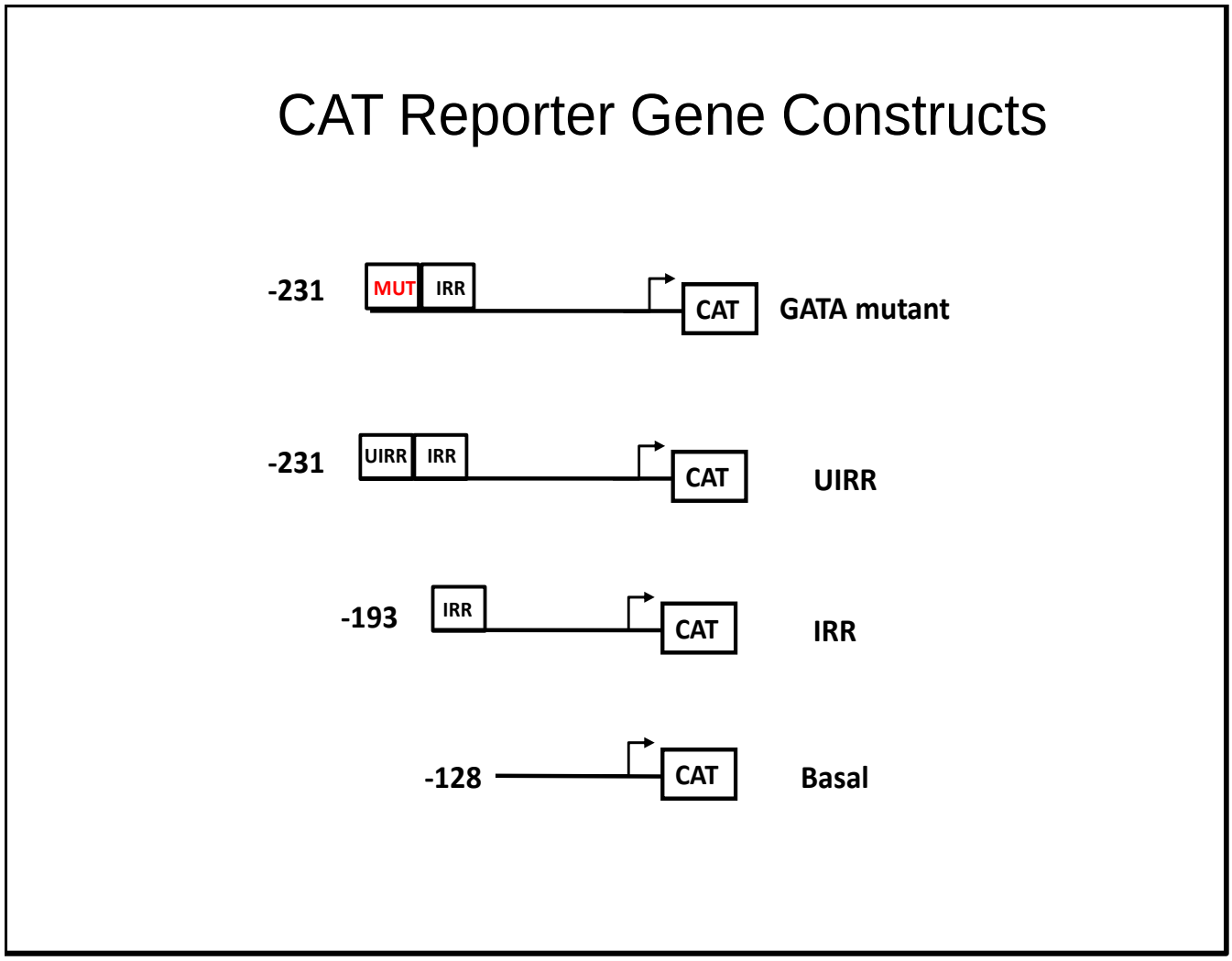



**Figure 4. Diagram of CAT reporter gene constructs used in this study.** Basal is the first 128 base pairs. The Interferon Response Region is located from -193 to -146 and the UIRR is located from -231 to -194. GATA mutant plasmids have either the GA or the TA core nucleotides of the GATA binding sequence mutated to a CC.

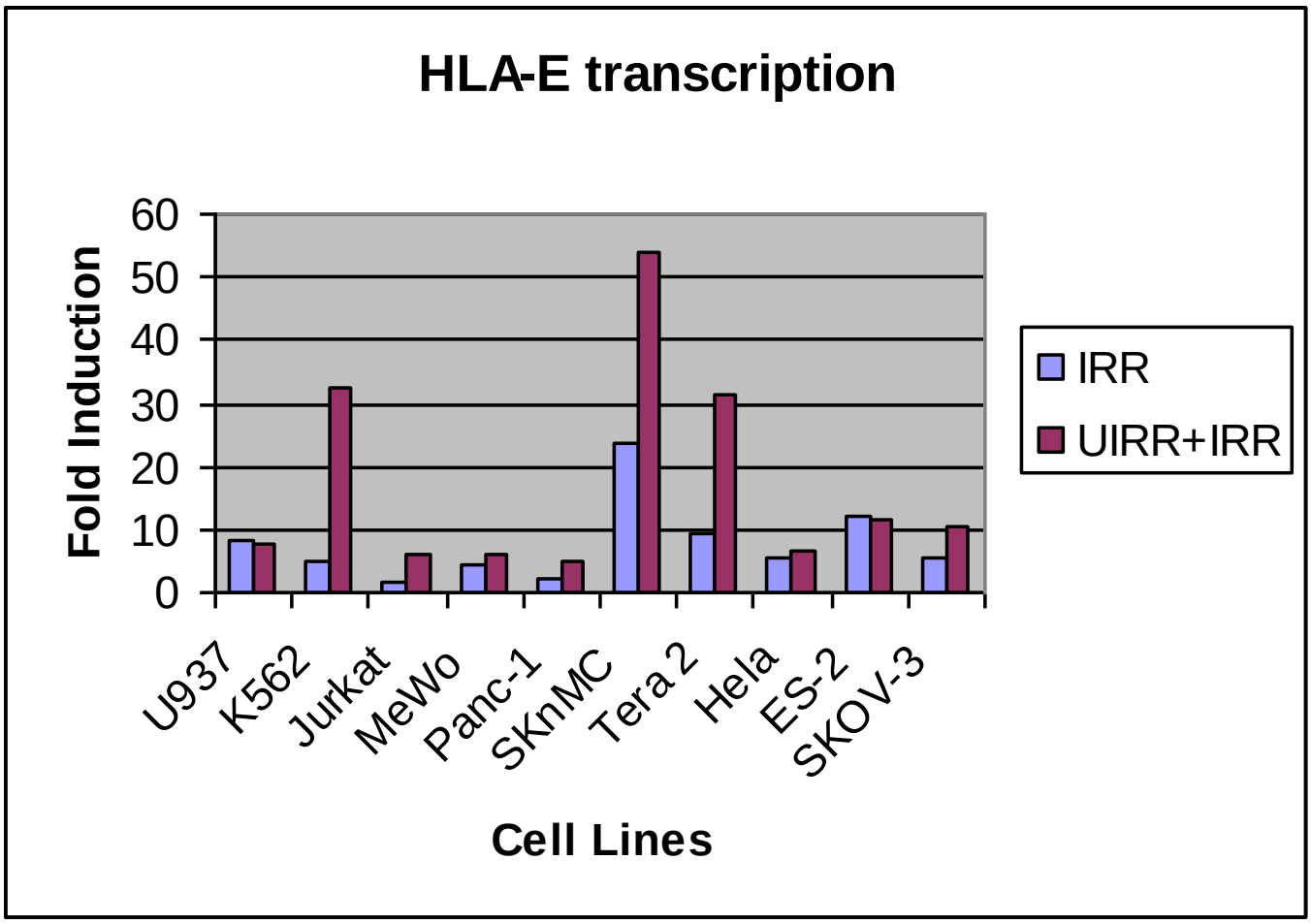



**Figure 5. Evaluation of UIRR Function in Various Tumor Cells Lines.** (Courtesy of D. Barrett). This preliminary work shows that tumor cells lines besides K562 can support UIRR functionality. Each cell line was transfected with either the IRR or UIRR HLA-E promoter driven plasmids for 3 hours prior to twenty-four stimulation with IFN-γ.

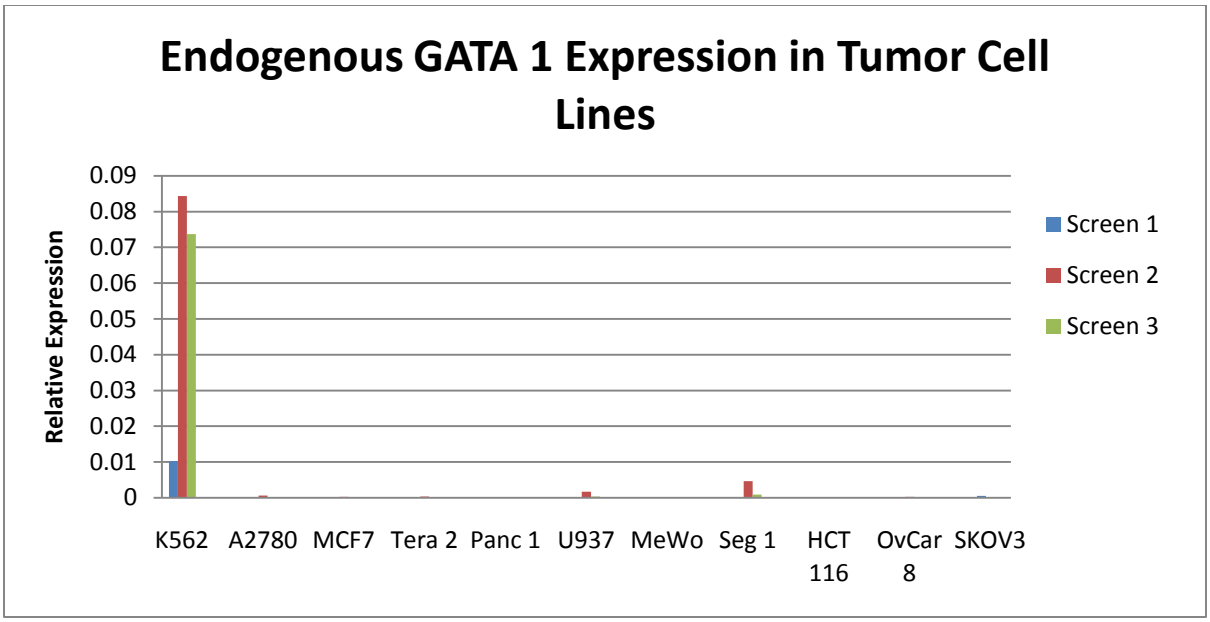



**Figure 6. Analysis of GATA-1 expression in various Tumor cells**. RNA was isolated from the indicated cells lines and screened for GATA-1 mRNA expression via qPCR. Expression of GATA-1 was normalized to Cyclophilin A. qPCR was run for 40 cycles.

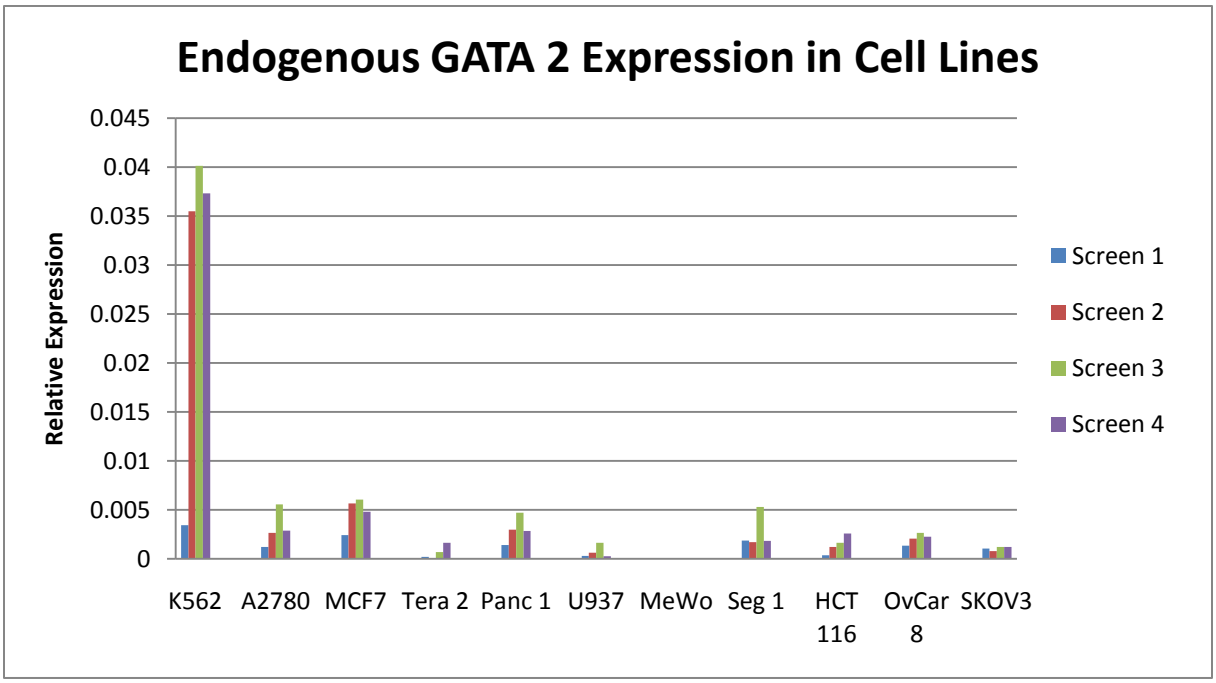



**Figure 7. Analysis of GATA-2 expression in various tumor cells.** RNA was isolated from the indicated cell line and screened for GATA-2 mRNA expression via qPCR. Expression was normalized to Cyclophilin A. qPCR was run for 40 cycles.

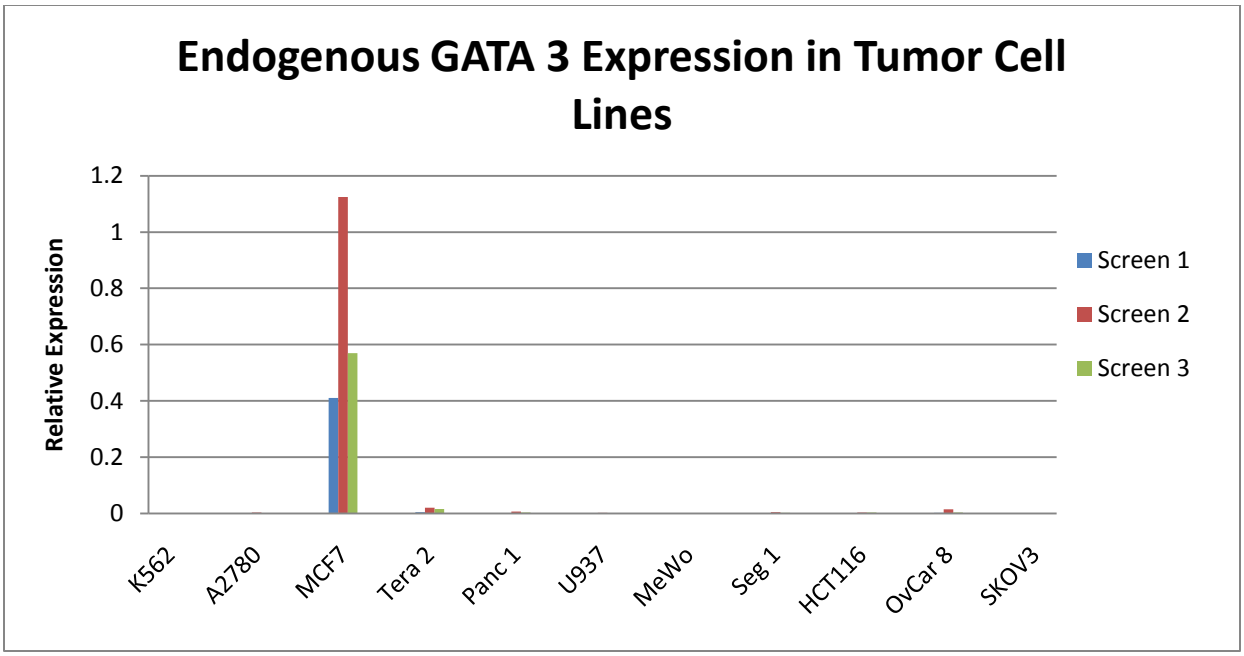



**Figure 8. Analysis of GATA-3 expression in various tumor cell lines.** RNA was isolated from the cell lines above and screened for GATA-3 message via qPCR. qPCR was run for 40 cycles and GATA 3 expression was normalized to Cyclophilin A. MCF 7 was the only cell line examined that was positive for GATA-3 expression.

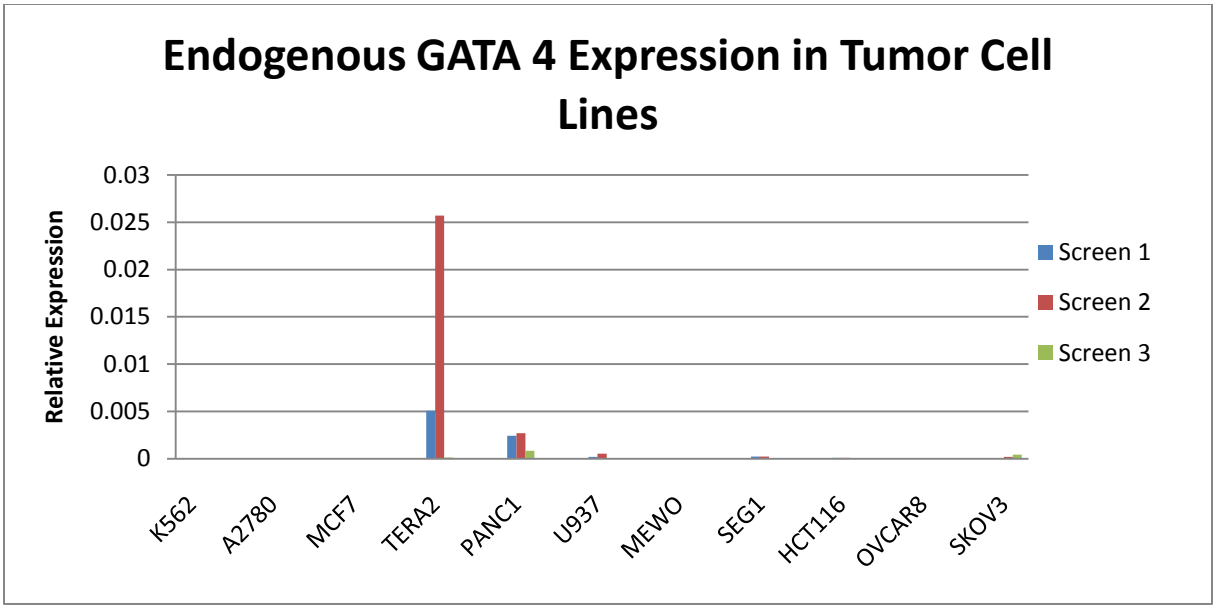



**Figure 9. Analysis of GATA-4 expression in tumor cell lines.** RNA was isolated from the cell lines above and screened for GATA-4 message via qPCR. 40 cycles of qPCR was performed and GATA-4 expression was normalized to Cyclophilin A. Tera2 and Panc1 were positive for GATA-4 expression.

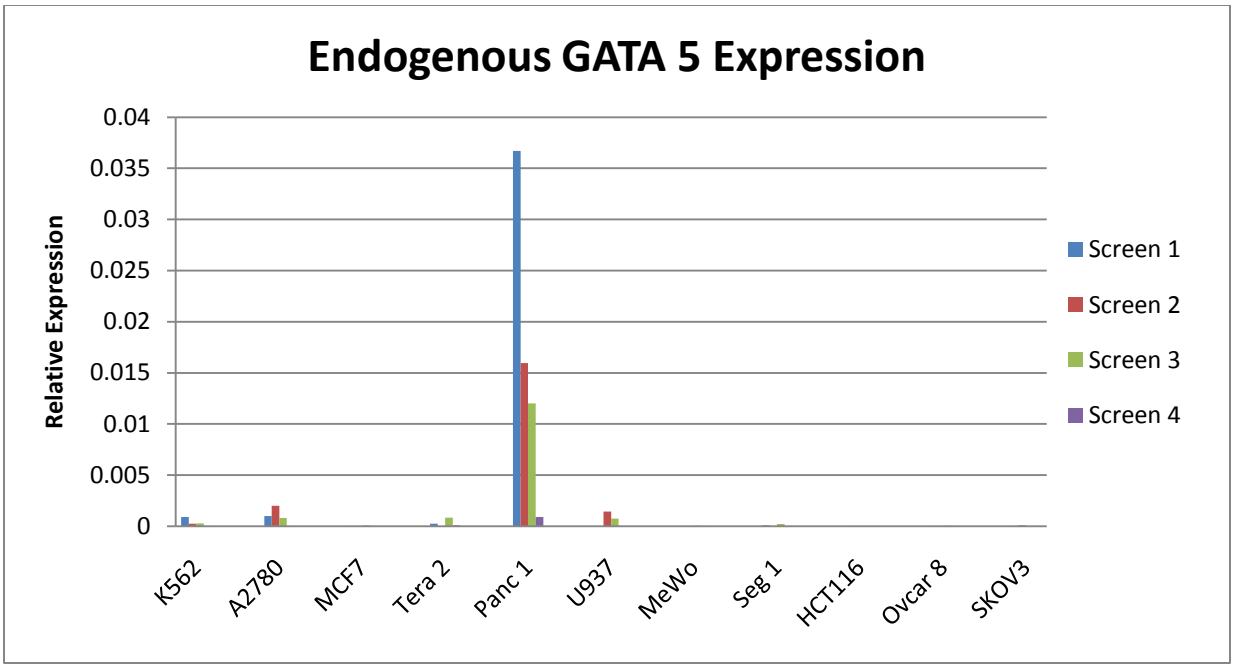



**Figure 10. Evaluation of GATA-5 expression in various tumor cell lines.** RNA was isolated from the cell tumor cell lines indicated and analyzed for GATA-5 message. 40 cycles of qPCR was performed and GATA-5 expression was normalized to Cyclophilin A. Panc 1 expressed the highest amounts of GATA-5 mRNA transcript.

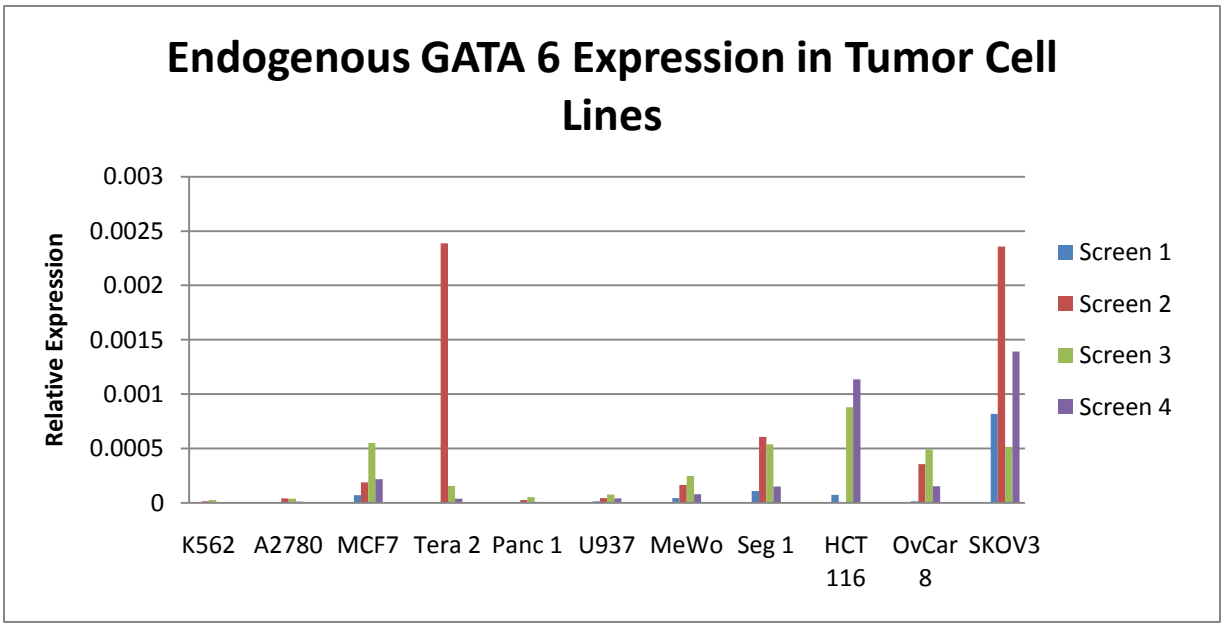



**Figure 11. Evaluation of GATA-6 expression in various tumor cell lines.** RNA was isolated from tumor cell lines above and analyzed for GATA-6 expression. 40 cycles of qPCR was performed and GATA-6 expression was normalized to Cyclophilin A. Tera 2, HCT 116, SKOV3, and Seg1 are positive for GATA-6 message.

These cell lines were transfected with plasmids containing CAT reporter gene driven by the HLA-E promoter. When expressed in HCT 116 cells, a 17.8 fold increase and a 47.9 fold increase in CAT activity versus a control CAT plasmid was observed from the IRR plasmid and the UIRR plasmid, respectively (Figure 12). In Seg1 cells, a 7.2 fold increase in CAT activity was measured from the IRR plasmid and a 28.7 fold increase with the UIRR plasmid (Figure13). The SKOV3 cell line demonstrated a 20.6 fold increase in CAT activity with the IRR plasmid compared to a 63.9 fold increase in CAT activity with the UIRR plasmid (Figure 14). The ovarian carcinoma A2780 cell line was shown to express low amounts of GATA factors 2 and 5(Figures 7, 10). When transfected into A2780 cells, the IRR plasmid resulted in a 4.7 fold CAT activity and the UIRR plasmid resulted in a 14.3 fold CAT activity (Figure 15). Therefore in each of these cell lines there was a 2.4 fold to a 4.0 fold increase in CAT activity measured from the UIRR above that of the IRR plasmid as wells as GATA transcription factor expression.

The melanoma cell line, MeWo, and the ovarian carcinoma, OvCar 8, were also screened for GATA transcription factor expression. The MeWo cell had no detectable GATA family transcript levels as measured by qPCR (Figures 6-11), whereas the OvCar 8 cell line had very low expression of GATA-2 and GATA-6 transcripts (Figures 7,11). When these cell lines were transfected with CAT reporter gene plasmids there was no appreciable difference in CAT activity when comparing the IRR plasmid measurement to the UIRR plasmid measurement. The IRR and UIRR reporters had a 6.8 fold and a 9.9 fold increase in CAT activity over control when expressed in MeWo cells (Figure 16), respectively. Similarly, the OvCar 8 cells exhibited a 9.7 fold CAT activity from the

IRR plasmid and a 9.3 fold CAT activity from the UIRR plasmid (Figure 17). These results suggest that other GATA family members have the ability to support UIRR function and that these factors are necessary for the response via the UIRR.

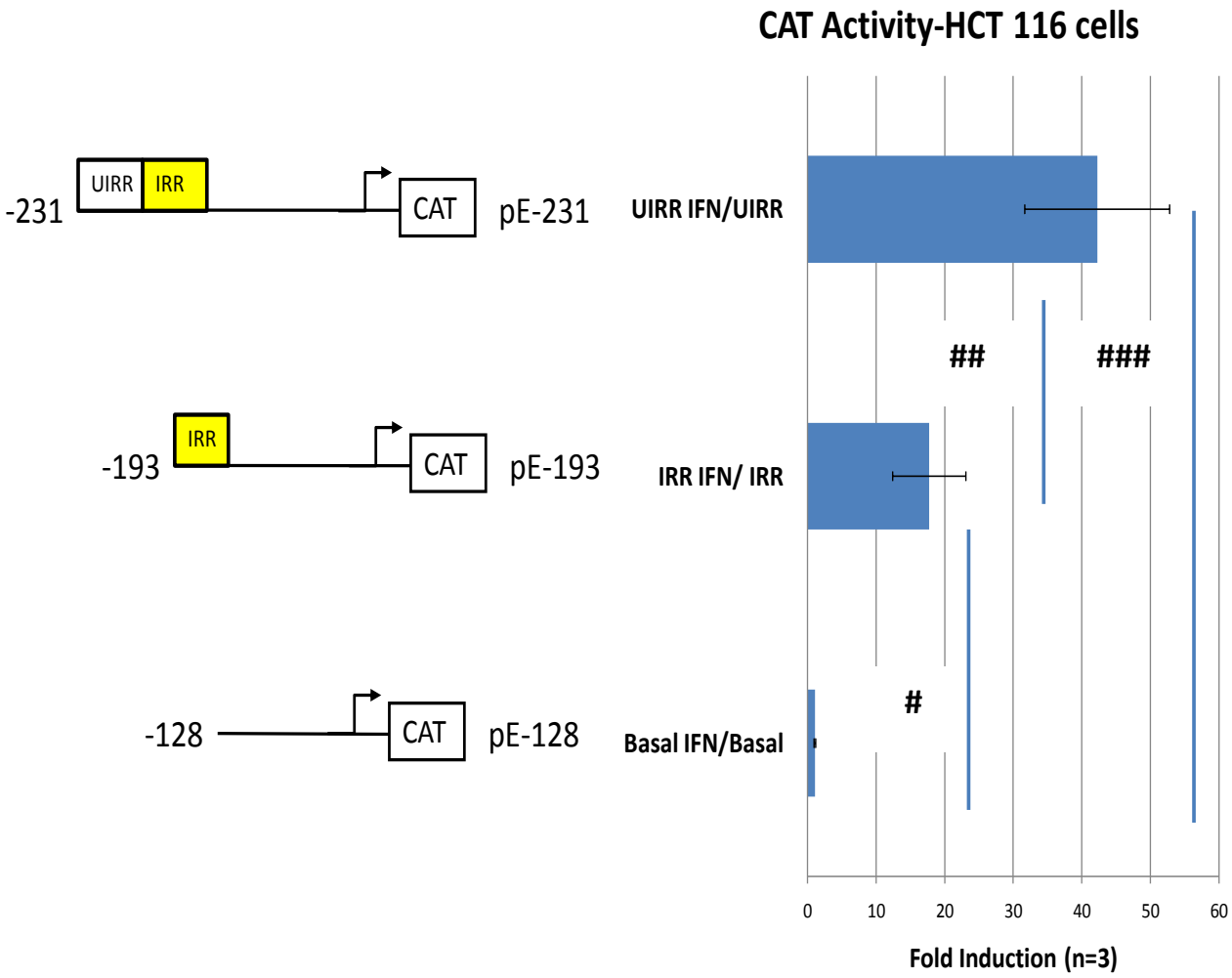



**Figure 12. HCT 116 colon carcinoma cells display an increase in HLA-E promoter driven CAT reporter gene activity via the UIRR.** HCT 116 cells were plated overnight and then transfected with CAT reporter gene plasmids as indicated in Figure 4. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. # p=0.09; ## p=0.13, ### p=0.06

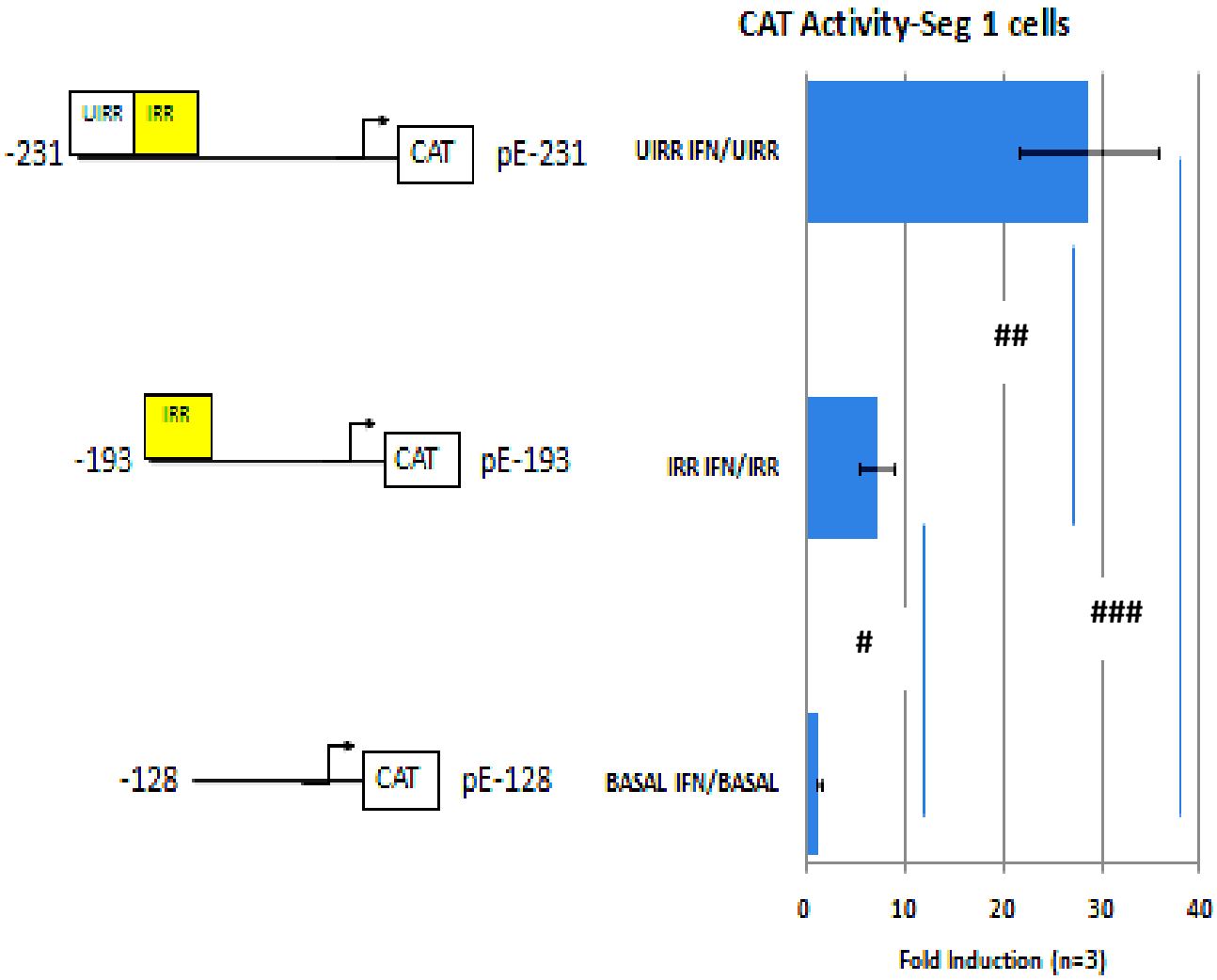



**Figure 13. Seg1 esophageal carcinoma cells exhibit UIRR functionality.** Seg1 cells were transfected with CAT reporter gene plasmids prior were plated overnight and then transfected with CAT reporter gene plasmids as indicated in Figure 4. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. # p=0.08; ## p=0.09, ### p=0.06

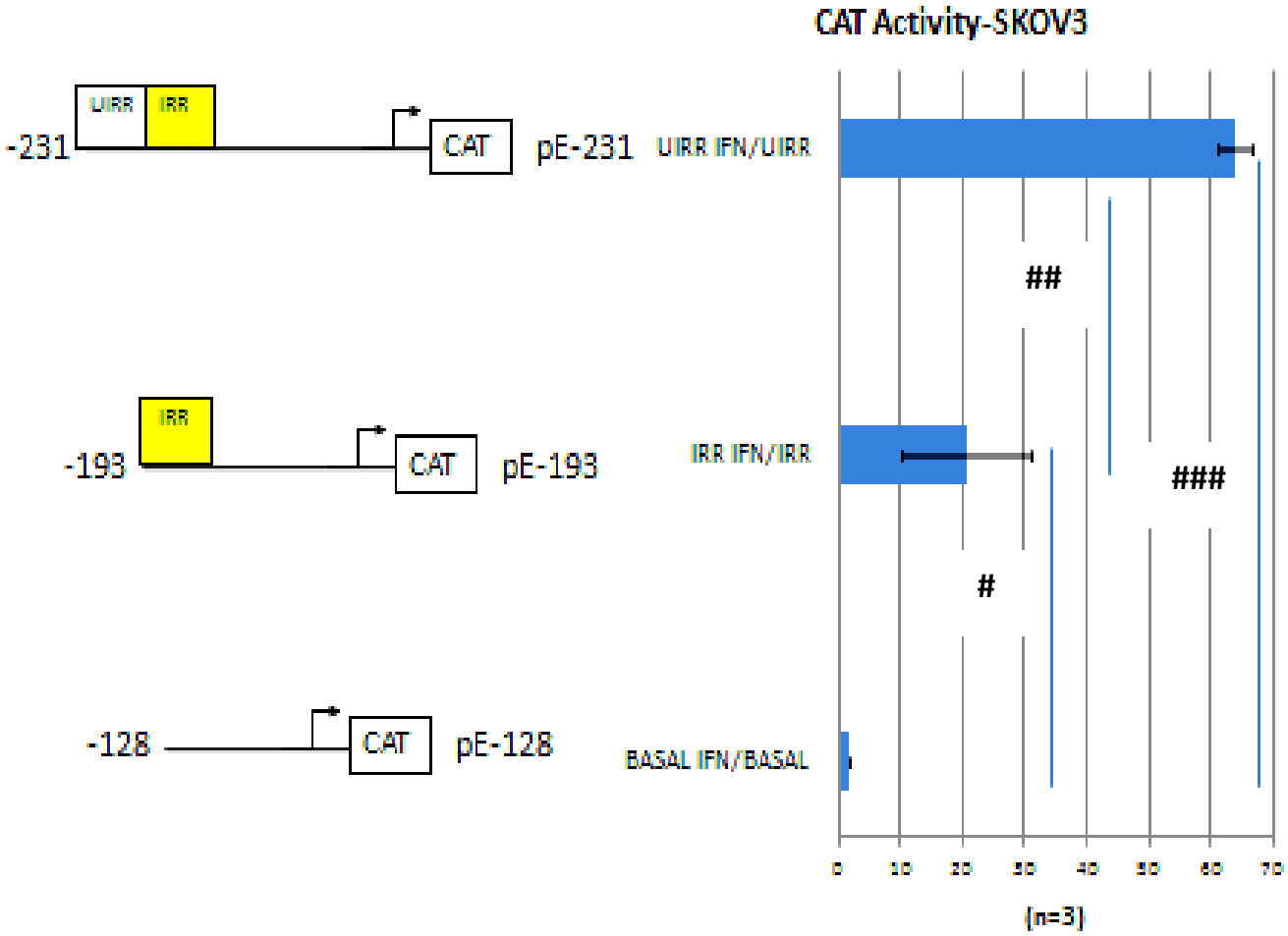



**Figure 14. SKOV3 ovarian carcinoma cells exhibit UIRR functionality.** SKOV3 cells were transfected with CAT reporter gene plasmids prior were plated overnight and then transfected with CAT reporter gene plasmids as indicated in Figure 4. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. # p=0.21; ## p= 0.21, ###p= 0.13

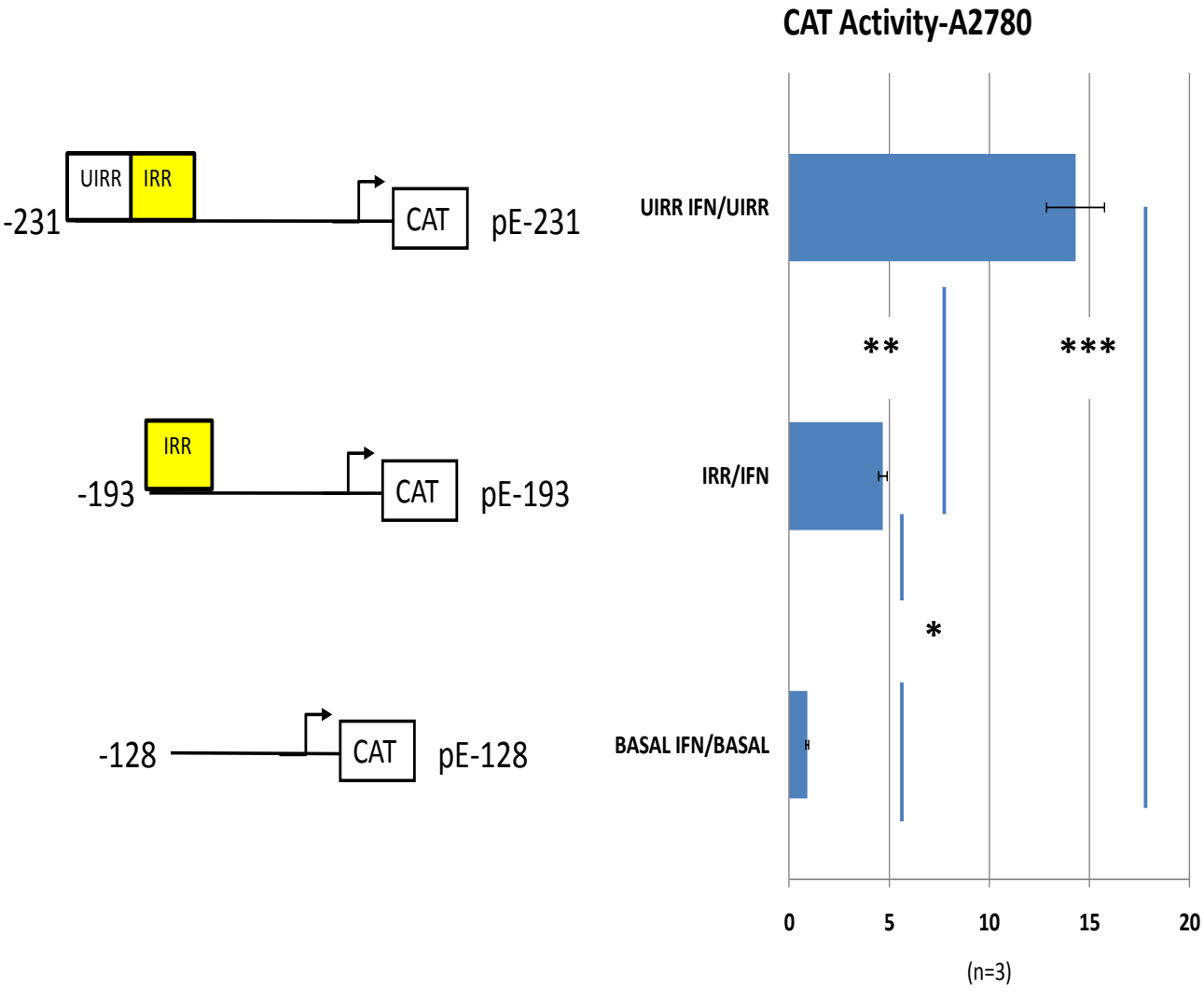



**Figure 15. A2780 ovarian carcinoma cells exhibit UIRR functionality.** A2780 cells were transfected with CAT reporter gene plasmids prior were plated overnight and then transfected with CAT reporter gene plasmids as indicated in Figure 4. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. * p= 0.0007; ** p= 0.007, *** p= 0.002

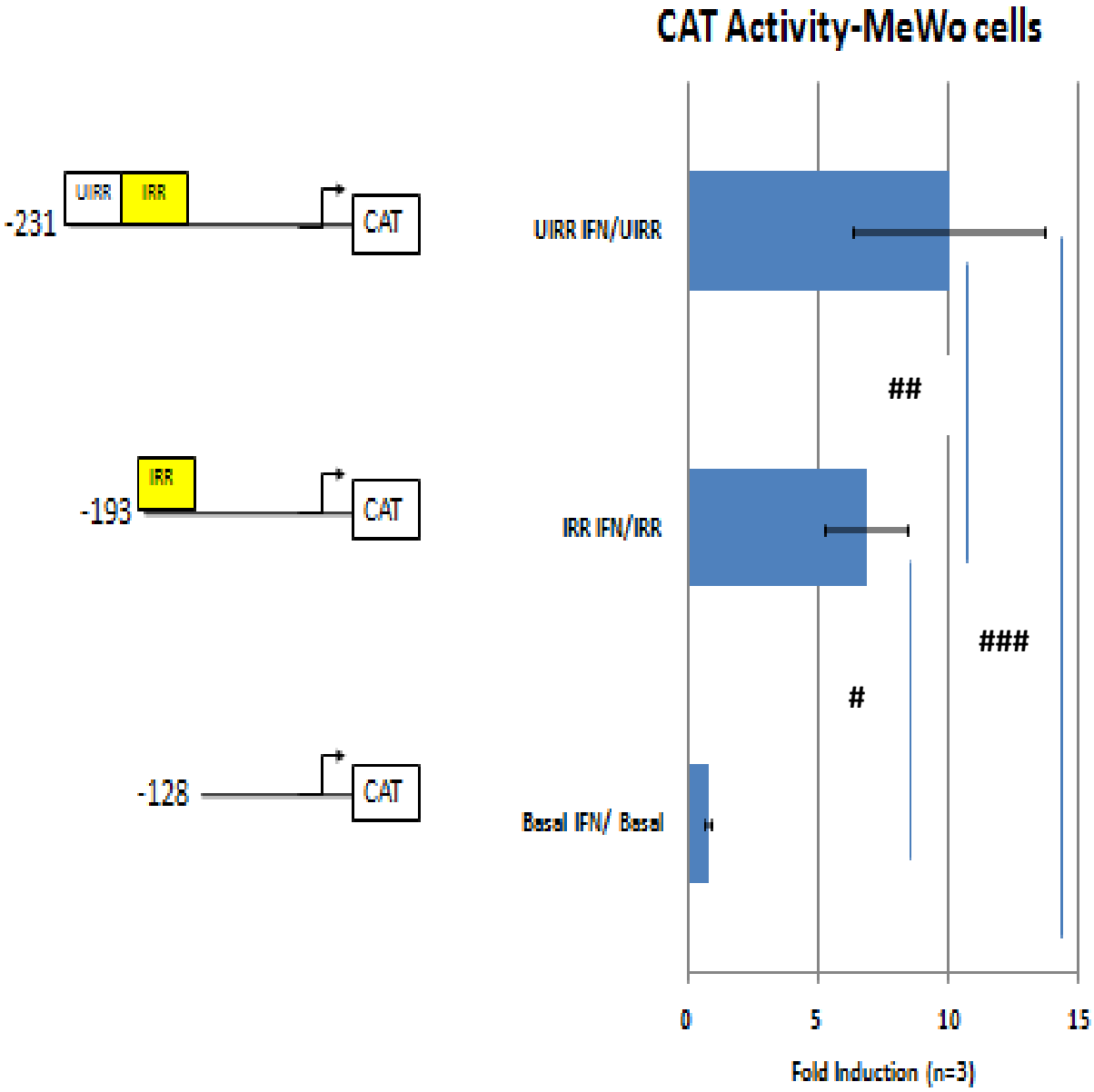



**Figure 16. MeWo cells do not support UIRR function.** MeWo cells were transfected with CAT reporter gene plasmids prior were plated overnight and then transfected with CAT reporter gene plasmids as indicated in Figure 4. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. # p= 0.06; ## p= 0.48; p= 0.12

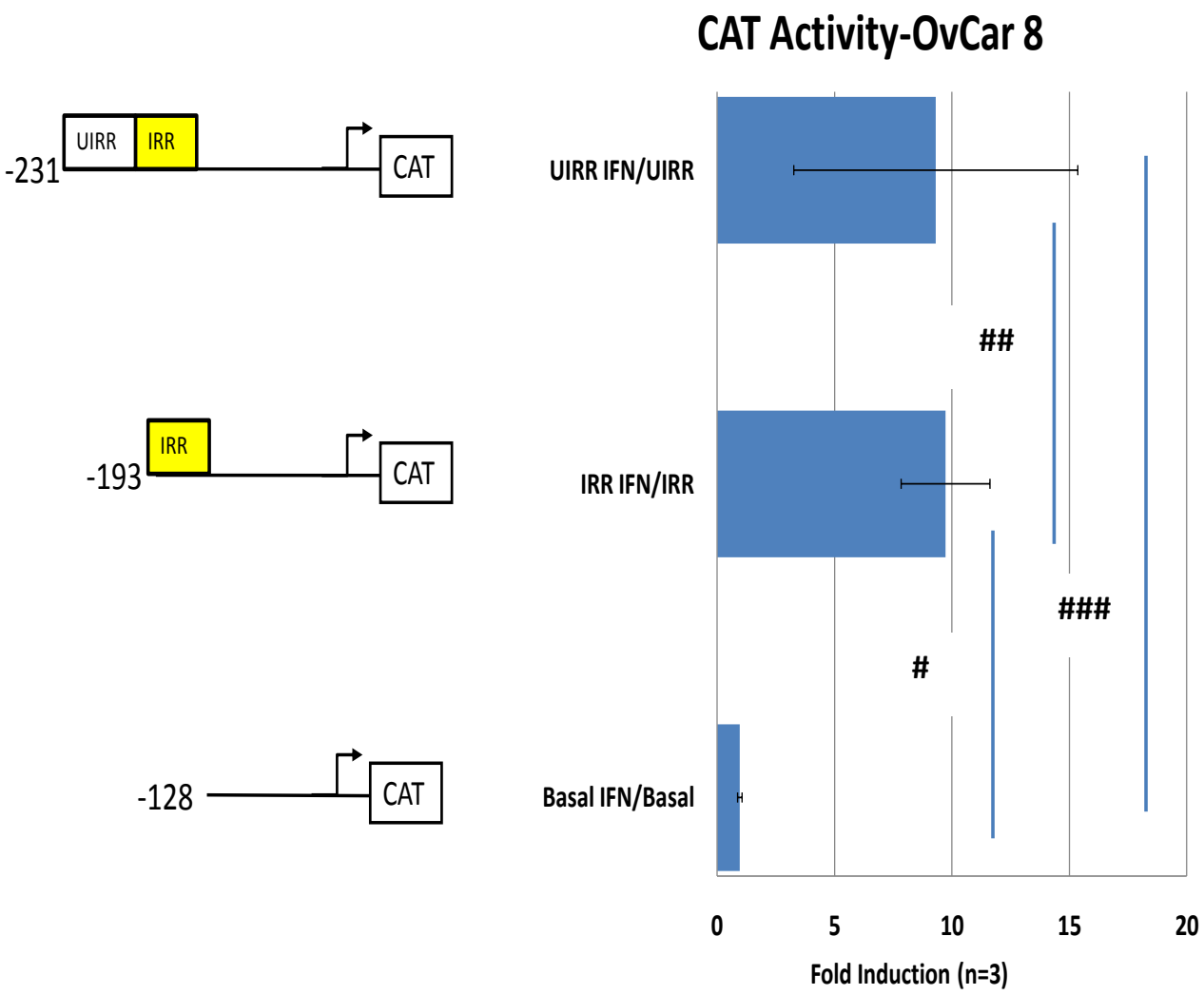



**Figure 17. OvCar 8 cells do not support UIRR function.** OvCar 8cells were transfected with CAT reporter gene plasmids prior were plated overnight and then transfected with CAT reporter gene plasmids as indicated in Figure 4. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. # p= 0.04; ## p= 0.95; ### p=0.1

**Mutation of the GATA binding site**

The A2780 and the SKOV3 cell lines which have been shown to express GATA-2 and GATA-5 (A2780) or GATA-6 (SKOV3) were transfected with plasmids in which the GATA binding site was mutated. Specifically, the GA nucleotides or TA nucleotides were mutated to a CC. In the case of A2780, the mutated binding site resulted in a reduction of CAT activity to a 2.9 fold increase in CAT activity when the GA nucleotides were altered or a 3.0 fold increase in CAT activity when the TA nucleotides were substituted. This amount of CAT activity was similar to the CAT activity when the IRR plasmid was transfected alone. Thus mutating the GATA binding site caused a 4 fold decrease in CAT activity when compared to wild type UIRR (Figure 18). When the SKOV3 cell line was transfected with CAT reporter plasmids containing GATA binding site mutations there was a significant decrease in CAT activity. Specifically mutating the GA binding site caused an eight fold decrease whereas mutating the TA portion caused a fourteen fold decrease in interferon induced CAT reporter gene activity (Figure 19). This was particularly interesting since there was only a 3 fold increase in CAT reporter gene activity when comparing interferon induction between the IRR plasmid and the UIRR plasmid. Mutation of the GATA element should prevent binding of the GATA transcription family members. Binding partners may exist that facilitate protein-protein interactions between the GATA factor on the UIRR element and the STAT homodimer on the IRR element. Perhaps the lack of GATA transcription factor binding prevents interaction with the STAT molecules and therefore causes an even lower interferon induction than with the IRR alone. This large decrease could also indicate the need for other binding partners in the A2780 cell line as compared to the SKOV3 cell line.

Another possibility is that the cell lines express the same binding partners but that these factors do not have the ability to interact with GATA-2 or GATA-5. Note that SKOV3 expresses GATA-6.

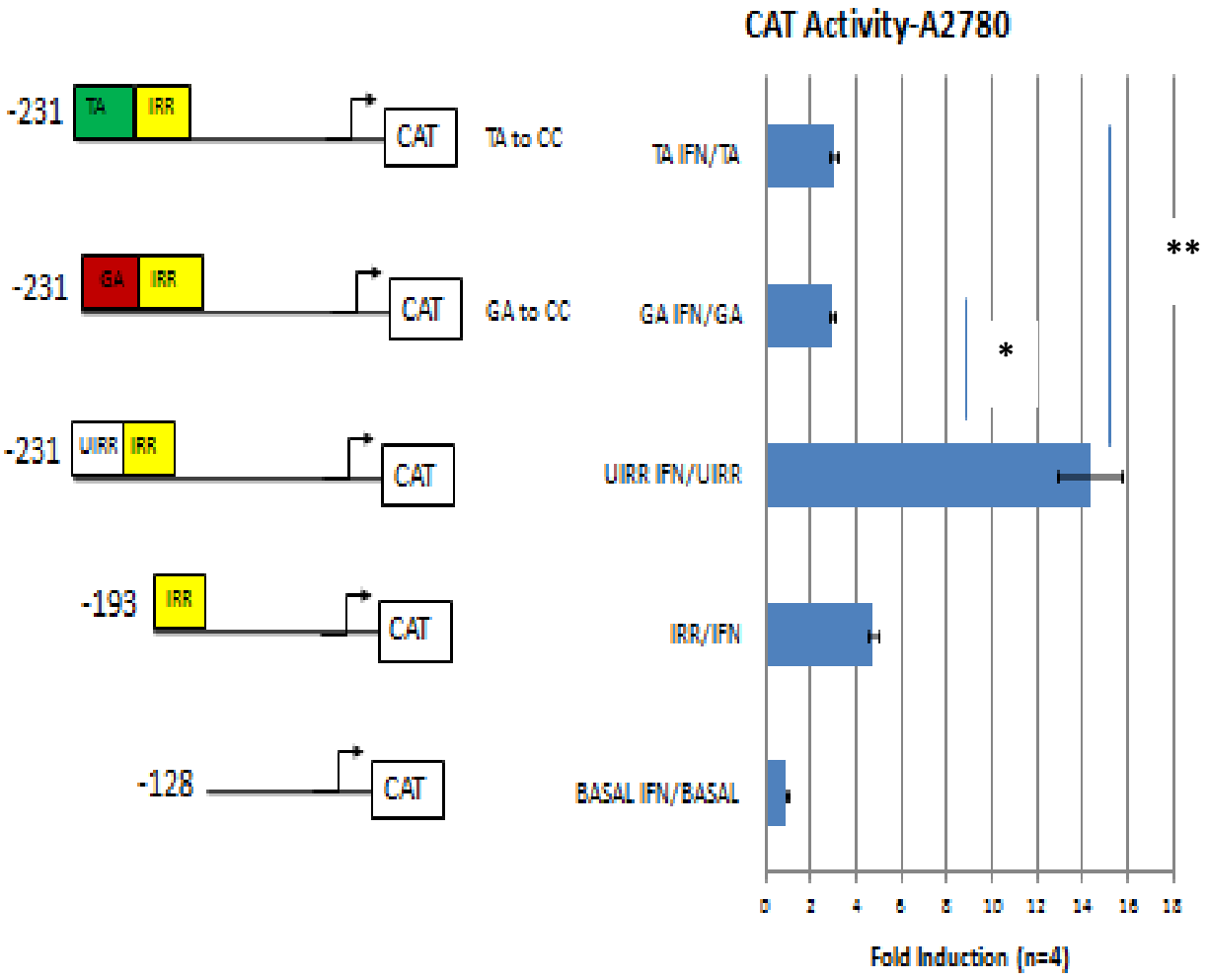



**Figure 18. Mutation of the GATA binding element eliminates the ability of A2780 cells to support UIRR function.** A2780 cells were transfect with CAT reporter gene plasmids. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. Expression was normalized to β-gal. * p= 0.004; ** p= 0.004

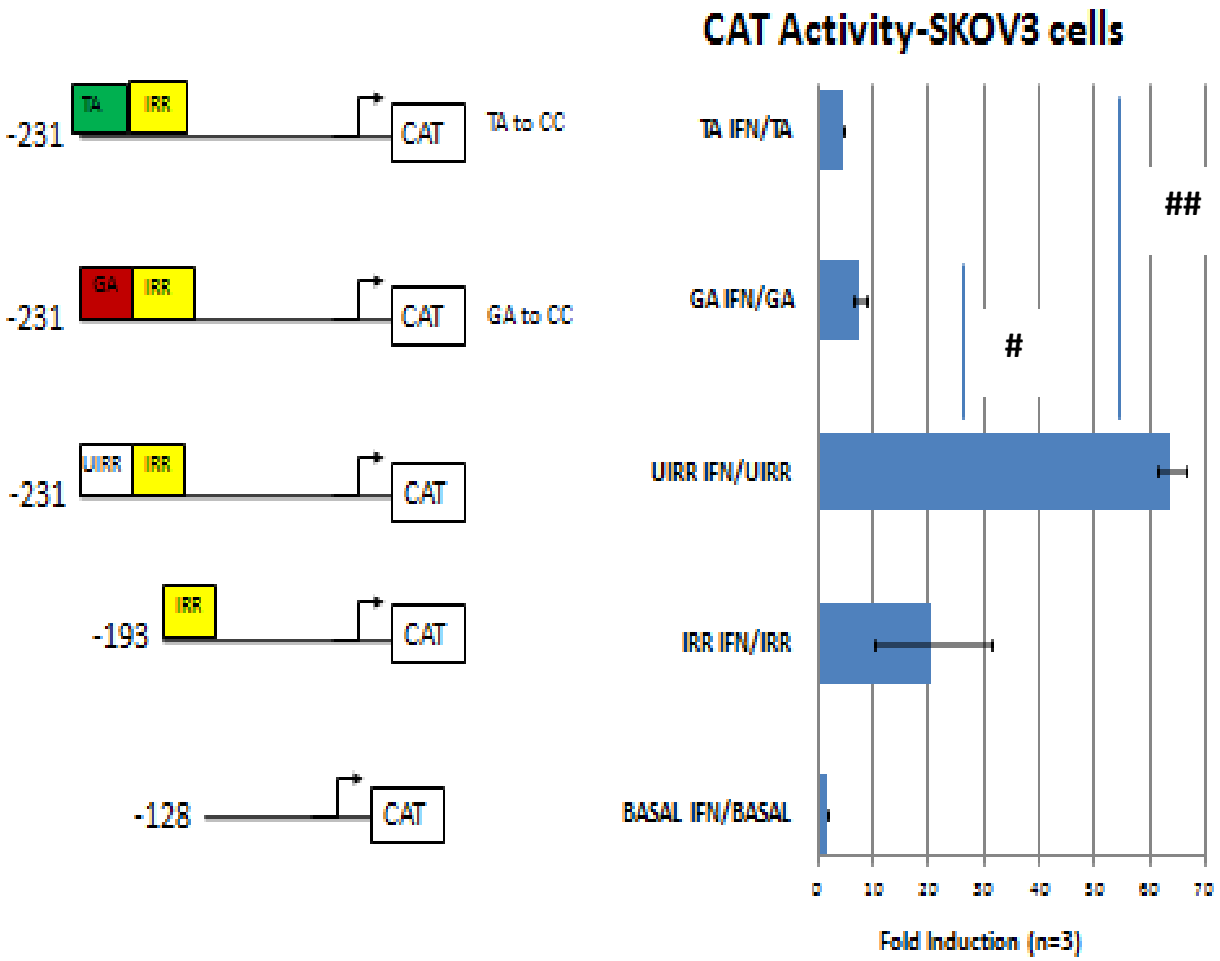



**Figure 19. Mutation of the GATA binding element eliminates the ability of SKOV3 cells to support UIRR function.** SKOV3 cells were transfect with CAT reporter gene plasmids as indicated in Figure 4. Cells were stimulated with 200 units/ml of IFN-γ for 24 hours. Cells were then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid. Expression normalized to β-gal. # p= 0.15; ## p= 0.16

**Knockdown of GATA Transcription Factor Expression**

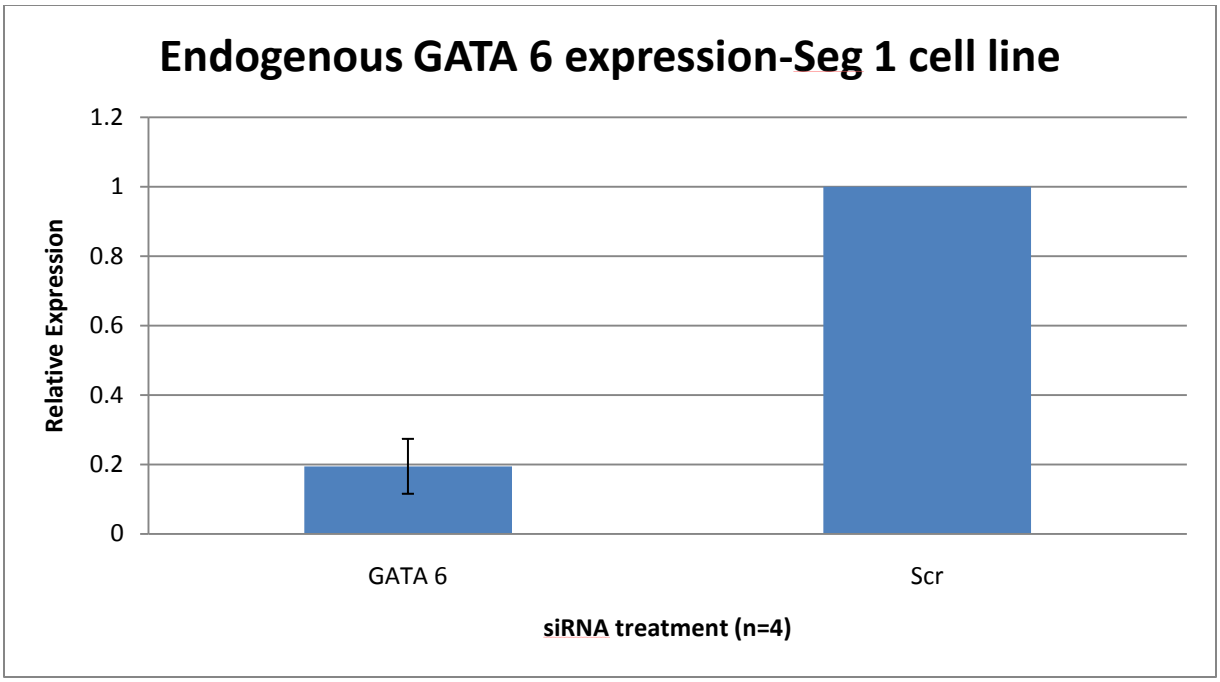
As a complement to the mutation studies we ablated the expression of GATA expression to determine the effect on transient and endogenous HLA-E transcription. The Seg 1 cell line was previously shown to express GATA-6. This cell line was transfected with a GATA-6 specific siRNA targeting. There was an 80% knockdown of the GATA-6 message (Figure 20) which resulted in a 40% reduction in the induction of HLA-E gene transcription when compared to wild type (Figure 21). STAT 1 alpha siRNA, which is required for HLA-E induction, was knocked down with a specific siRNA as a positive control. The expression of STAT 1 alpha was decreased by 55% and resulted in approximately a 44% reduction in HLA-E transcription (Figure 20).

The colon carcinoma, HCT 116, was shown to express GATA-6 as well. The expression of GATA-6 was decreased by 60% (Figure 22) while the expression of STAT 1 alpha was decreased by 40% (Figure 22). The decrease of each transcription factor resulted in a 32% decrease in the expression of endogenous HLA-E induction (GATA-6) or no appreciable decrease (STAT1$\alpha$), respectively (Figure 23). The lack of decrease in HLA-E induction is most likely due to an inadequate decrease in STAT expression.

The effect of GATA-6 gene targeting on HLA-E transcription was also examined in the SKOV3 carcinoma cell line. The cells were transfected with a pSuperior.neo vector which contained Tet Repressor binding sites in its promoter which therefore renders the expression of GATA-6 shRNA to be tetracycline regulated. SKOV3TR/GATA6 cells were selected with 500 mg/ml of tetracycline for approximately three weeks. Single cell clones were then selected and expanded. The ability to knockdown the expression of

GATA-6 was determined using qPCR. Clones #1, #34, #49, #58 and #81 were treated with 2 ug/ml of doxycyline for 72 hours prior to quantitation of GATA-6 mRNA. There was a 46.7% decrease in GATA-6 message in clone #1 when treated with doxycyline, an 81.6% decrease in clone #34, a 63.3% decrease in clone #49, and a 79.3% decrease in clone #81 (Figure 24). Some of the clones were also transfected with CAT reporter gene constructs to determine the functional effects of GATA-6 knockdown on the HLA-E promoter. For the clones #1, #8, #32, and #58, a 3 fold decrease in CAT reporter gene activity was detected only when clone #1 was treated with doxycycline for 72 hours followed by stimulation with interferon gamma for 24 hours (Figure 25). In summary, the knockdown of GATA-6 in the cell lines Seg 1, HCT 116, and SKOV3 resulted in a decrease in GATA-6 expression. The decrease in endogenous HLA-E induction was determined to be 40% in Seg 1 cells and 30% in HCT 116 cells. Analysis of HLA-E induction in the SKOV3 GATA-6 knockdown clones compared to their wild-type counterparts did not demonstrate a difference in the induction of HLA-E transcription (Figure 26- 29).

A

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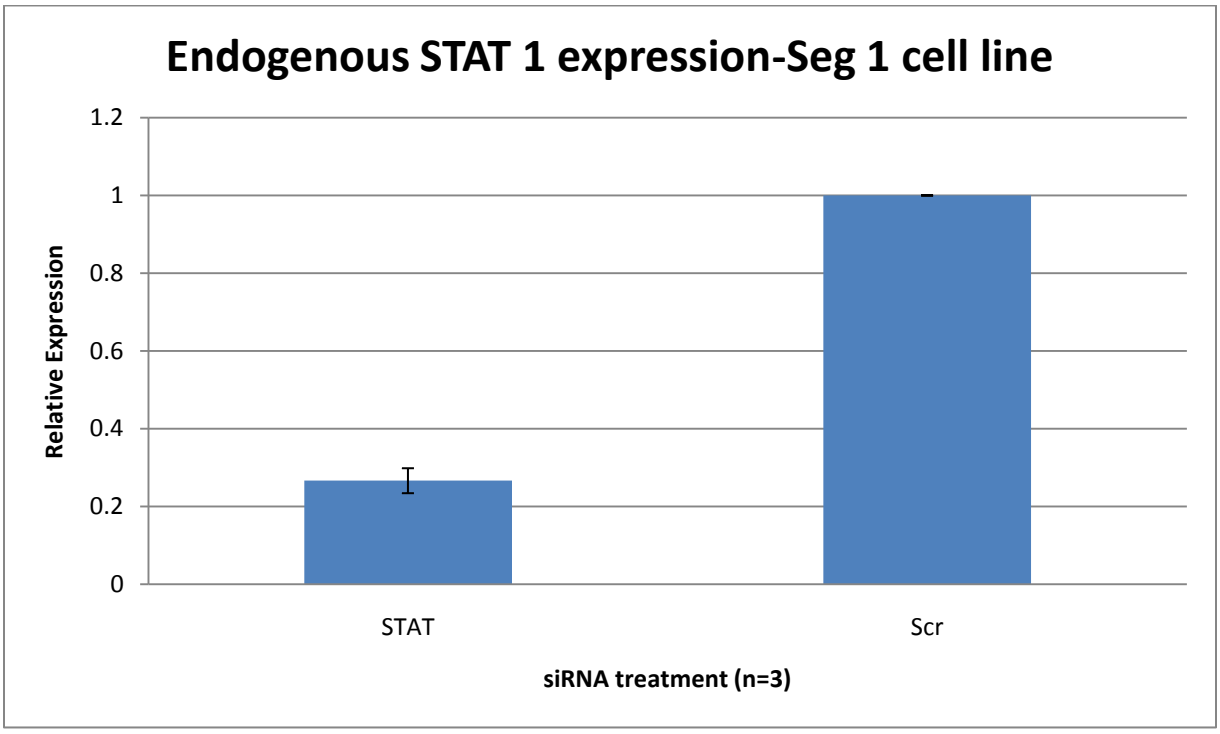



**Figure 20. Gene targeting causes a decrease in GATA-6 and STAT 1α expression in Seg esophageal carcinoma cells.** Seg1 cells were plated overnight and then transfected with siRNA specific for GATA-6, STAT 1α, or scramble control. After 72 hours of exposure to siRNA , the cells were stimulated with 200 units/ml of IFN-γ. Total RNA was obtained from cells and the expression of GATA-6 (A) and STAT 1 α was determined. Target gene expression was normalized to human Cyclophilin A.

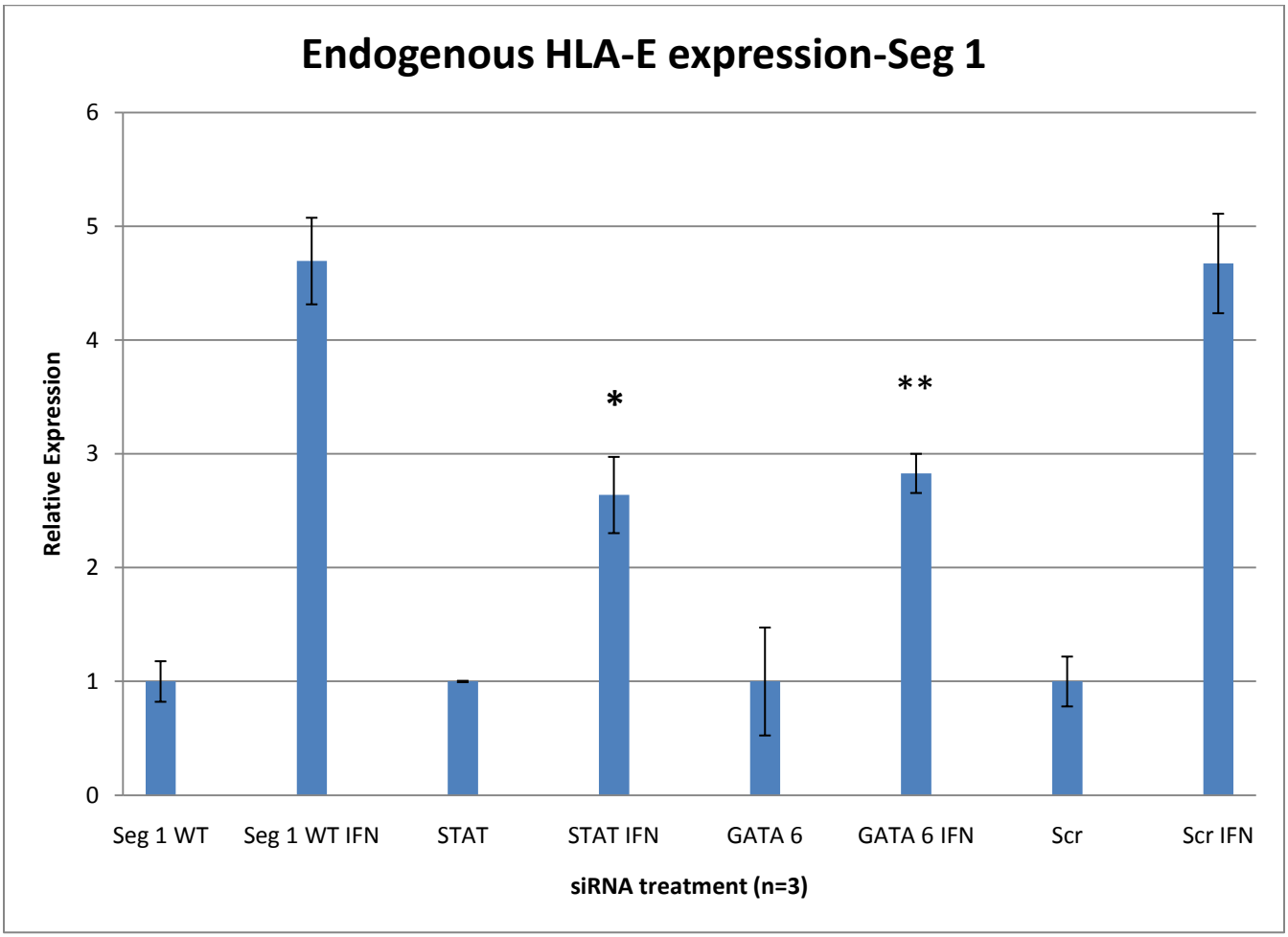
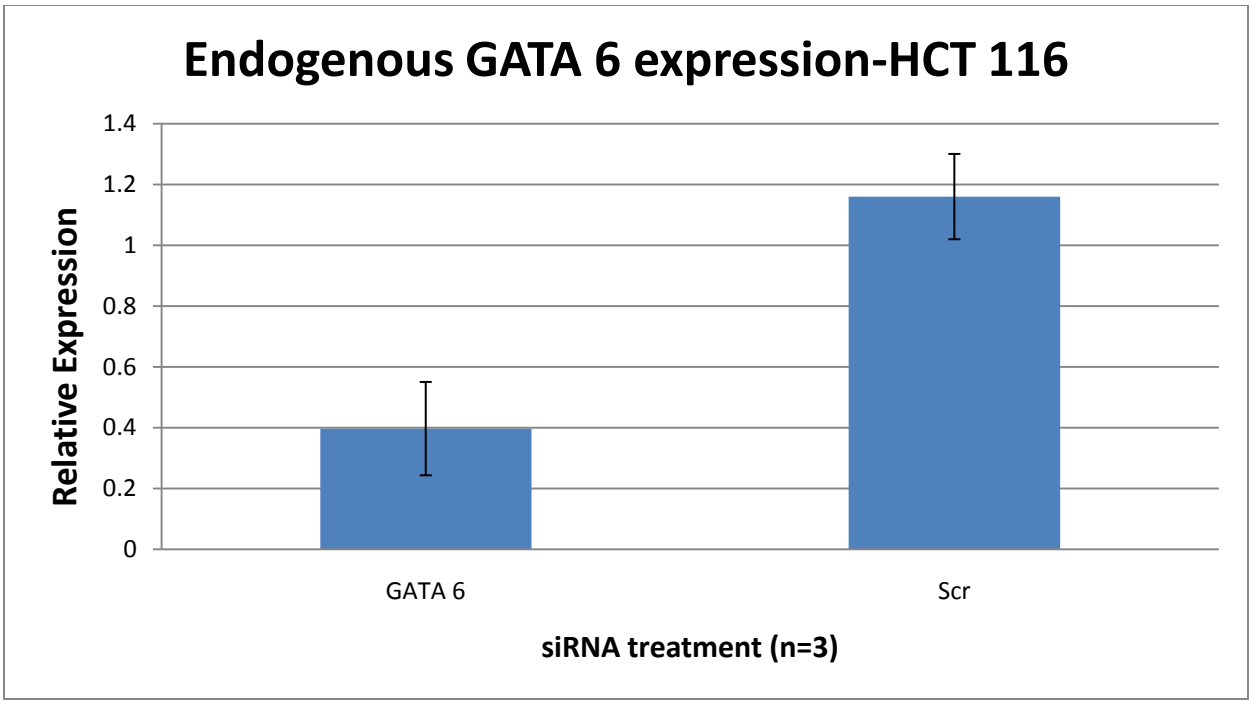



**Figure 21. Effect of gene targeting on Endogenous HLA-E Expression in Seg 1 esophageal carcinoma cells.** Cells were treated as described in Figure 20. Expression was normalized to human Cyclophilin A. The amount of induction was compared to scramble control. * p=0.01; ** p= 0.03

A

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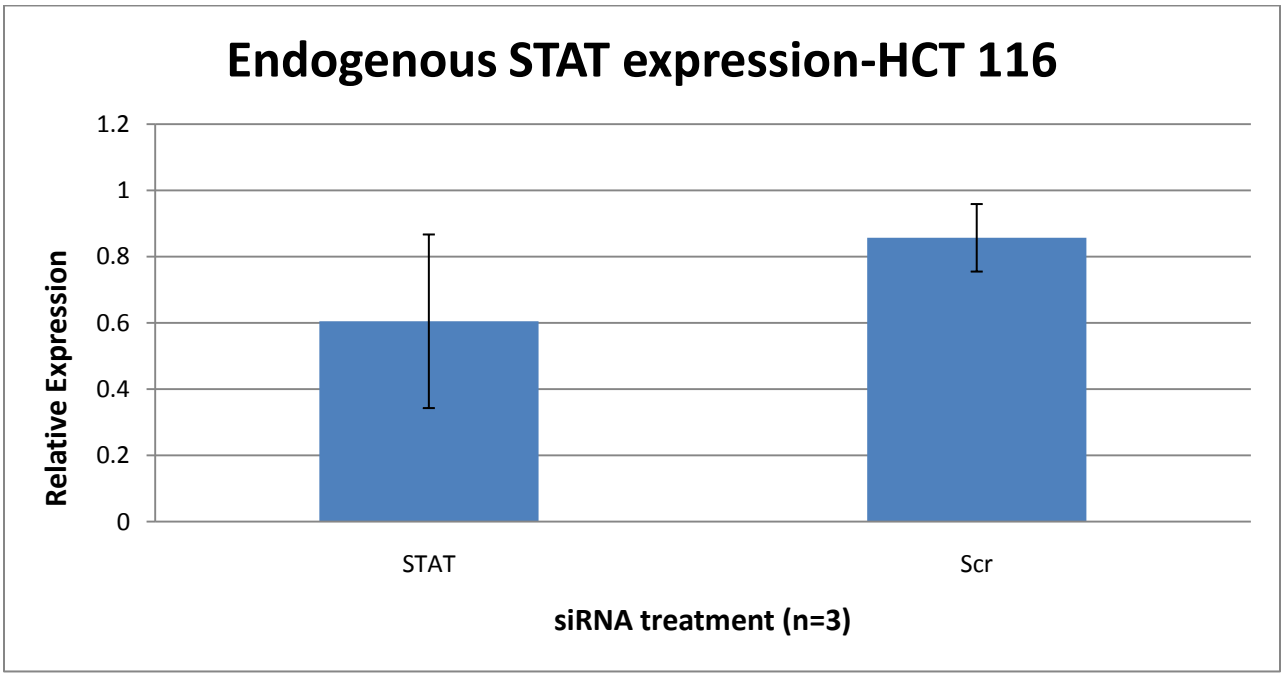


**Figure 22. Gene targeting causes a decrease in GATA-6 and STAT 1α expression in HCT116 colon carcinoma cells.** HCT116 cells were plated overnight and then transfected with siRNA specific for GATA-6, STAT 1α, or scramble control. After 72 hours of exposure to siRNA , the cells were stimulated with 200 units/ml of IFN-γ. Total RNA was obtained from cells and the expression of GATA-6 (A) and STAT 1 α was determined. Target gene expression was normalized to human Cyclophilin A.

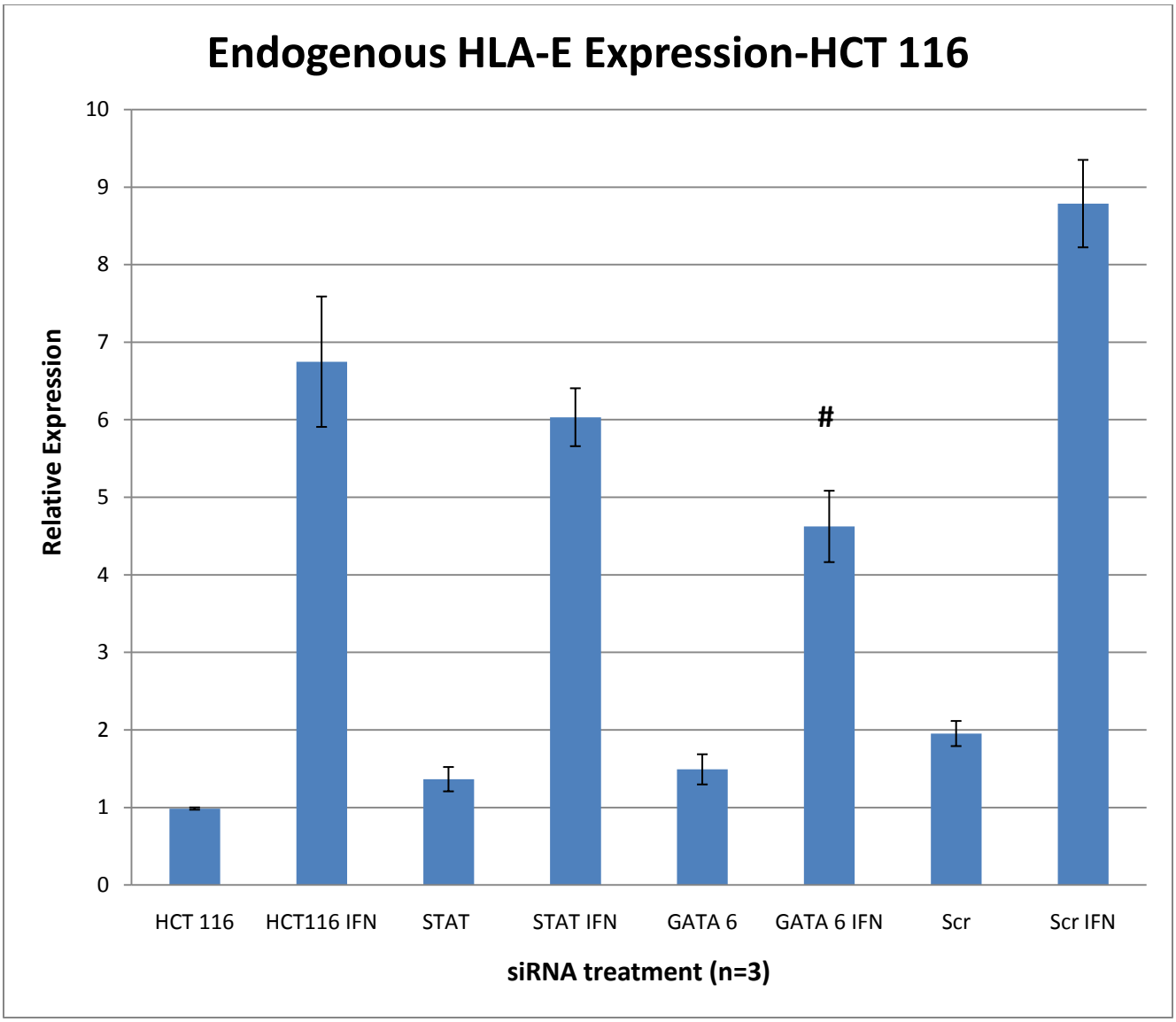


**Figure 23. Effect of gene targeting on HLA-E expression in HCT 116 colon carcinoma cells.** Cells were treated as described in Figure 22. Expression was normalized to human Cyclophilin A. The amount of induction was compared to scramble control. #p= 0.06

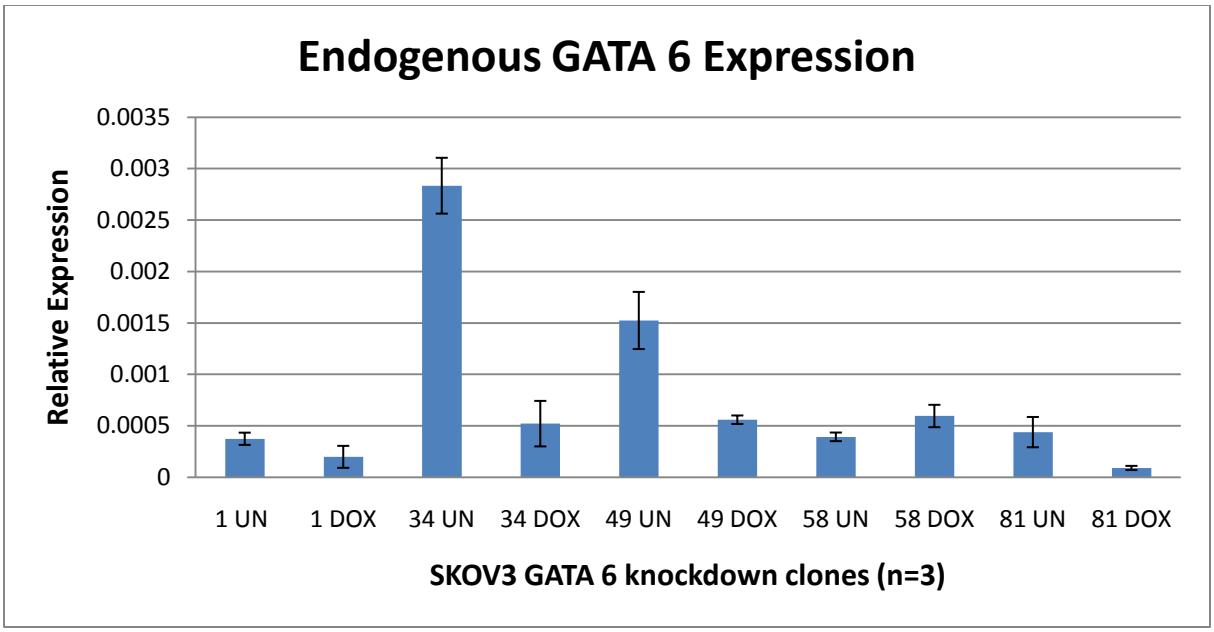


**Figure 24. Analysis of GATA-6 expression in SKOV3 knockdown clones.** Clones were treated for 72 hours with 2ug/ml of doxycyline prior to RNA isolation. qPCR was used to determine GATA-6 expression. Expression was normalized to Cyclophilin A.

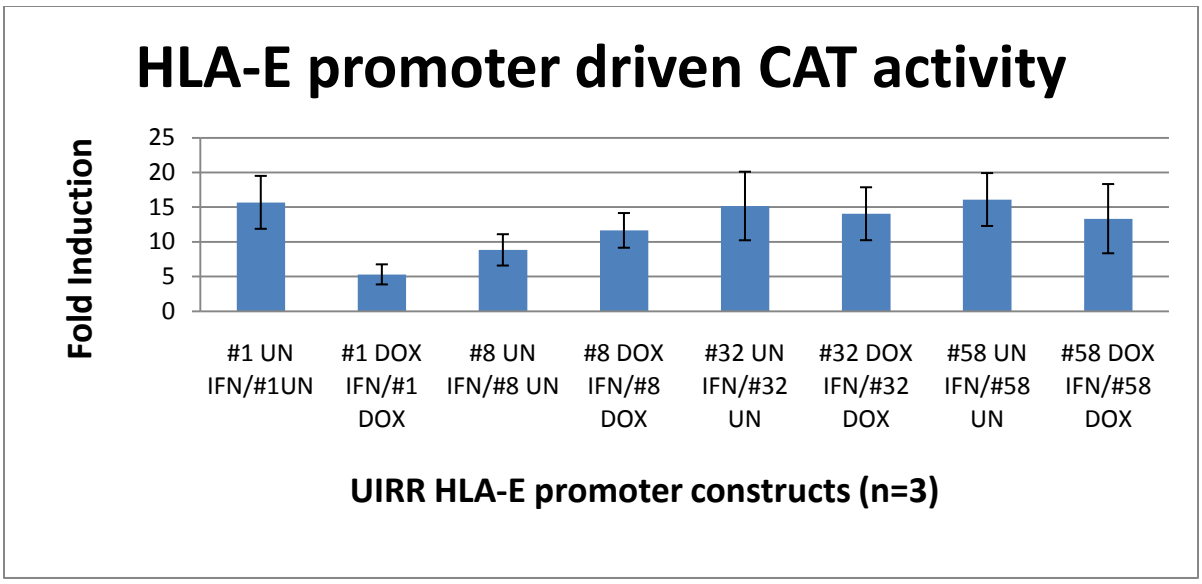


**Figure 25. CAT Activity in SKOV3 Knockdown clones.** Each clone was treated with 2ug/ml of doxycycline prior to transfection with CAT reporter gene plasmids. Six hours later cells were stimulated with IFN-γ. Cells were stimulated for 24 hrs and then harvested using the freeze-thaw method and the resulting supernatant was used to determine CAT activity. The fold induction is the ratio of IFN stimulated cells compared to unstimulated cells that were transfected with the same plasmid.

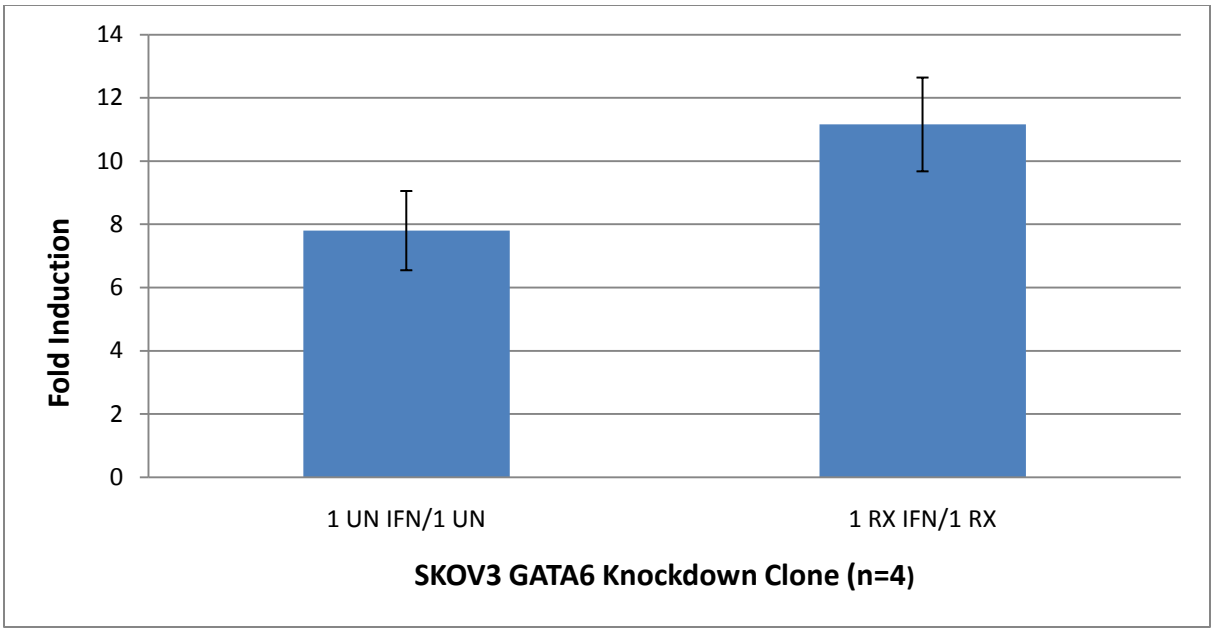


**Figure 26. Analysis of Endogenous HLA-E induction in SKOV3 GATA-6 knockdown clone #1.** Clones were treated for 72 hours with 2ug/ml of doxycycline prior to stimulation with IFN-γ for an additional 24 hr. Total RNA was isolated and qPCR was run for 40 cycles. Expression was normalized to Cyclophilin A.

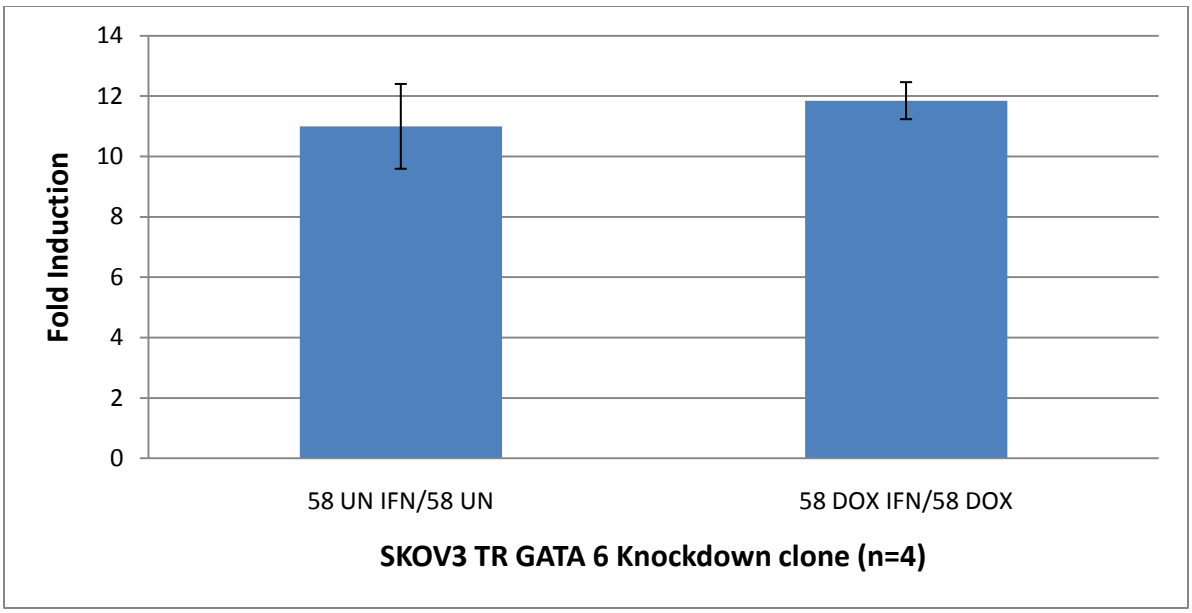


**Figure 27. Analysis of Endogenous HLA-E induction in SKOV3 GATA-6 knockdown clone #58.** Clones were treated for 72 hours with 2ug/ml of doxycycline prior to stimulation with IFN-γ for an additional 24 hr. Total RNA was isolated and qPCR was run for 40 cycles. Expression was normalized to Cyclophilin A.

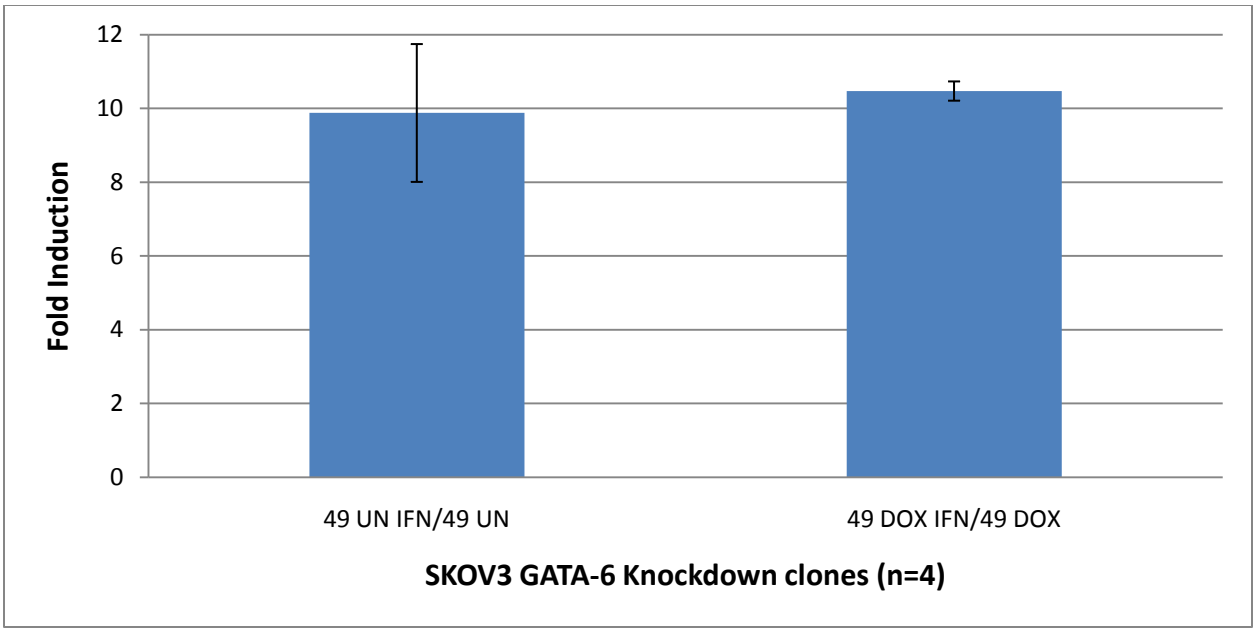


**Figure 28. Analysis of Endogenous HLA-E induction in SKOV3 GATA-6 knockdown clone #49.** Clones were treated for 72 hours with 2ug/ml of doxycycline prior to stimulation with IFN-γ for an additional 24 hr. Total RNA was isolated and qPCR was run for 40 cycles. Expression was normalized to Cyclophilin A.

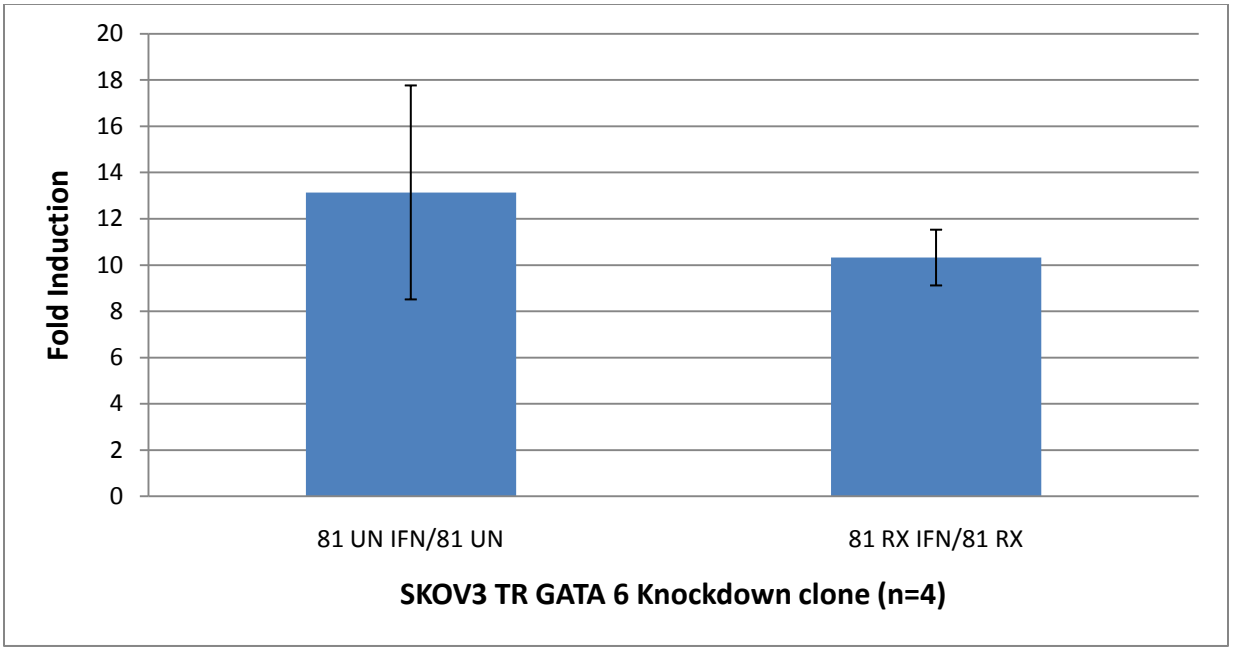


**Figure 29. Analysis of Endogenous HLA-E induction in SKOV3 GATA-6 knockdown clone #81.** Clones were treated for 72 hours with 2ug/ml of doxycycline prior to stimulation with IFN-γ for an additional 24 hr. Total RNA was isolated and qPCR was run for 40 cycles. Expression was normalized to Cyclophilin A.

**Overexpression of GATA Factors**

The parallel gain of function experiment was also performed in the GATA-6 null cell lines OvCar 8 and MeWo. These cell lines were transfected with pCDNA4/TO overexpression plasmids which contained cDNA for mGATA4 or hGATA6. The transfected cells were selected with 100 ug/ml of Zeocin and then pooled. Each cell line was tested for GATA factor expression via qPCR and Western Blot analysis.

The OvCar 8 cell line was shown to express very low levels of GATA-2 and GATA-6 message, but was incapable of supporting UIRR function. Therefore we wanted to determine if UIRR functionality could be achieved by ectopically expressing GATA-4 and/or GATA-6 in the OvCar 8 cell line. The resulting overexpression pools were designated OvCar M4, OvCar H6 or OvCar M4H6 and were examined for GATA-4 and GATA-6 expression via qPCR. The OvCar M4 pools and the OvCarM4H6 pools showed an 8 fold and 14 fold increase in GATA4 expression compared to vector alone (Figure 31 A). Additionally, expression of GATA6 transcript was 4 fold higher in the OvCar H6 pools compared to vector alone (Figure 32 A). Examination of protein expression showed no discernable difference in GATA4 expression between the OvCar vector, OvCar M4 and OvCar M4H6 cell lines (Figure 33A). In fact, GATA-4 expression was detected in all three overexpression pools despite the lack of detectable GATA4 transcript in the OvCar vector pool or wild-type OvCar 8. Similarly GATA-6 protein was detected in the OvCar vector pool. However there was a clear increase in GATA-6 protein expression in both the OvCar H6 and OvCar M4H6 overexpression pools (Figure 33B).

Each overexpression pool was transfected with a dual CAT reporter gene plasmid and then CAT gene expression was determined by qPCR. Gene expression was normalized to the Cyclophilin A housekeeping gene and transfection efficiency was determined by dividing the expression of the CAT gene to the expression of the GFP gene. There was a 3.8 fold increase in CAT gene expression read from the UIRR plasmid above that of the IRR plasmid detected in the OvCar M4 cell line (Figure 31C). Mutation of the GATA binding site resulted in a reciprocal decrease in CAT gene expression. We were unable to detect a difference in CAT gene activity in the OvCar H6 or OvCar M4H6 cell lines (Figures 32C and 33C). Finally we analyzed the effect of GATA-4 and GATA-6 overexpression on endogenous HLA-E gene induction. We did not observe a difference in HLA-E induction when comparing the overexpression pools to the vector control (Figure 34).

The melanoma cell line MeWo was also transfected with overexpression plasmids for GATA-4, GATA-6, and GATA-4 plus GATA-6. After a three week selection period, the cells were pooled and analyzed for mRNA transcript levels and protein expression. The expression of GATA-4 and GATA-6 was evaluated via qPCR. Analysis of GATA-4 gene expression in the MeWo vector, MeWo H6, and the MeWo M4H6 cell lines revealed a 40 fold increase in GATA-4 expression in the MeWo M4 cell line above that of the vector control (Figure 35A). Similarly, GATA6 transcript levels were similar 4 fold higher and 30 fold higher in the MeWo H6 and MeWo M4H6 cell lines, respectively (Figure 36A). We also determined that GATA-4 protein levels were 2 fold higher in the MeWo M4 and MeWo M4H6 cell lines (Figure 37A). GATA-6 protein levels were also approximately two fold higher in the MeWo H6 and MeWo M4H6 cell lines (Figure 38B).

The MeWo overexpression pools were transfected with CAT reporter gene plasmids, stimulated with 200 units/ml of IFN-γ, and the analyzed for CAT gene expression via qPCR. MeWo H6 cells showed a 3.5 fold induction in CAT gene expression when cells were transfected with the IRR plasmid and a 3.0 fold increase in CAT gene expression with the UIRR plasmid (Figure 36C). However, we were unable to detect IFN induction in the either the MeWo M4 or MeWo M4H6 cells (Figure 35C and Figure 37C). The endogenous expression of HLA-E was also examined in the MeWo overexpression pools. The MeWo M4H6 cell line exhibited a 3 fold higher induction than MeWo vector, but surprisingly the MeWo M4 cell line showed no enhancement in HLA-E transcription when compared to the MeWo vector control (Figure 38).

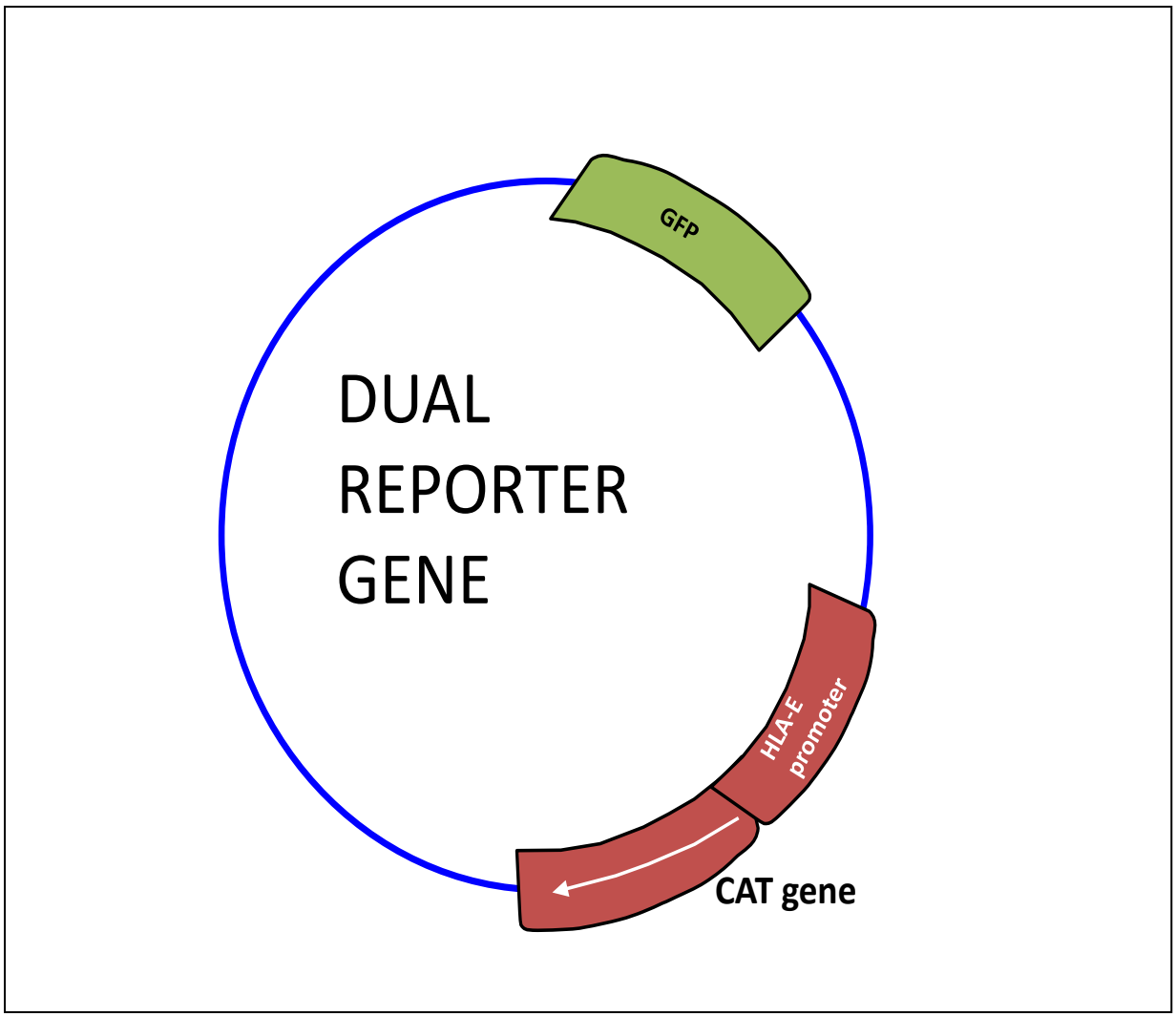


**Figure 30. Schematic of the HLA-E promoter driven dual reporter gene.** Overexpression pools were transfected with plasmids designated as Basal, IRR, UIRR, or MUT. CAT expression was normalized to GFP expression to determine transfection efficiency.

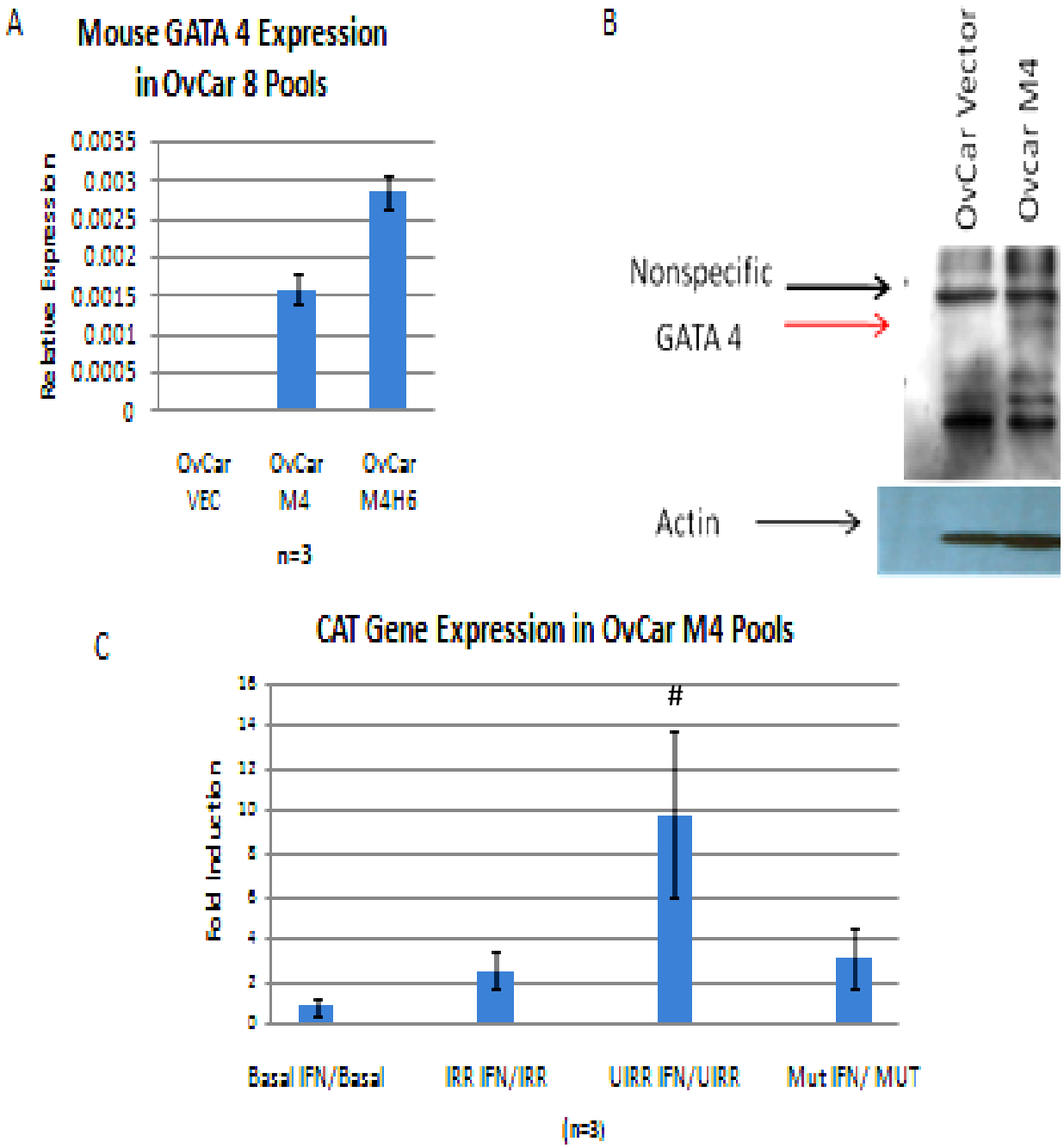


**Figure 31. Evaluation of GATA4 Overexpression in OvCar ovarian cancer cells.** A. Total RNA was harvested from OvCar 8 cells transfected with vector alone, GATA 4 or GATA4 and GATA6. Expression was normalized to human Cyclophilin A. B. GATA 4 protein expression was analyzed by western blot. C. HLA-E promoter driven gene expression was analyzed as described earlier. # p= 0.20 (Comparison of UIRR induction to IRR induction).

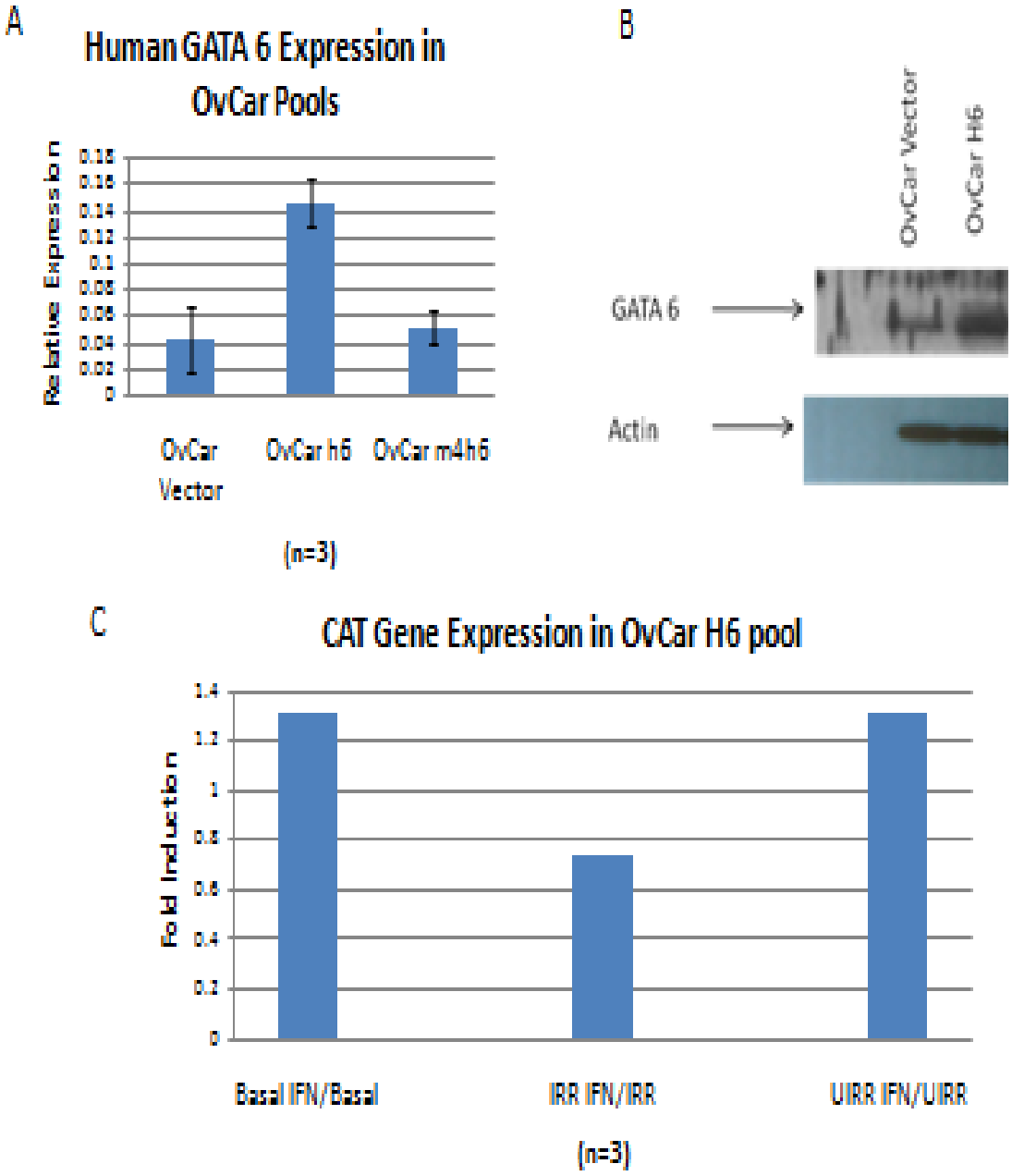


**Figure 32. Evaluation of GATA6 Overexpression in OvCar ovarian cancer cells.** A. Total RNA was harvested from OvCar 8 cells transfected with vector alone, GATA 6 or GATA4 and GATA6. Expression was normalized to human Cyclophilin A. B. GATA 6 protein expression was analyzed by western blot. C. HLA-E promoter driven gene expression was analyzed as described earlier.

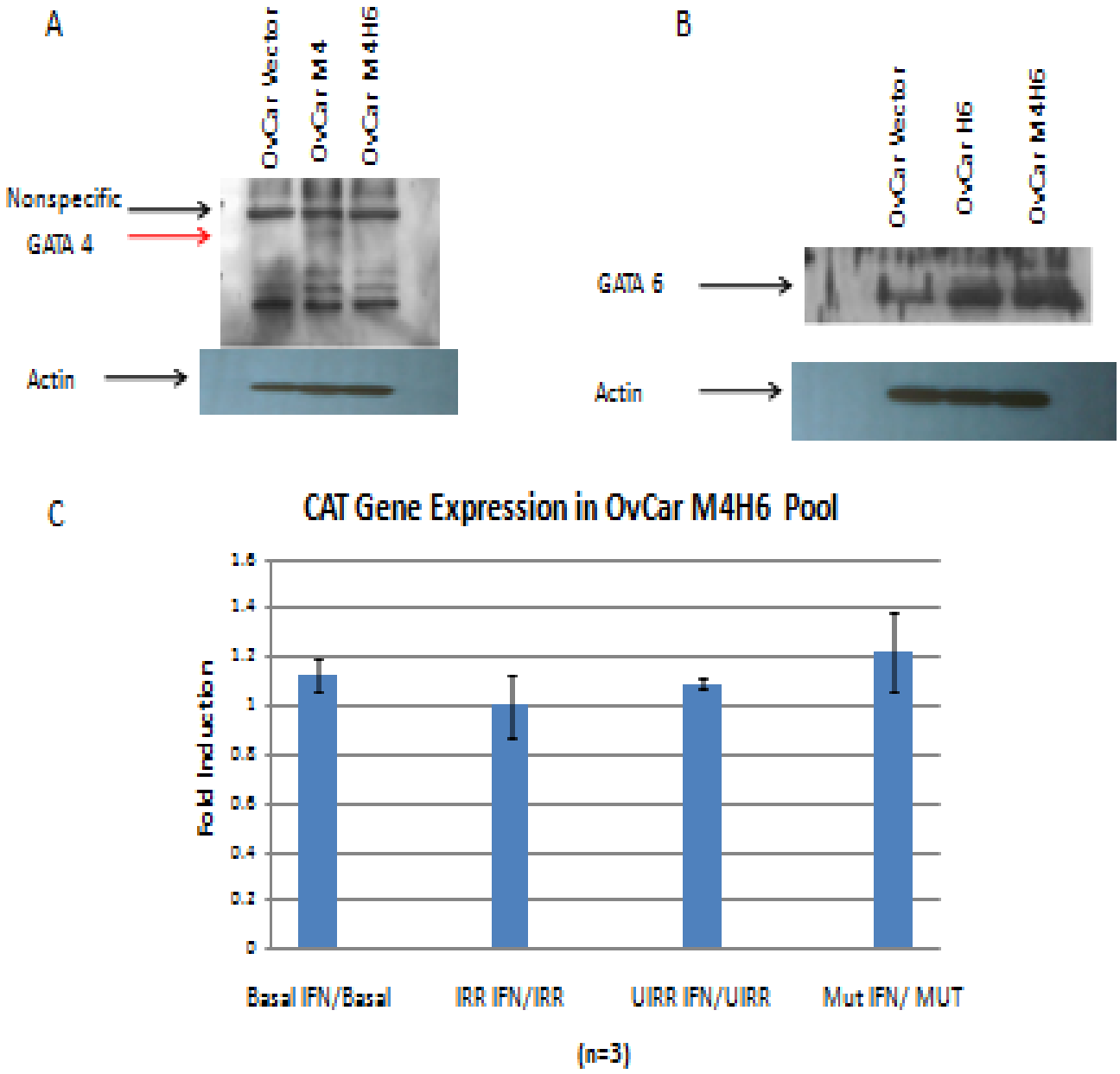


**Figure 33. Evaluation of GATA4 and GATA6 Overexpression in OvCar ovarian cancer cells.** A. GATA-4 protein expression was examined in OvCar M4H6 pools by western blot. B. GATA-6 protein expression was analyzed in OvCar M4H6 cells by western blot. C. HLA-E promoter driven gene expression was analyzed as described earlier.

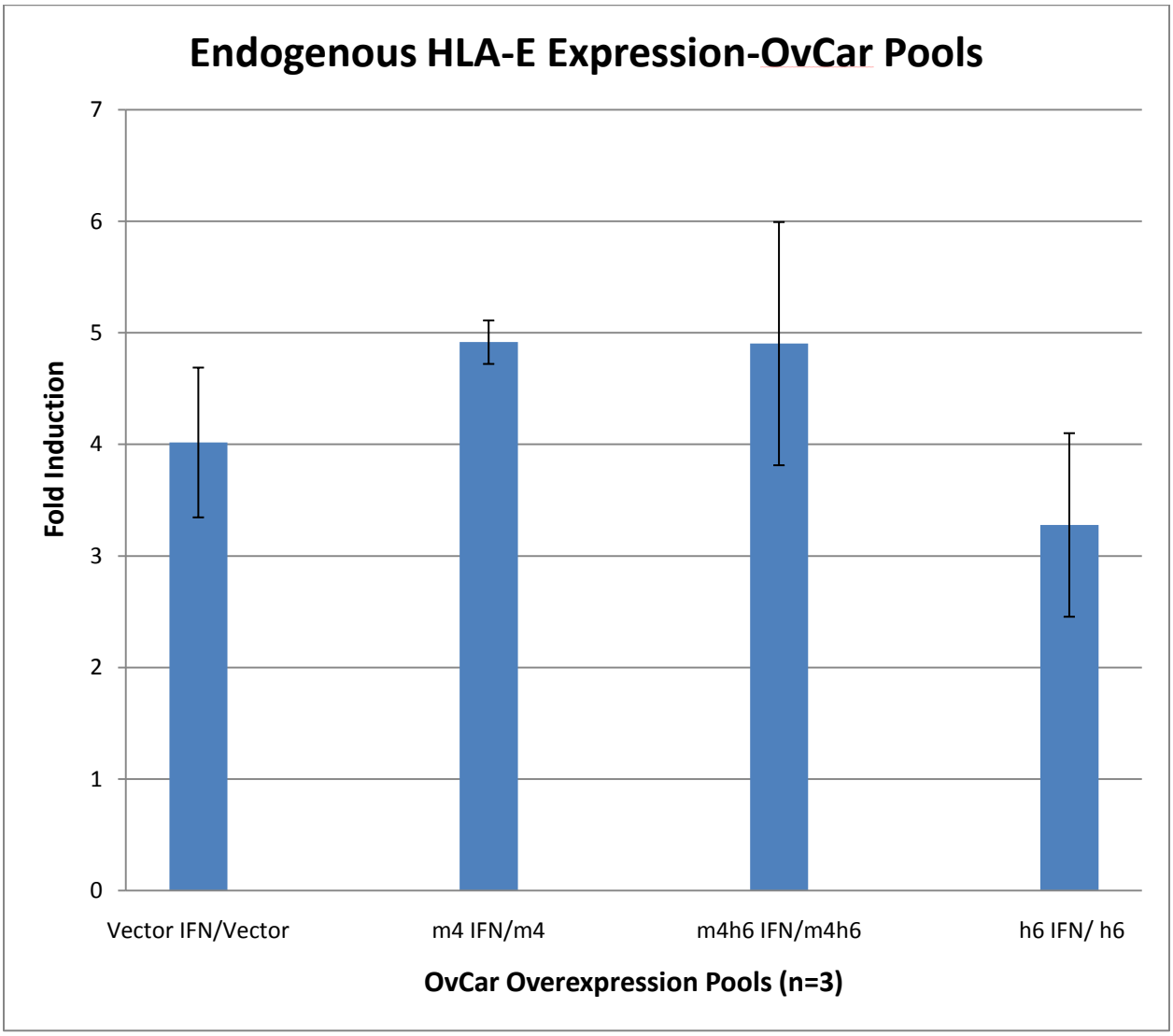


**Figure 34. Analysis of HLA-E induction in OvCar Overexpression Pools.** Cells were treated for 24 hours with 200 units/ml of IFN-γ prior to RNA isolation. Expression was normalized to human Cyclophilin A.

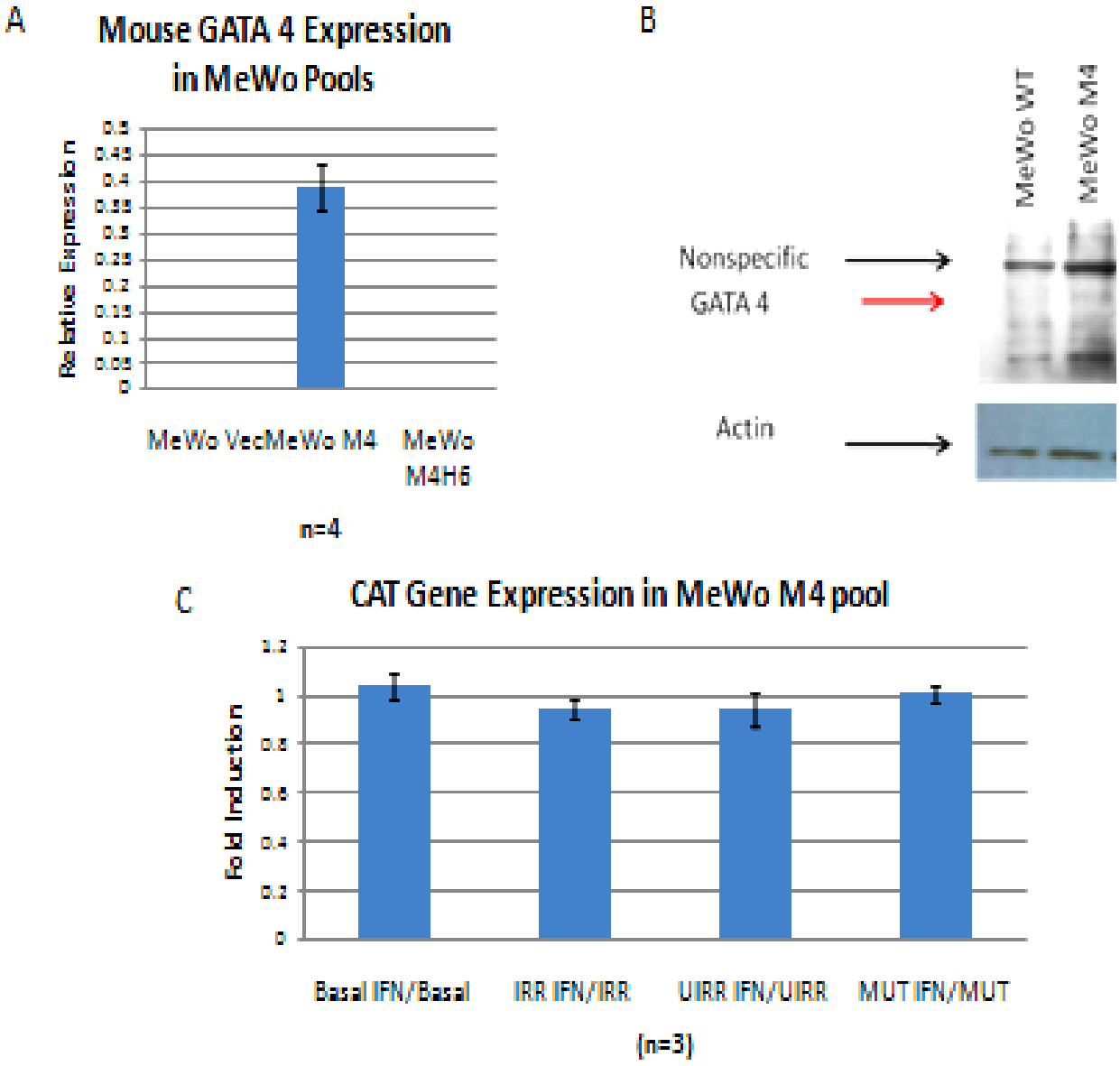


**Figure 35. Evaluation of GATA4 Overexpression in MeWo melanoma cells.** A. Total RNA was harvested from MeWo cells transfected with vector alone, GATA 4 or GATA4 and GATA6. Expression was normalized to human Cylophilin A. B. GATA 4 protein expression was analyzed by western blot. C. HLA-E promoter driven gene expression was analyzed as described earlier.

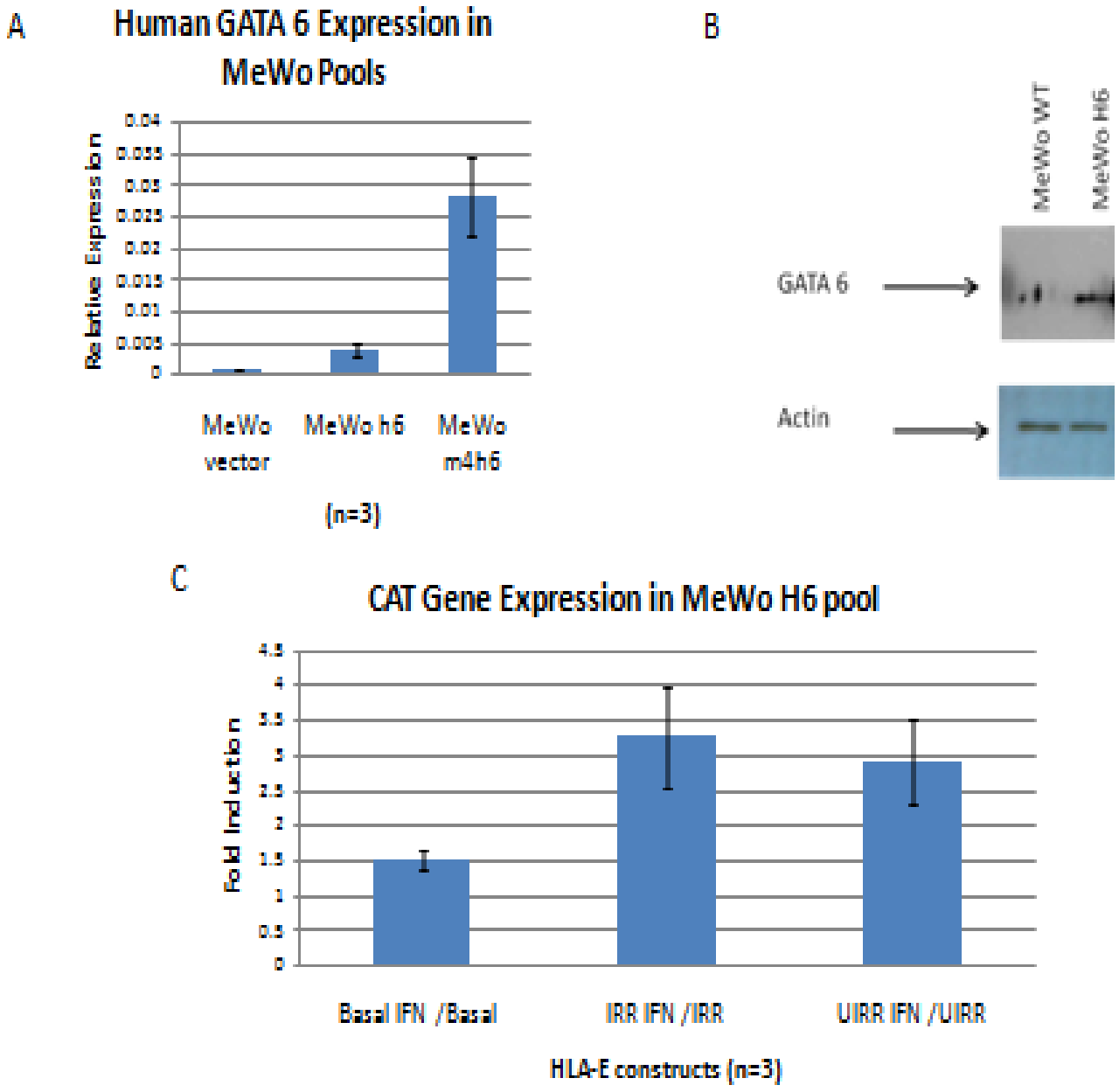


**Figure 36. Evaluation of GATA6 Overexpression in MeWo melanoma cells.** A. Total RNA was harvested from OvCar 8 cells transfected with vector alone, GATA 4 or GATA4 and GATA6. Expression was normalized to human Cylophilin A. B. GATA 6 protein expression was analyzed by western blot. C. HLA-E promoter driven gene expression was analyzed as described earlier.

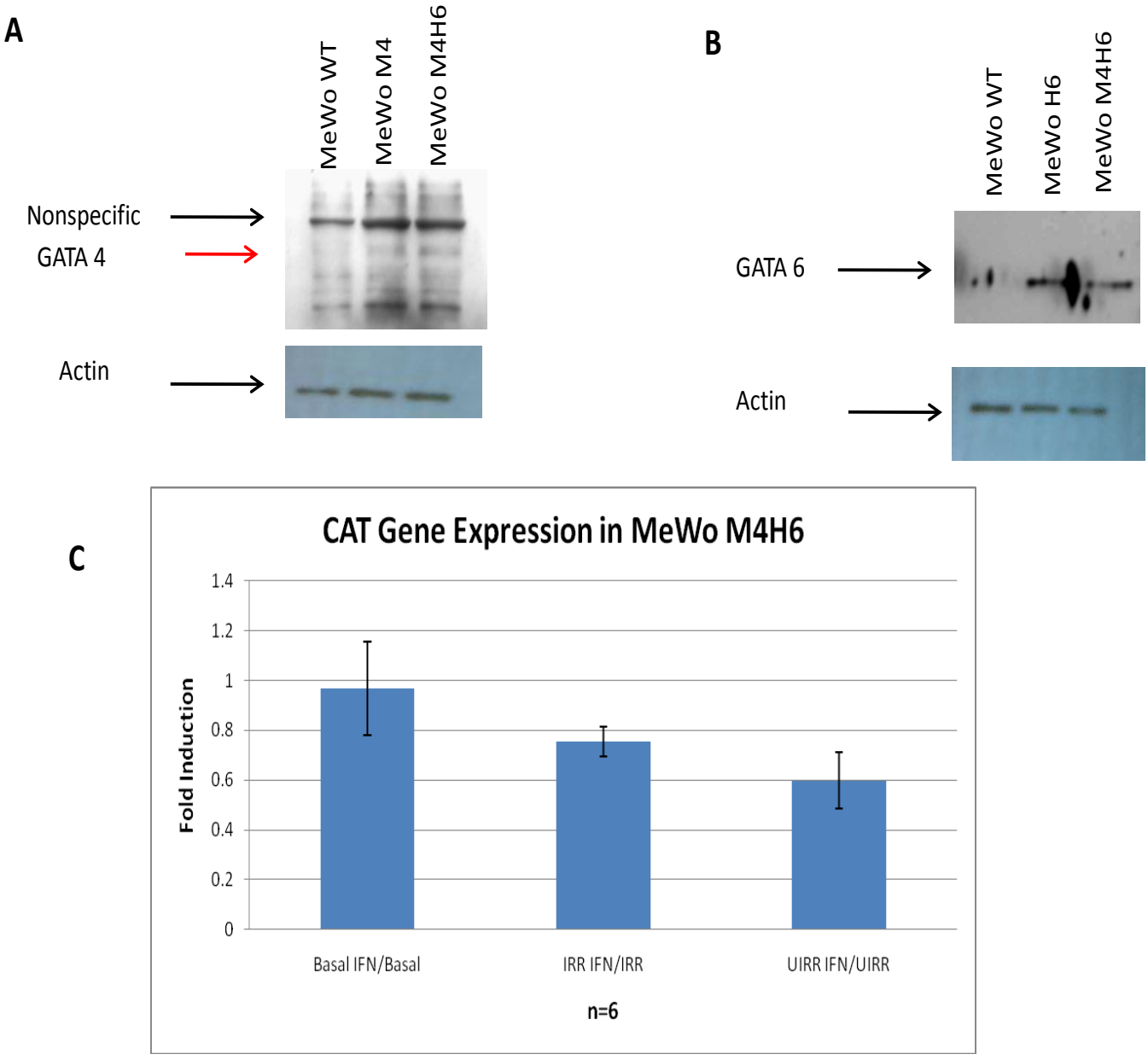


**Figure 37. Evaluation of GATA4 and GATA6 Overexpression in MeWo melanoma cells.** A. GATA-4 protein expression was examined in MeWo M4H6 pools by western blot. B. GATA-6 protein expression was analyzed in MeWo M4H6 cells by western blot. C. HLA-E promoter driven gene expression was analyzed as described earlier.

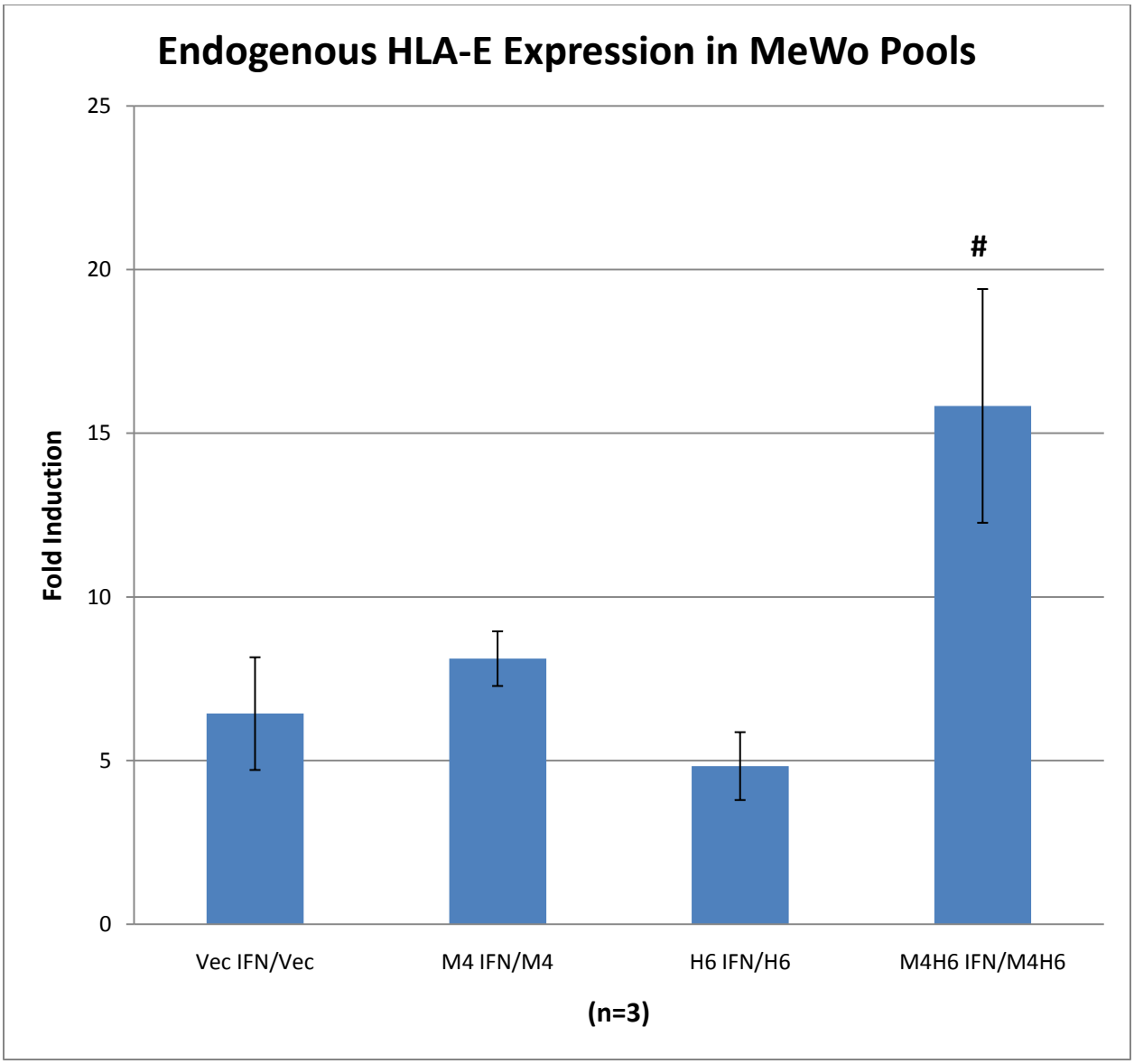


**Figure 38. Analysis of HLA-E induction in MeWo Overexpression Pools.** Cells were treated for 24 hours with 200 units/ml of IFN-γ prior to RNA isolation. Expression was normalized to human Cyclophilin A. MeWo M4H6 exhibited an increased response to IFN-γ compared to vector control. # p=0.26

## Discussion

In this study we have shown that GATA transcription family members other than GATA-1 can promote enhancement of HLA-E gene expression in response to IFN-γ. We evaluated the ability of various tumor cell lines to support UIRR function and screened these cell lines for GATA gene expression via qPCR. We determined that the HCT116, Seg1, SKOV3 and A2780 cell lines supported a 2.4 to 3.0 fold increase in CAT reporter gene activity via the UIRR plasmid above that of the IRR plasmid alone. Some of our strongest supporting data is the GATA binding site mutation studies. We demonstrated that alteration of either the GA or TA portion causes a decrease in CAT reporter gene activity in the SKOV3 and A2780 cell lines. Furthermore, we targeted GATA-6 expression in order to ascertain the effect on HLA-E promoter driven CAT reporter gene activity and endogenous HLA-E expression. We used siRNA gene knockdown to decrease the expression of GATA6 in the Seg1 and HCT 116 cell lines. Although we obtained a similar level of transfection efficiency in both cell lines as visualized with an Alexa fluorophore labeled negative siRNA control (data not shown), we were able to achieve greater gene knockdown in the Seg1 cell line. We are currently developing a GATA-6 miRNA construct that will be packaged into a lentivirus vector in hopes that we will be able to achieve a higher level of GATA6 knockdown and therefore observe a greater effect on HLA-E induction.

We also created a stable, tetracycline inducible GATA-6 knockdown cell line, SKOV3TR/GATA6. We selected single cell clones to expand for future studies. One of the benefits of a tetracycline inducible cell line is that the untreated cells are an ideal

negative control. One of the difficulties we experienced with this technology is variable knockdown in the same clones between treatment periods. We demonstrated the decrease of GATA-6 gene expression from a range of 46% to 80% but saw no effect on endogenous HLA-E expression in any of the clones. This suggests that a higher amount of knockdown is needed to see an effect on HLA-E induction. It also raises the question of compensation by another GATA family member. However, this is unlikely since a previous GATA factor protein screen showed that only GATA-6 is expressed in the SKOV3 cell line. Furthermore, there have been no studies that implicate GATA-6 as a negative regulator of the other GATA family members.

We demonstrated the ability to overexpress GATA-4 and GATA-6 proteins in the OvCar 8 and MeWo cell lines. We saw an increase in GATA4 transcript levels in the OvCar M4, OvCar M4H6 and the MeWo M4 cell lines. We also saw an increase in GATA-4 protein expression however the total amount of protein was low. There was also a slightly nonspecific band that ran slight higher than GATA-4. This raises the question if there was recognition of another GATA family member or an antigenically similar 50 kDa protein by the GATA-4 antibody. If so, there may have been an increase in GATA-4 protein levels that is masked by the expression of the other protein. Despite the detection of increased GATA6 protein levels in the OvCar H6 and the OvCar M4H6 cell lines, we saw no effect on CAT reporter gene activity in these cells.

However, it is important to point out that no clear conclusion of CAT gene expression could be made due to subsequent difficulties in visualizing CAT gene induction. Overexpression pools were transfected with 3 ug of CAT reporter gene plasmids prior to

IFN-γ stimulation. However the detection of CAT gene expression in the unstimulated cells was observed at 14-16 cycles of qPCR. This rapid detection suggests very high levels of gene expression and further illustrated the need for transfection of lower amounts of reporter gene plasmid. We repeated our studies using 1ug of plasmid but obtained similar results (data not shown). It is important to note that there were no difficulties in obtaining induction in our initial studies in the OvCar M4 cell line. Future attempts will be made to examine the effect of GATA overexpression on CAT reporter gene expression. We plan to further decrease the amount of DNA transfected into cells. Alternatively, if these experiments prove unsuccessful, we will switch back to evaluating CAT protein activity using our single reporter gene plasmids.

We also demonstrated the ability to increase GATA-4 protein expression in the MeWo M4 and MeWo M4H6 cell lines as well as GATA-6 protein expression in the MeWo H6 and MeWo M4H6 cell lines. Although there was an obvious increase in GATA6 protein levels the absolute amount of protein detected in both cell lines was low. We observed a 3 fold increase of endogenous HLA-E induction in the MeWo M4H6 cell line. The protein data suggests that this effect may be attributed more to GATA-4 overexpression than GATA-6.

GATA transcription family members were named based upon the ability to recognize and bind to WGATAR sequences in the promoters of target genes. There are several examples of different GATA family members regulating the expression of the same gene. For example, both GATA-4 and GATA-5 can regulate the expression of the Atrial Natriuretic Factor 1 in cardiac cells (Takaya 2008). However there are examples where

one GATA factor regulates gene expression, while another does not. For example, only GATA-6 regulates the expression of the Indian hedgehog gene (Ihh) which is required for proper gut development (Haveri 2008). Our present studies indicate that GATA-4, GATA-5, and GATA-6 can regulate HLA-E gene expression. Previous studies indicate that GATA1 binds to the HLA-E promoter in vivo. Although we have observed GATA-4 and GATA-6 interaction with radiolabeled UIRR sequences in vitro, it still remains to be seen if GATA-4 or GATA-6 can bind to the HLA-E promoter in vivo.

# Chapter 3: Identification of Putative Components of the Interferon Response Region Activation Complex

## Introduction

Interferon gamma is a soluble cytokine that is secreted by a variety of cells including CD8+ T cells and NK cells. It has the ability to activate macrophages and promotes intracellular viral activity by increasing the expression of both Class Ia and Ib molecules. The mechanism of action of this cytokine has been shown to be mediated primarily through tyrosine phosphorylation of STAT1α by JAK kinases upon binding to the interferon gamma receptor. Phosphorylated STAT 1α then forms a homodimer, which translocates to the nucleus and binds to the gamma activation site (GAS) of various target genes. In the case of the HLA Class Ia genes, IFN-γ induces the transcription of the interferon response factor-1 (IRF-1), which binds to the interferon stimulated response element (ISRE) in the promoters of HLA-B or HLA-C, thereby inducing transcription.

Similarly, the transcription of the HLA Class Ib gene, HLA-E, is also stimulated by IFN-γ despite the lack of a functional ISRE in its promoter. Previous studies in our laboratory show that HLA-E is induced via a variant STAT1α binding element, named the Interferon Response Region (Gustafason et al 1996). This binding element consists of two half sites which are similar to but are not consensus matches of the GAS and ISRE, respectively. Experiments using the drugs Distamycin A and Netropsin, which interact with the minor groove, were able to disrupt the formation of the IRR-AC, suggesting that a minor groove binding protein was present in the complex.

High mobility group A (HMGA1) is a nuclear, non-histone chromosomal protein that binds to AT rich sequences in the minor groove binding protein region. The four members of the HMG family, HMGA1a, HMGA1b, HMGA1c, and HMGA2, have been shown to play a role in transcriptional activation and modulation of chromatin structure (Chau 2005). HMGA1a, HMGA1b, and HMGA1c are splice variants that are transcribed from the same gene. All members of this family contain a Pro-Arg-Gly-Pro AT hook domain (Reeves 2001). The binding of these factors to the minor groove results in bending of the DNA molecule and permits interactions of transcription factors on adjacent parts of the promoter, which is illustrated in IFN-γ gene expression (Chau 2009). Additionally, overexpression of HMGA1 has been linked to metastatic progression and poor prognosis in pancreatic cancer (Cai 2009).

The polypyrimidine binding protein-associated factor (PSF) is another potential candidate of the Interferon Response Region Activation Complex. PSF, which also contains an AT-hook domain, has been shown to bind DNA and to function in coordination with known transcription factors, including SP-1 and Sin3A (Mathur, Tucker, & Samuels, 2001; Urban & Bodenburg, 2002). Furthermore, PSF was identified by mass spectroscopy of the purified complex. The previous findings as well as the inherent characteristics of proteins suggest that they could play a role in transactivation of the HLA-E gene. However, further studies are needed in order to definitively show what proteins bind to the interferon response region of the HLA-E promoter.

The paradigm with interferon stimulated gene expression has always been that IFN-α/β induces ISGF3 complex formation, while IFN-γ induces GAF complex formation. The Interferon Regulatory Factor (IRF) Family plays in the cellular response to viral

infections. IRF9, or p48, was identified as a component of the ISGF3 complex that binds to the Interferon Stimulated Response Element in response to IFN-α. However, Blussyen et al showed that the ISRE of the ISG15 gene could be activated by STAT1α homodimer/IRF9 complex when interferon gamma stimulation was preceded by IFN-stimulation (1996). Recent studies show that other STAT containing complexes can be activated in response to IFN-γ signaling and bind to the GAS of target genes, such as a STAT 3 homodimer or a STAT1:STAT3 heterodimer (Wesoly 2007). The Interferon Response Region consists of an ISRE-like half site and a GAS-like half site. Based upon these studies, we decided to investigate the role of IRF-9 in HLA-E induction.

There is also the possibility of a bridging factor that interacts with the STAT 1α molecule that binds to the Interferon Response Region and the GATA factor that binds to the Upstream Interferon Response Region. This hypothesis was developed based upon the observation that the IRR is not a consensus STAT binding site and the UIRR is not a consensus binding sites GATA. Since p300/CBP has been shown to interact with STAT1 and some members of the GATA transcription family, we decided to test if p300 activity was required for the full induction of the HLA-E gene.

The p300/CREB-binding protein (CBP) family functions as transcriptional coactivators that promote an active chromatin state by acetylating histones and allowing RNA polymerase II access to gene promoters (Chan 2001, Giordano 1999, Zhang 1996). The p300 protein was discovered due to its ability to interact with Adenovirus E1a oncoprotein (Whyte 1989). Likewise, the CBP protein was identified based upon its interaction with the cAMP responsive element binding protein (Chrivia 1993). CBP and p300 share sequence homology in five separate protein domains: the three cysteine-

histidine rich regions, the KIX domain (binding site for CREB), the bromodomain, the HAT domain and the steroid receptor coactivator 1 interaction (SID) domain (Kalkhoven 2004). These similar domains suggest the ability to interact with the same proteins and thus a similarity in function. For example, both proteins interact with the general transcription factors TFIIB, TBP, and RNA polymerase II, RNA helicase A (Blobel 2002 and Giordano 1999).

The association of p300 with gene specific transcription factors has been shown to enhance their transactivation capabilities. For example, p300 was shown to interact with STAT 1 at both the N-terminus and the C-terminus in response to IFN-γ (Zhang 1996). Additionally, p300 has been shown to interact with GATA-1, GATA-4, GATA-5, and GATA-6 (Boyes 1998, Kakita 1999, Wada 2000, Takaya 2008, Chen 2009). Miyomato et al showed that overexpression of p300 in transgenic mice resulted in acetylation of GATA-4 and myocardial cell hypertrophy, while a mutant lacking p300 histone acetyltransferase activity could not support either of these activities (2006). Takaya et al further investigated the interaction between GATA-4 and p300 via mutation of target lysine residues in the C-terminal motif and discovered that GATA-4's ability to transactivate the atrial natriuretic factor 1 (ANF-1) and the endothelial 1 promoters was reduced (2008). Additionally, p300 has been shown to associate with GATA-6 to help promote activation of the smooth muscle-myosin heavy chain gene (Wada 2000). Furthermore, Adenovirus E1a transfection into vascular smooth muscle cells or cardiac muscle cells downregulated the expression of the smooth muscle myosin heavy chain gene and the atrial natriuretic factor gene (Wada 2000 and Kakita 1999).

In order to determine the role of p300 in HLA-E gene activation, we decided to co-transfect wild type and mutant Adenovirus E1a with CAT reporter gene plasmids containing the IRR or the UIRR+IRR. It was determined that both wild-type E1a and a mutant that was only able to bind p300/CBP caused a 6 fold decrease in interferon induction via the UIRR in K562 cells. We decided to evaluate this interaction further by siRNA targeting of p300 expression in the SKOV3 cell line.

## Material and Methods

### Cell Culture

The ovarian carcinoma, SKOV3 was maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen, Carlsbad, CA) which was supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 ug/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were induced with interferon-gamma (RnD systems) at 200 units/ml for times indicated.

### Transient Transfection and siRNA treatment

Cells were plated in a 12-well dish at a density of $2x10^4$ cells per well. 40 picomoles of siRNA were diluted in 100 ul of serum free media that was combined with 100 ul of serum free media plus 5 ul of Lipofectamine. The mixture was incubated at room temperature before addition of 200 ul to the appropriate well. After 72 hours of siRNA treatment, 200 units/ml of IFN-y was added to the appropriate wells. Cells were then incubated for twenty-four hours at 37˚C and 5% $CO_2$ before being harvested.

### qRT-PCR

Cytoplasmic RNA was isolated using the Trizol reagent system (Invitrogen, Carlsbad, California). 1 ml or 0.5 ml of Trizol reagent was added to approximately 1 x$10^6$ (six well dishes) or 3 x $10^5$ (twelve well dishes), respectively, for ten minutes. The cells were collected an added to a 1.5 ml microcentrifuge tube prior to the addition of 200 microliters of chloroform. Cell lysate suspensions were shaken for thirty seconds and allowed to settle for three minutes followed by centrifugation at 12000 x g at 4˚C. The upper phase was removed to a fresh tube and 550 microliters of isopropanol was added before ten minute incubation at room temperature. The samples were then spun at 12000 x g at 4˚C for fifteen minutes. The supernatant was removed and the RNA pellet was washed with 600 microliters of 70% ethanol/30% DEPC treated water. Samples were spun at 7500 x g at 4˚C for five minutes. The supernatant was removed and pellets were allowed to air dry for ten minutes. The pellet was resuspended in 100 microliters of 100% DEPC treated water. RNA was quantified by spectrophotometry. Each RNA sample was then DNAse treated to remove any contaminated genomic DNA. Specifically, 0.5 ul of DNase I, 0.5 ul of RNase Inhibin, plus water added to a total volume of 20ul was added to 2 ug of RNA. The samples were incubated at 37˚C for thirty minutes, and 75˚C for ten minutes to cause inactivation of the enzymes. Next, cDNA was synthesized using the i-Script cDNA synthesis kit by Biorad. 500 ng of DNAse I treated RNA was used for cDNA synthesis. The reaction mix consisted of 2 ul of 5x iScript reaction mix, 0.5 ul of iScript reverse transcriptase, and nuclease free water to ten microliters. The samples were then incubated as follows: 5 minutes at 25˚C, 30 minutes at 42˚C, 5 minutes at 85˚C, and 10 minutes at 4˚C. Each qPCR reaction was

performed using 20ng of cDNA. 12.5 ul of 2x Sybr Green Mix (Roche) and 7.5 ul of 1 nM primer mix containing forward and reverse primers (IDT) were used in each sample.

The primer sequences were as follows:

| Primers | Forward | Reverse |
|---|---|---|
| HLA-E | TTGCAAGGGCCTCTGAATCTGTCT | AGGAACACAGGTCAGTGTGAGGAA |
| STAT 1α | GTGCATCATGGGCTTCATCAGCAA | TAGGGTTCAACCGCATGGAAGTCA |
| Cyclophilin A | AGACAAGGTCCCAAAGACAGCAGA | TGTGAAGTCACCACCCTGACACAT |
| p300 | TACCAAATGCTGCAGGCATGGTTC | TACAAGTCATTCCTGGTTGCGGCT |
| CBP | TTCGTTGATTGCAAGGAGTGTGGC | TGTTCACTCGGTCTTCCAAGTGGT |
| PSF | ACAGCGATGTCGGTTGTTTGTTGG | TGGGTGTATCATCCAGTTCGGCTT |
| HMGA1 | ACTTATTGTCCAGGTGAGGCCCAA | AGTGGCGGAAGCAAAGTAGGGTTA |
| IRF9 | TCCATTCAGACATTGGGAGCAGCA | AGATGAAGGTGAGCAGCAGTGAGT |

**Statistical Analysis**

As described in Chapter 2.

## Results

The effect of these transcription factors on HLA-E transcription was investigated via siRNA gene targeting. STAT 1α was used as positive control since it is critical for the HLA-E interferon response. SKOV3 cells were transfected with siRNA directed against STAT 1α, PSF, HMGA1, and p300. The percent knockdown for each gene was determined as well as the overall effect on HLA-E induction by IFN-γ. Gene expression was normalized by dividing the expression of the target gene by expression of human Cyclophilin A. Cells were treated for 72 hours prior to stimulation with 200 units/ml of

IFN- γ. The total knockdown of the target genes in comparison to a scrambled siRNA control were as follows: STAT 1α expression was decreased by 82% (Figure 39), PSF expression wad decreased by 66% (Figure 40), HMGA1 expression was decreased by 82% (Figure 41), and p300 expression was decreased by 49% (Figure 42). As expected there was approximately a 50% reduction in HLA-E induction when STAT 1 α expression was reduced (Figure 43). However there was no appreciable decrease in HLA-E induction when PSF, HMGA1, or p300 was knocked down (Figure 43). In the case of HMGA1 it can be concluded that this transcription factor is not necessary for HLA-E transcription in SKOV3 cells. However it can be argued that a greater reduction in p300 or PSF is needed before the effect on HLA-E can be determined.

We also determined the effect of reducing the expression of CBP and IRF9 on HLA-E induction. SKOV3 cells were transfected with siRNA for 72 hours and then stimulated with 250 units/ml of IFN-γ. The gene expression was normalized to Cyclophilin A and compared to a scrambled control. The expression of the p300 family member, CBP, was only decreased by 33% (Figure 45) but surprisingly resulted in a 54% reduction in IFN- γ stimulated transcription (Figure 47). Additionally, we reduced IRF9 gene expression by 76% (Figure 46), resulting in a 43% decrease in HLA-E induction (Figure 47). These results suggest that both CBP and IRF9 could be components of the IRR-AC.

Since we were unable to obtain adequate knockdown of p300 and PSF, we wanted to determine if a shorter siRNA treatment time world allow us to see an effect on HLA-E induction. We transfected SKOV3 cells with 40 pmol of gene specific siRNA for 48 hours and then stimulated the cells with 200 units/ml of IFN- γ. STAT 1α expression

was decreased by 92% compared to a scramble control (Figure 48). Endogenous HLA-E induction was reduced by 72% compared to wild type (Figure 49). PSF expression was decreased by 37% which did not have any significant difference in HLA-E induction (Figure 49 and Figure 51). The expression of p300 was not decreased and had no effect on HLA-E (Figure 50 and Figure 51).

Therefore the knockdown of p300 was repeated with two new p300 targeting siRNAs. The first was labeled as p300_95 and the second as p300_97. Once again STAT 1α was also knocked down to serve as a positive control and a non-specific scrambled siRNA was used as a negative control. Gene expression was normalized to the housekeeping gene Cyclophilin A. STAT 1 α was decreased by 88% (Figure 52), p300 expression was decreased by 64% when p300_95 siRNA was transfected, and by 62% when a combination of p300_95 and p300_97 was used (Figure 53). The total induction of wild-type samples was 7.2 fold. This increase in transcription was diminished by 67% when STAT 1 α expression was decreased (Figure 54). Also there was a 32% or 42% decrease in HLA-E induction when p300_95 or p300_95/97 was used respectively (Figure 54). This result was interesting because in addition to initial experiments that suggested that p300 played a role in HLA-E induction, it has also been shown that p300 acetyltransferase activity has helped to increase the transactivation of GATA-1, GATA-4, GATA-5 and GATA-6 (Chen 2009, Wada 2000, Kakita 1999, Takaya 2008).

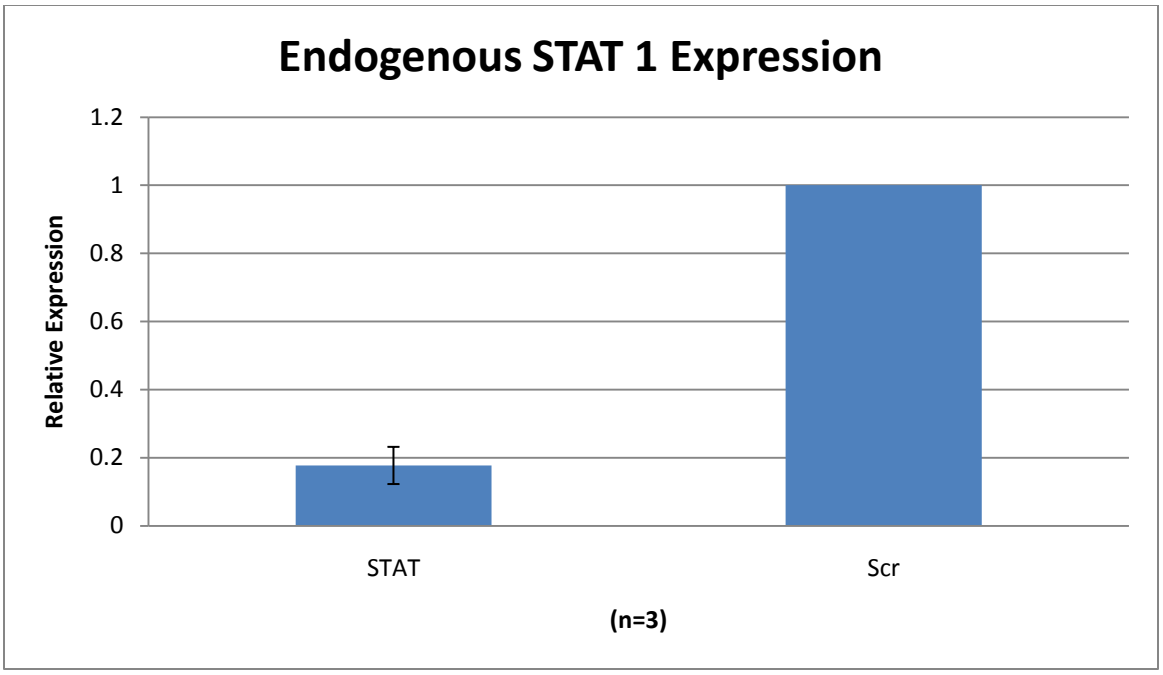


**Figure 39. STAT 1 α expression is decreased by gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific siRNA and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ. Gene expression was normalized to Cyclophilin A.

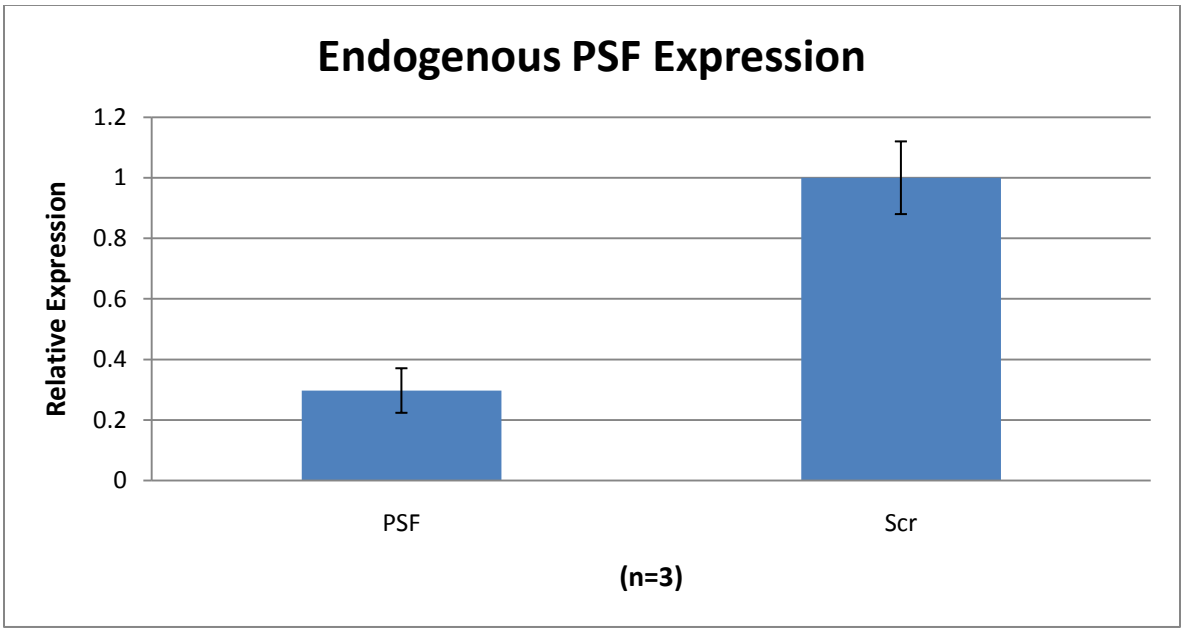


**Figure 40. PSF expression is decreased by gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ. Gene expression was normalized to Cyclophilin A.

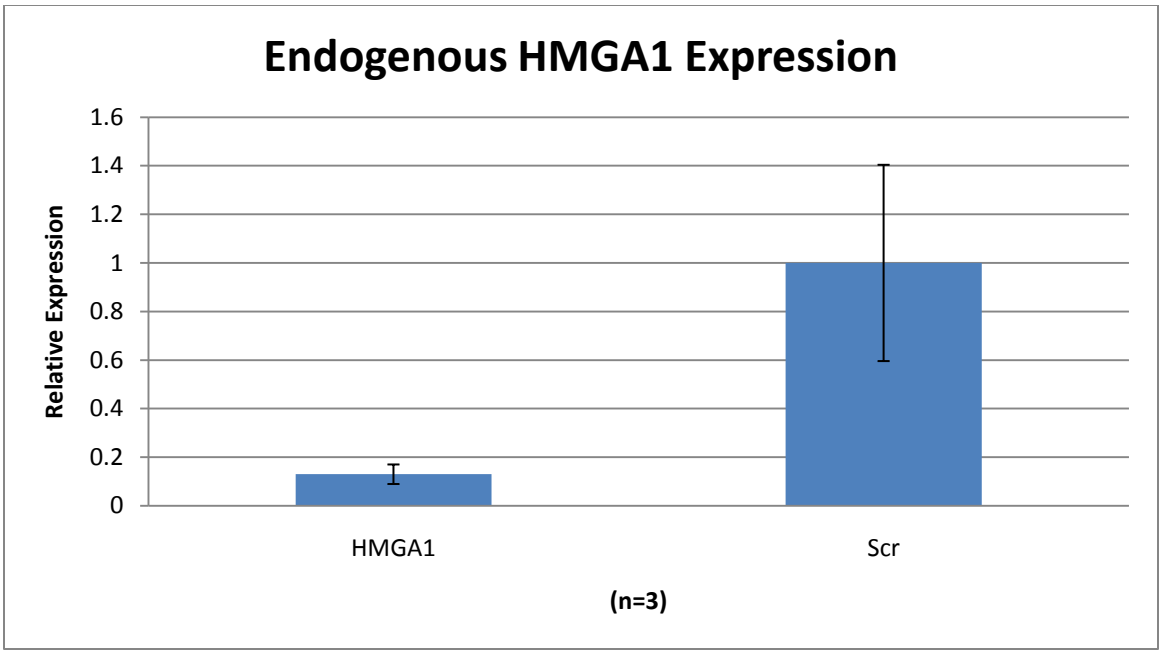


**Figure 41. HMGA1 expression is decreased by gene targeting in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ. Gene expression was normalized to Cyclophilin A.

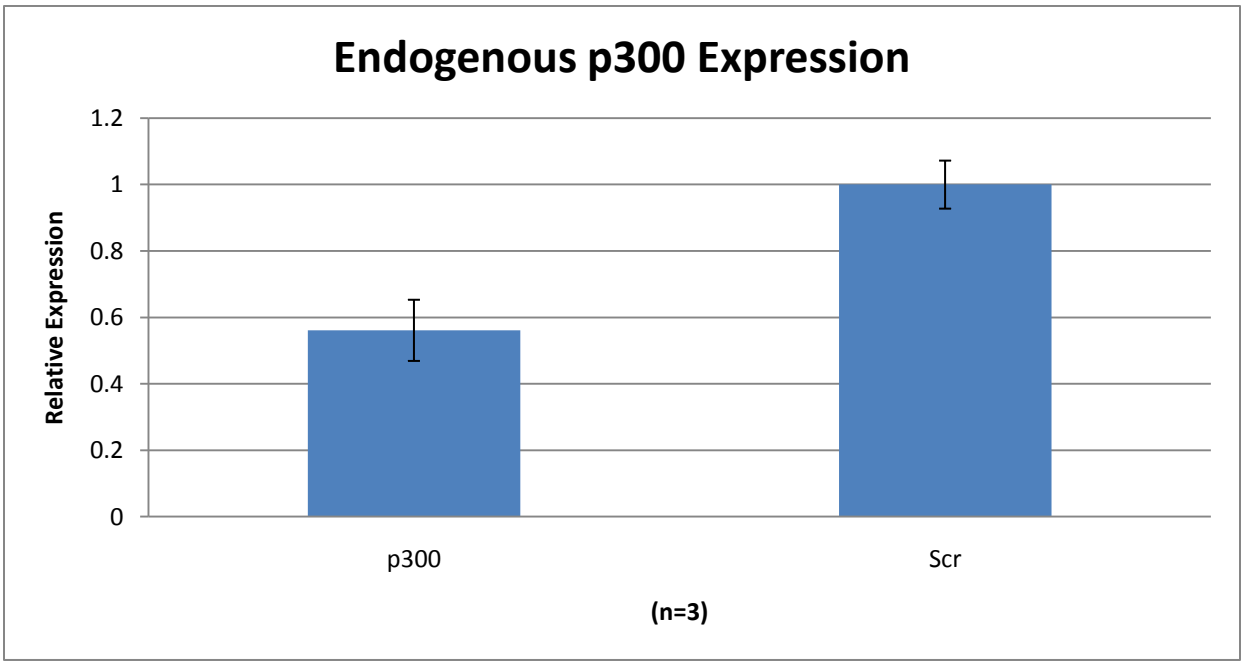


**Figure 42. p300 expression is slightly decreased by gene targeting in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ. Gene expression was normalized to Cyclophilin A.

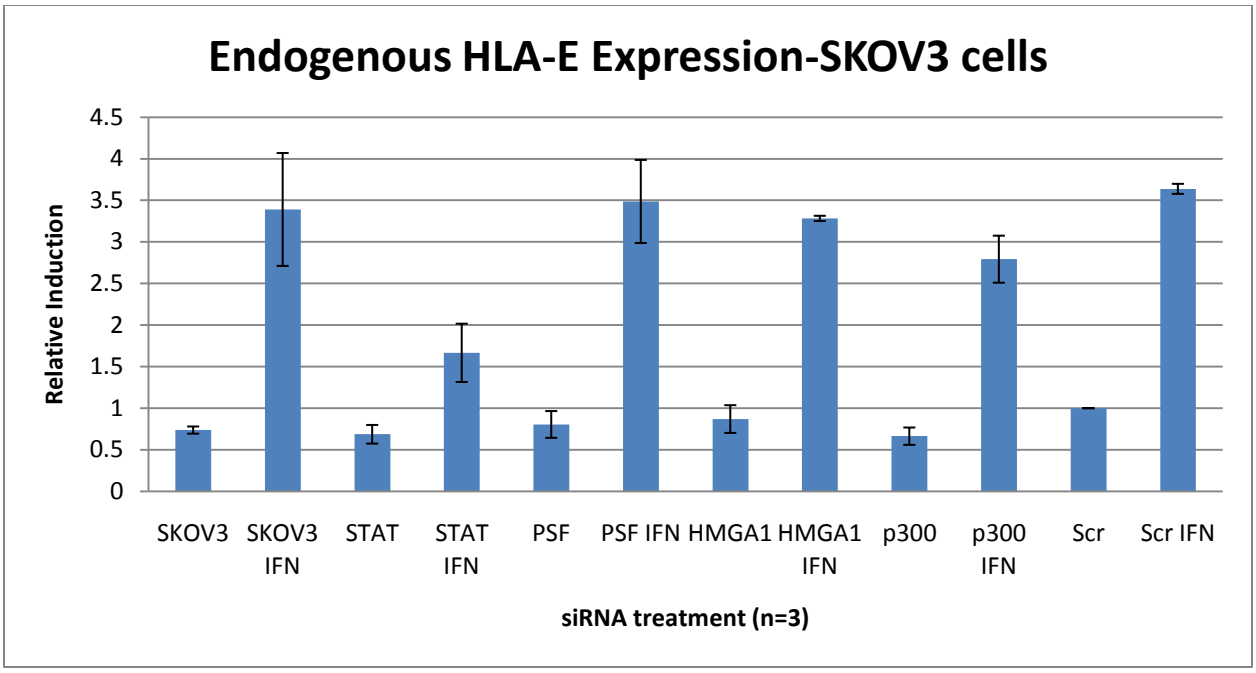


**Figure 43. Analysis of endogenous HLA-E Expression after gene specific knockdown in SKOV3.** Cells were treated as described in Figures 39-42. Expression was normalized to human Cyclophilin A. The amount of induction was compared to scramble control.

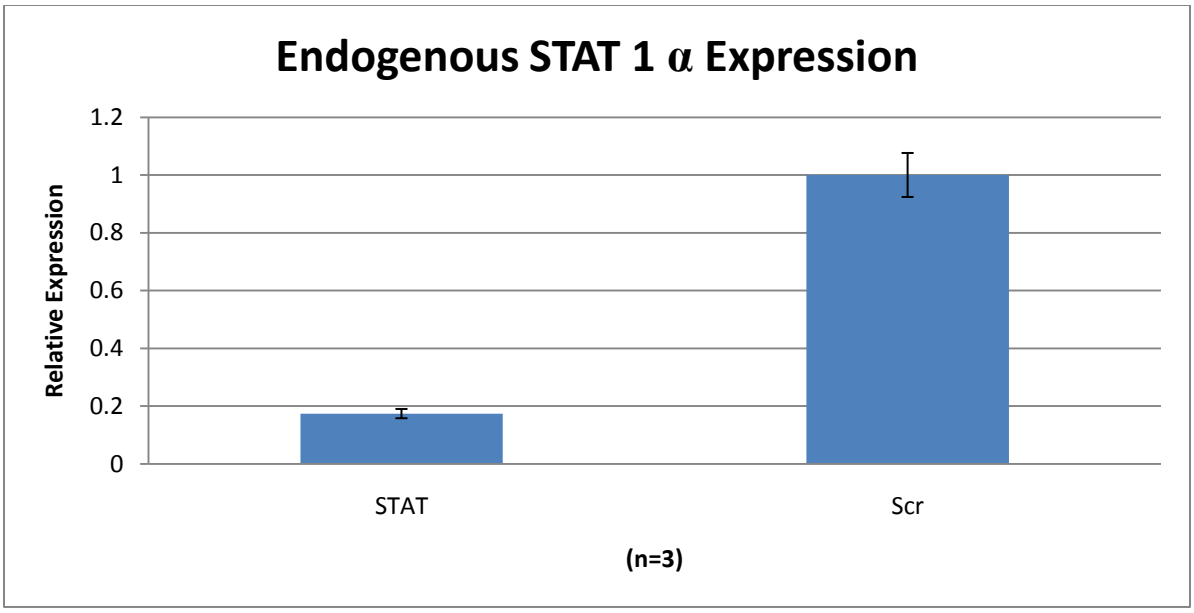


**Figure 44. STAT 1α expression is decreased by gene targeting in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ.

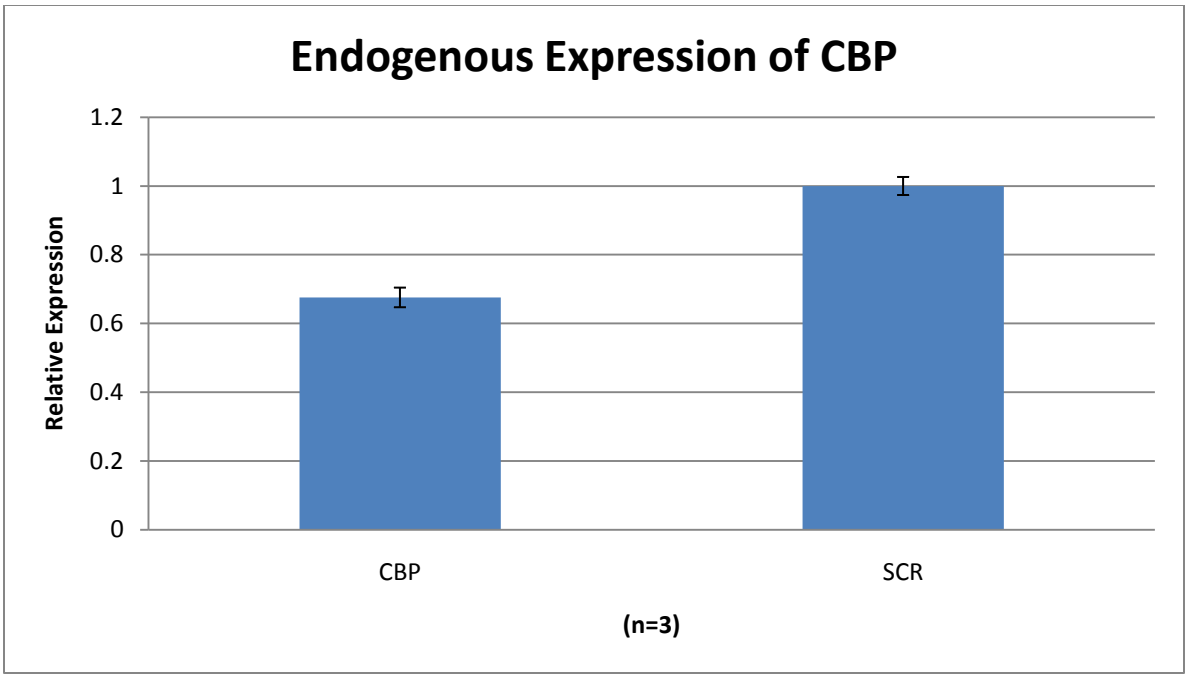


**Figure 45. Effect of CBP gene knockdown in SKOV3 cells.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ.

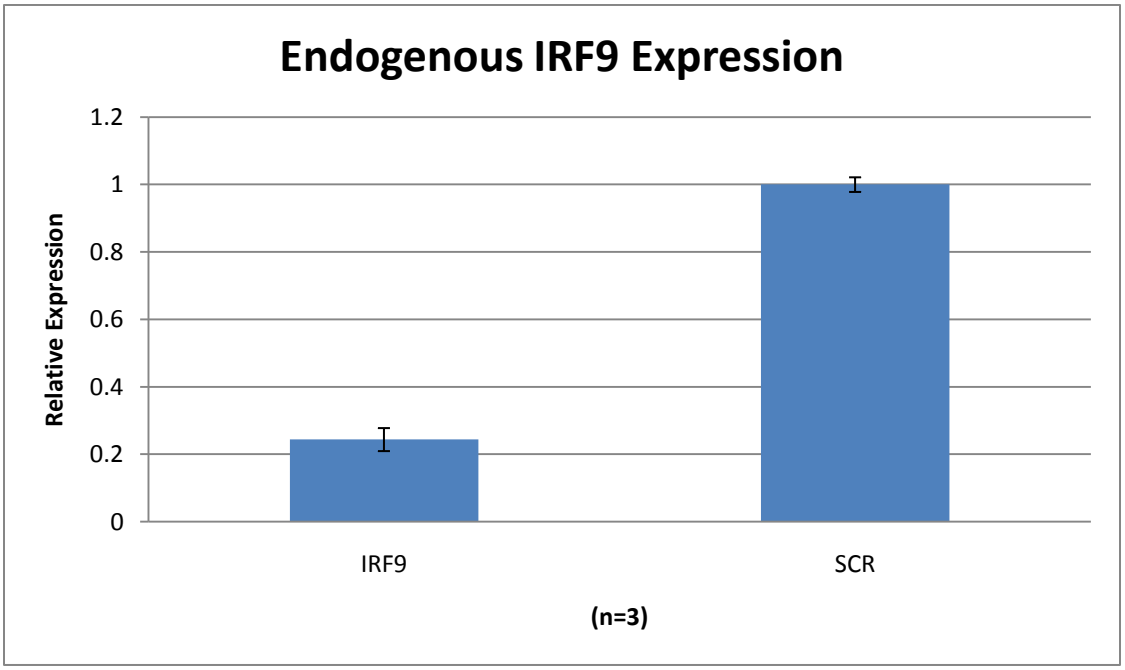


**Figure 46. IRF9 expression is decreased by gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ.

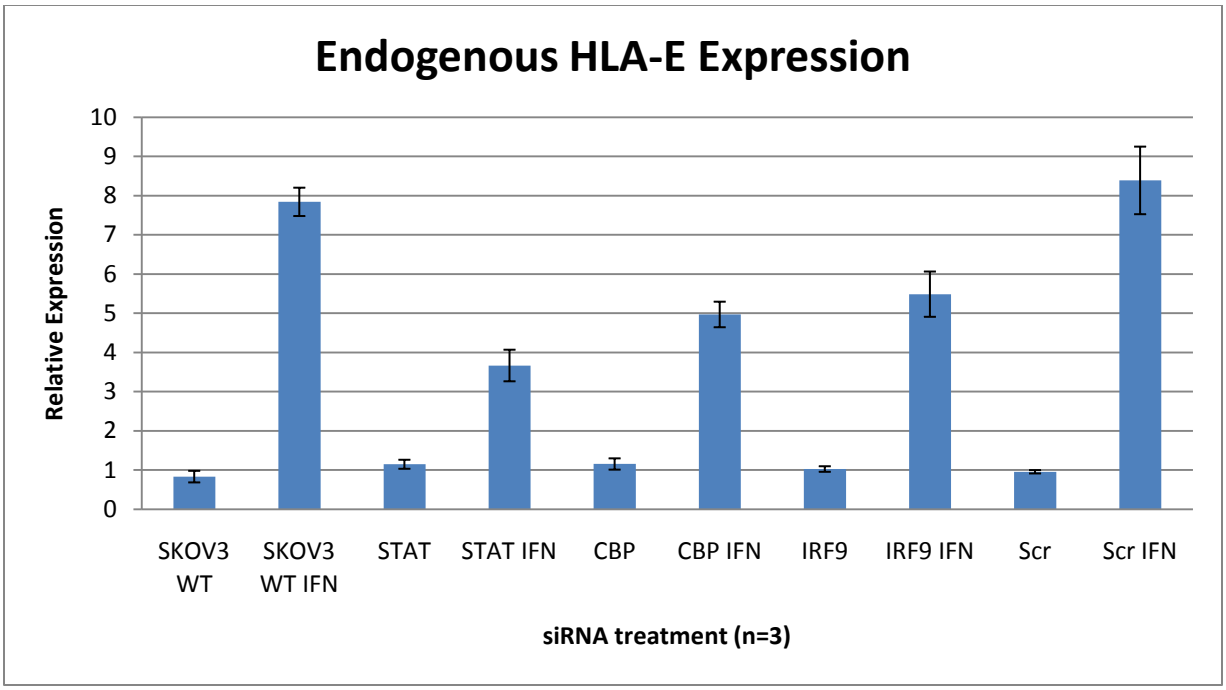


**Figure 47. Analysis of CBP and IRF9 Knockdown on HLA-E induction.** Cells were treated as described in Figures 44-46. The amount of induction was compared to scramble control. Expression was normalized to the Cyclophilin A housekeeping gene.

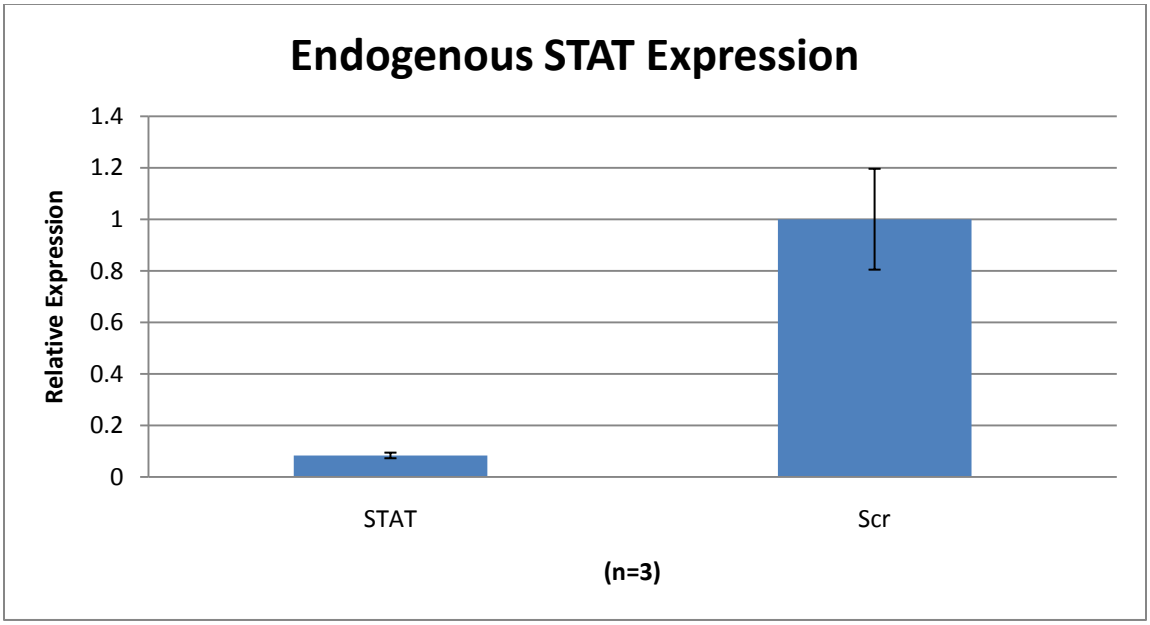


**Figure 48. STAT 1 α expression is decreased by gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 48 hours prior to 24 hour stimulation with IFN-γ.

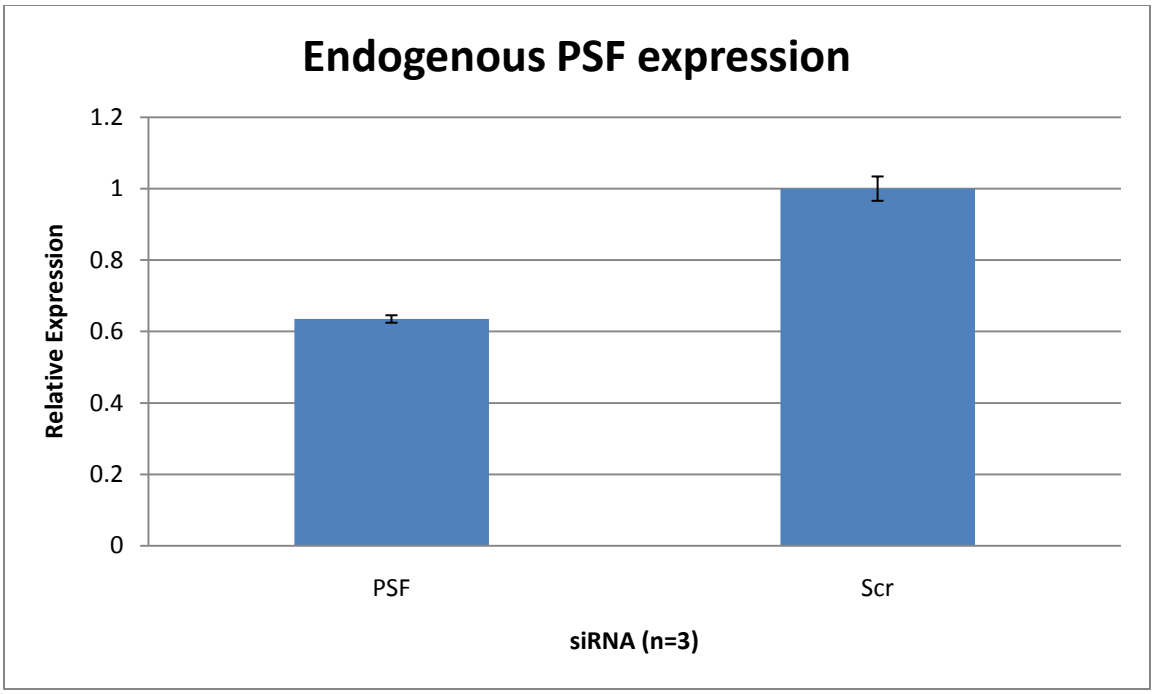


**Figure 49. Effect of PSF expression gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 48 hours prior to 24 hour stimulation with IFN-γ.

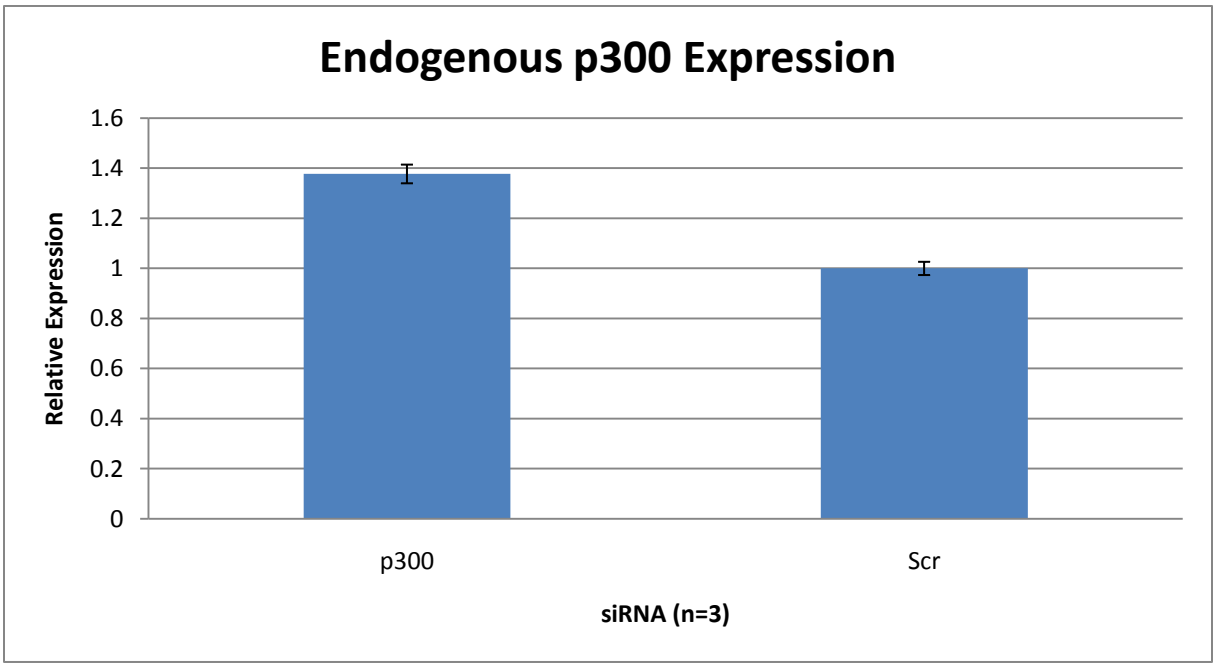


**Figure 50. Effect of p300 expression gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 48 hours prior to 24 hour stimulation with IFN-γ.

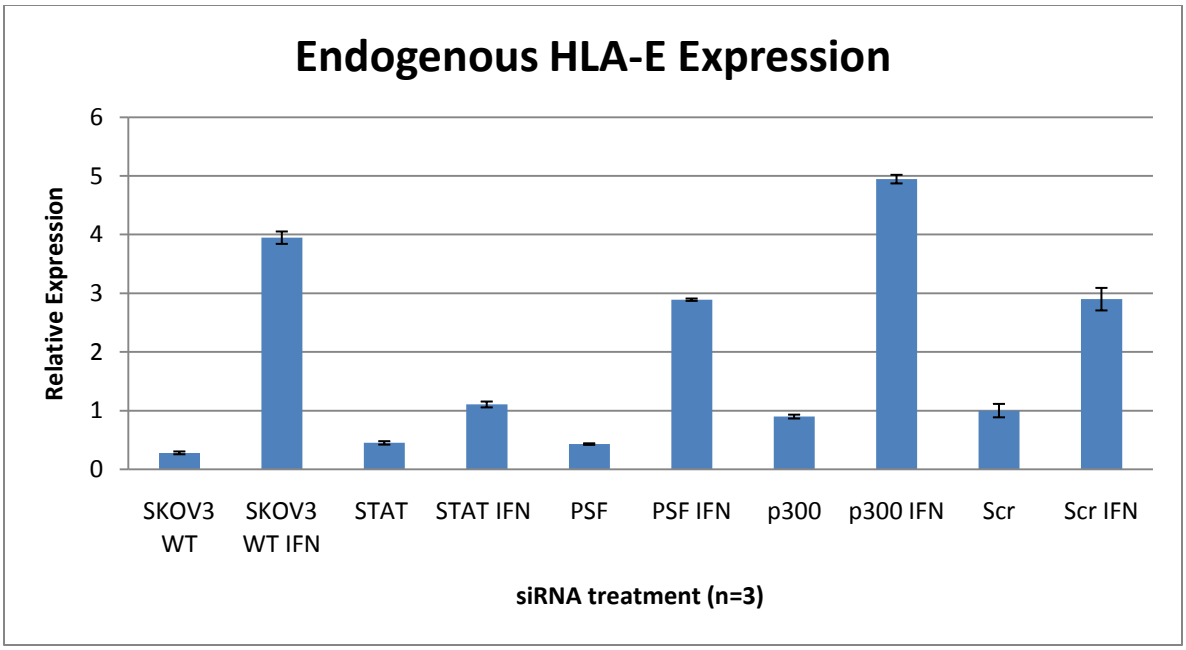


**Figure 51. Analysis of endogenous HLA-E expression after siRNA knockdown.** Cells were treated as described in Figures 48-50. Expression was normalized to human Cyclophilin A. The amount of induction was compared to scramble control.

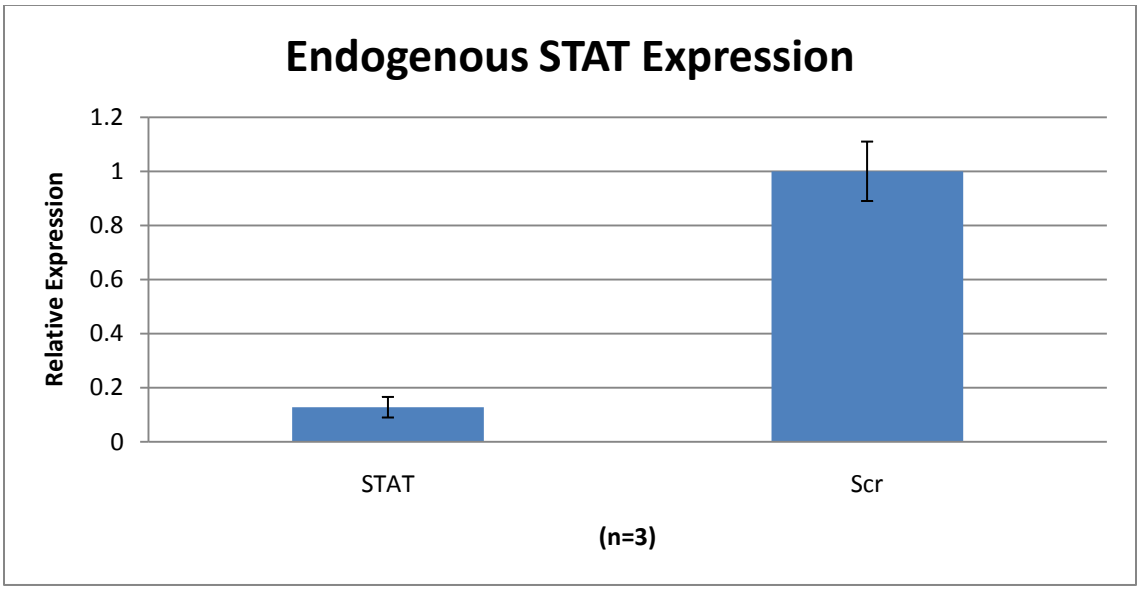


**Figure 52. Effect of STAT 1 α expression gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ.

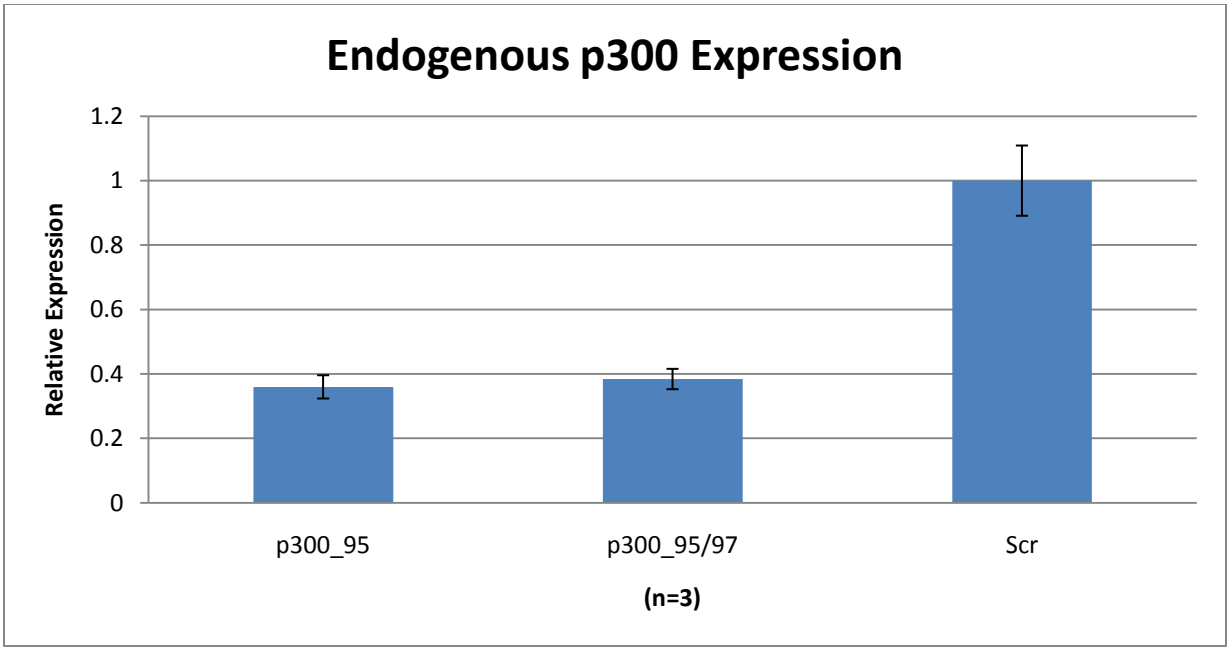


**Figure 53. Effect of p300 expression gene knockdown in the SKOV3 cell line.** SKOV3 cells were transfected with gene specific and nonspecific scramble control for 72 hours prior to 24 hour stimulation with IFN-γ

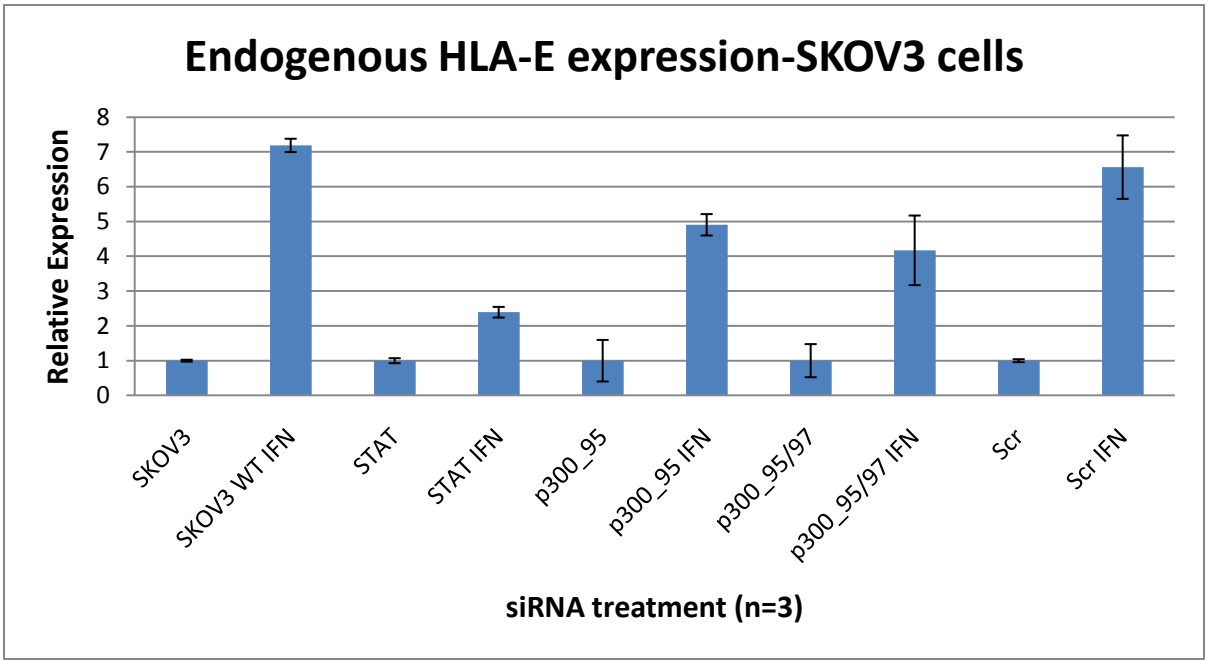


**Figure 54. Analysis of HLA-E Expression in SKOV3 cells after gene specific knockdown of p300.** Cells were treated as described in Figures 52 and 53. Expression was normalized to human Cyclophilin A. The amount of induction was compared to scramble control.

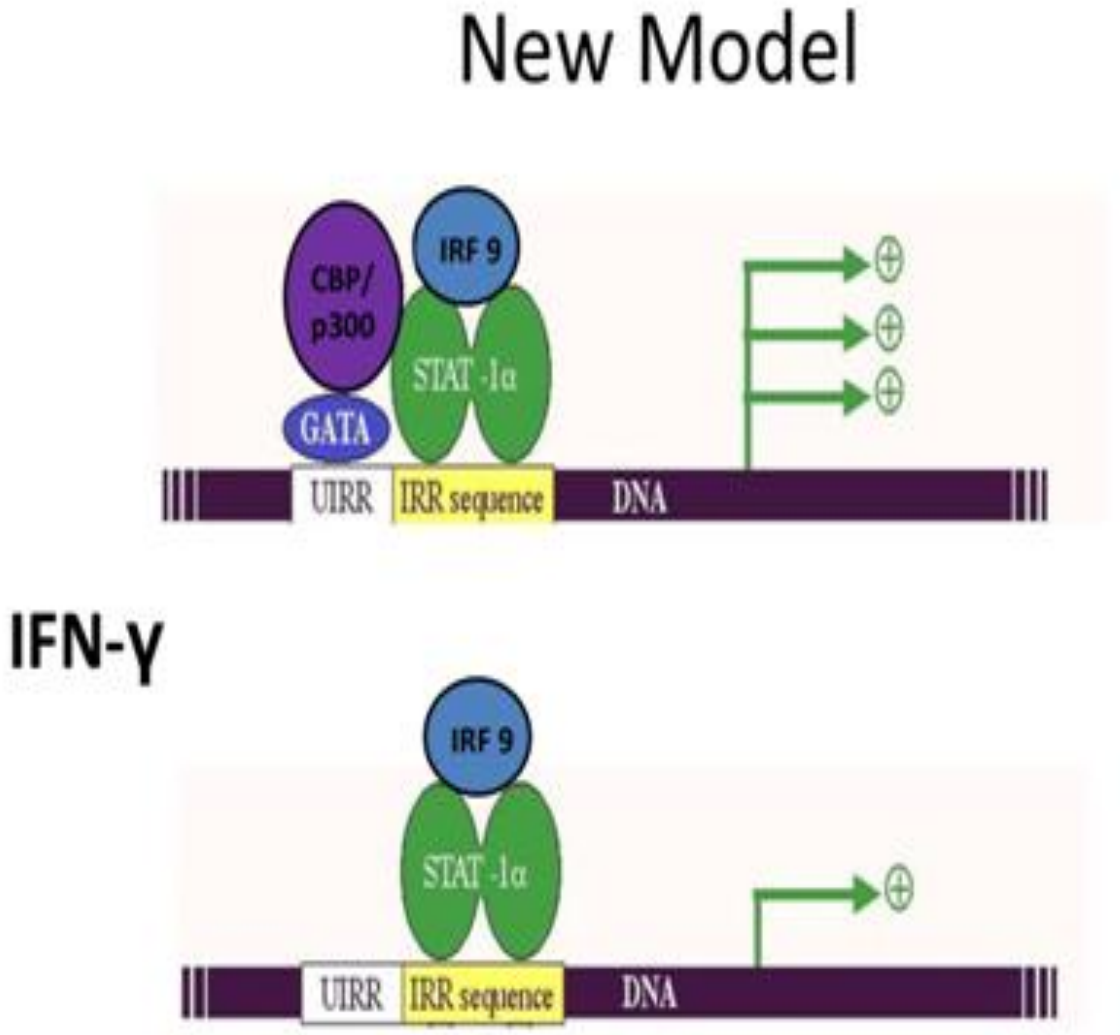


**Figure 55. Model of transcriptional regulation on the HLA-E promoter in response to IFN-γ.** In the first panel , we have illustrated a cell line that expresses a GATA transcription family member which has the ability to recruit CBP or p300 to the transcriptional activation complex. In the lower panel, we have illustrated a cell line that does not express a GATA factor and also does not recruit CBP or p300. We are also illustrating the interaction of IRF-9 with the STAT 1 α homodimer.

## Discussion

We previously reported that the Interferon Response Region is a novel interferon response element consisting of two half sites which are similar to but are not consensus to the Gamma Activation Site (5' half) or the Interferon Stimulated Response Element (3' half). Despite the lack of homology to known binding sites, each portion of the IRR is recognized and bound by a STAT 1α homodimer. Thus we hypothesized that additional proteins may bind to the IRR and facilitate the interaction of the STAT molecules with the HLA-E promoter. Our laboratory also demonstrated that the interactions of the activation complex with a radiolabeled IRR sequences is diminished in the presence of the minor groove binding drugs, Nystatin and Distamycin A. In the present study, we have attempted to verify potential components of the IRR-AC via gene knockdown studies.

HMGA1 was the principal factor implicated as a member of the Interferon Response Region Activation Complex. Using siRNA to target HMGA1 gene expression, we achieved 82% knockdown of target gene expression. We next examined HLA-E induction but saw no effect on gene transcription. We also targeted the expression of PSF, which is another protein that interacts with the minor groove. Target gene expression was decreased by 66% when treated for 72 hours and by 37% when treated for 48 hours, but showed no effect on HLA-E transcriptional activation. If either HMGA1 or PSF were components of the activation complex, we would have expected to see a similar decrease of HLA-E transcription as when STAT1α expression is reduced.

Previous studies also suggested that the p300/CBP family functions at the HLA-E promoter. Adenovirus E1a was transfected in to K562 cells to determine the effect of p300/CBP sequestration on HLA-E induction by IFN-γ. This resulted in a 6-fold decrease in HLA-E induction and essentially reduced HLA-E promoter CAT activity measure from the UIRR plasmid to the measurement from the IRR plasmid alone. We targeted the expression of both p300 and CBP using siRNA. Our maximal knockdown of p300 expression was 62-65% which caused a 32-42% decrease in IFN-γ stimulation of HLA-E gene transcription. These results suggest that p300 plays a role in HLA-E induction and that the mechanism involves p300 acetyltransferase activity. In order to fully ascertain the effect of p300/CBP on HLA-E induction it is necessary to rule out compensation from other family members. This can be achieved by performing a dual knockdown of both p300 and CBP activity. We also slightly decreased the expression of CBP. Despite approximately a 30% decrease in CBP gene expression there was a 54% reduction in HLA-E induction. This suggests CBP plays a bigger role in HLA-E induction than p300. Experiments are underway to simultaneously knockdown both factors.

The ISGF3 protein complex, which consists of STAT1, STAT2, and IRF9, binds to the Interferon Stimulated Response Element in response to IFN-α or IFN-β. The Interferon Response Region of the HLA-E promoter consists of two half sites that are similar to the GAS and the ISRE, respectively. Additionally, it has been shown that IRF9 can associate with a STAT 1 homodimer and bind to the ISRE in response to IFN-γ (Bluyssen1996). As a result of these studies we decided to target IRF-9 expression. We successfully reduced IRF-9 expression by 76% and saw a subsequent 43%

reduction in HLA-E induction. This observation suggests that IRF9 is able to bind to the IRR in response to IFN-γ. Future studies will focus on demonstrating IRF9 binding to the HLA-E promoter.

The Interferon Response Region represents one example of a unique interferon response element. Other groups have described novel elements as well. A recent report by Merraro et al describes the ETS/Interferon Response Element (EIRE) in the promoter of the Interferon Stimulated Gene 15 (ISG15). They show that the ISG15 promoter sequence differs from the classical ISRE element in the MxA, IFN-β, GBP, and IFN-α genes, which are bound by interferon regulatory factors but not by PU.1. The ISRE element in the ISG15 gene (5' CGGGAAAGGGAAACCGAAACTGA3') is bound by PU.1, IRF4, and IRF8, therefore the group proposes renaming the sequence as the EIRE. Furthermore they demonstrate that similar sequences can be found in other genes such as the human ISG54, p69, and Factor B promoters (2002). Additionally Marechi et al report a LPS inducible composite PU.1/IRF element that is located in the IL-1β promoter and is bound by PU.1, IRF4, IRF1, and IRF2 (2001).

Another inducible element was discovered by Weihu et al during their investigation of IFN-γ stimulation of the IRF 9 gene. The IFN-γ activated transcriptional element (GATE) is located upstream of the translational start site between the -351 and -1045 bp of the IRF9 promoter (3' GGGCCTCTCTTAACTTTGAATCCC 5') and shows no homology to either the GAS or ISRE. This element was further shown to be activated by the IFN-γ activated transcriptional element binding factor 1 (GBF1) and C/EBPβ (Meng 2005). These studies along with our own prove that the concept of interferon

response elements should be expanded beyond GAS or the ISRE and furthermore that the protein complexes that interact with these novel sequences do not require STAT.

# Chapter 4: Summary and Future Directions

The goal of this project is to identify molecular targets that cause the differential induction of Class Ia and Class Ib genes in the presence of IFN-γ. Our interest in HLA-E centers on the observation that HLA-E has the ability to produce an immune response that is opposite of that induced by classical MHC molecules. In addition, we have shown that the promoter of HLA-E differs significantly from that of the Class Ia molecules. We have demonstrated that the HLA-E molecule responds to interferon gamma via a novel element termed the Interferon Response Region. Additionally, the induction of HLA-E can be further increased when an element immediately upstream of the IRR, termed the upstream interferon response region, is bound by a GATA transcription family member. Previous studies in our laboratory showed that the action of the upstream element was dependent upon the IRR. Studies in the K562 erythroleukemia cell line demonstrated the ability of GATA-1 to bind to the HLA-E promoter in vivo and that siRNA targeting of GATA caused a decrease in the induction of HLA-E by interferon gamma. Analysis of a number of other cell lines for UIRR functionality suggested that other GATA family member could support UIRR function as well.

This led to our hypothesis that all members of the GATA transcription family could interact with the HLA-E promoter at the UIRR and mediate an increase in HLA-E expression by interferon gamma. In this work, we wanted to focus on epithelial cells that would most likely express GATA family members 4, 5, or 6. We detected GATA-6 expression in the Seg 1 and HCT 116 cell lines and demonstrated that induction of HLA-E could be decreased via specific siRNA gene targeting of GATA-6 in these cell

lines. The reduction of CAT activity from HLA-E promoter-CAT fusion plasmids with mutated GATA binding elements further illustrated that GATA factors interact at the HLA-E promoter. Additionally, overexpression of GATA-4 and GATA-6 in the MeWo and OvCar 8 cell lines resulted in increased induction of HLA-E in some cases. This work has demonstrated the importance of GATA transcription factors in the control of HLA-E expression in specific tumor cell lines.

In this study, we analyzed the expression of GATA factors in various tumor cell lines via qPCR. We compared the expression of the specific GATA target genes (GATA1-6) to the expression of the housekeeping gene, Cyclophilin A. We screened hematopoietic cells, ovarian carcinoma cells, and breast cancer cells for GATA family expression. K562 cells are an erythroleukemia ell line that was previously demonstrated to express GATA-1 protein (Barrett et al., 2004) which correlates with our finding of GATA-1 mRNA. This finding is further supported by previous reports describing both GATA-1 and GATA-2 expression in red blood cells (Lowry & Mackay, 2006; Patient & McGhee, 2002; Simon, 1995). A2780 cells are ovarian carcinoma cells that express GATA2 and GATA5 transcripts. Previous examination of GATA-4 and GATA-6 protein expression in the A2780 cell line resulted in no detection of protein levels and led Capo-chichi et al to designate this cell line as “GATA-null” while our findings suggest that A2780 cells are not “GATA-null” but simply express different GATA factors than examined by this group (Capo-chichi et al., 2003). Similarly, the ovarian carcinoma, OvCar 8, expressed GATA-2 and GATA-6 transcripts. It has been previously reported that GATA factors -2, -4,-5, and -6 are expressed in the gonads (Viger, Guittot, Anttonen, Wilson, & Heikinheimo, 2008). The evaluation of MCF-7 breast cancer cells demonstrated high levels of GATA-

3 transcript expression. The role of GATA-3 in luminal cell development and the expression of GATA-3 in white adipocyte precursors in the mammary gland has previously been described (Chou, Provot, & Werb, 2010; Kouros-Mehr, Kim, Bechis, & Werb, 2008)

We also examined the expression of GATA factors in colon cancer, esophageal cancer, pancreatic cancer and skin cancer. We determined that the colon carcinoma, HCT 116, expressed GATA- 2 and GATA-6 transcripts. Examination of GATA-6 expression in the gastrointestinal tract demonstrated GATA-6 expression in both the small and large intestine. Interestingly, the expression of GATA-6 was reduced in colon carcinomas in comparison to normal epithelium (Haveri et al., 2008). Seg1 cells are an esophageal tumor cell line that express GATA-2 and GATA-6 transcript. Guo et al examined the expression of GATA-4, -5, and -6 in esophageal carcinomas and determined that GATA-6 expression was detectable via qPCR. However, GATA-4 and GATA-5 were not detectable and this lack of expression was a result of promoter hypermethylation of both of these genes (Guo et al., 2006)). We observed expression of GATA-4 and GATA-5 in Panc-1 cell line. This observation is supported by reports of GATA-4 and GATA-5 message in human pancreatic carcinomas (Fu et al., 2007). Our examination of GATA expression in the MeWo cell line revealed very low levels of GATA-6 transcript which is a novel finding.

Our observation of GATA-2 expression was the only finding that did not seem to correlate with previous observations in the literature. GATA-2 transcript was detected in several cell lines with the highest level of expression occurring in K562 erythroleukemia cells. The other positive cell lines were MCF-7 (breast carcinoma), SKOV-3 and OvCar

8 (ovarian carcinomas), Tera-2 (embryonal carcinoma), Panc-1 (pancreatic carcinoma), and MeWo (melanoma). Expression in the cell lines besides K562 may be example of aberrant protein expression in tumor cells since these tissues are mesoendodermal in nature and therefore would be expected to express GATA-4, -5, or -6. Interestingly, the expression of GATA-2 in these cell lines in comparison to K562 was at least 8-fold lower. It is necessary to examine each cell line for GATA-2 protein expression to determine if the low levels of GATA-2 transcript leads to GATA-2 protein.

We investigated the effect of GATA overexpression on UIRR functionality of the HLA-E promoter by transfecting the MeWo cell line with GATA-4, GATA-6, or both GATA-4 and GATA-6. Interestingly, we observed a decrease in pigmentation in cells that overexpressed GATA transcription factors as compared to wild-type. This decrease in color suggests de-differentiation of the MeWo cells. Similarly, overexpression of GATA-3 in white adipose tissue resulted in cellular arrest at the preadipocyte stage while downregulation of GATA-3 allowed progression from the preadipocyte stage to the adipocyte stage (Chou et al., 2010) Furthermore, Tong et al showed that GATA-3 controls the preadipocyte-adipocyte developmental transition by suppressing the expression of the peroxisome proliferator activated receptor gamma (PPAR- γ) gene expression. The expression of other molecular markers of adipogenesis such as Glut-4 and adipocyte fatty acid bind protein AP2 were also decreased in comparison to vector transfected controls (Tong et al., 2000)

In addition to screening the tumor cell lines for GATA mRNA expression, we also investigated the UIRR functionality in these cell lines using HLA-E promoter driven CAT reporter gene assays. Based on our findings we can conclude that expression of a

GATA transcription factor family member is necessary but not sufficient to cause an enhanced response in HLA-E transcription after IFN-γ treatment. We have observed this in the case of the ovarian carcinoma, Hela, and the pancreatic carcinoma, Panc -1 cell line. Hela cells have been shown to express GATA-2 and Panc-1 has been shown to express GATA-5. While it is tempting to speculate that these GATA-2 and GATA-5 are incapable of mediating UIRR function, UIRR activity was observed in the A2780 cell line, which expresses both of these factors. A possible explanation is that the GATA-2 and GATA-5 expressed in Hela and Panc-1 cells, respectively, are altered in some way or lack required cofactors and thus lack the ability to interact with the HLA-E promoter. Future experiments should include isolation and cloning of the specific GATA family member expressed in Hela or Panc-1 to determine if the proteins are truncated or are unable to bind to the HLA-E promoter. This could also be evaluated by the overexpression of a GATA family member in these cells. However previous attempts made by our laboratory to overexpress GATA-1 in Hela cells proved to be unsuccessful.

Another intriguing possibility is the analysis of cofactors that may be interacting with GATA family members bound to the UIRR. Interaction with other transcription factors, co-activators, or co-repressors can modulate the activity of GATA transcription factors. Furthermore, GATA transcription factors have been reported to interact with several different proteins. One of the best characterized associations is that between the GATA factor family and the Friend of GATA (FOG) family, which consists of FOG-1 and FOG-2. The FOG proteins can act as either activators or repressor of GATA activity. Tsang et al first described FOG-1 as a nuclear zinc finger protein that demonstrated a similar pattern of expression as GATA-1 (fetal liver, yolk sac, spleen, liver, and testis) and was

critical for erythroid cell and megakaryocyte differentiation ((Tsang et al., 1997)(Robert, Tremblay, & Viger, 2002).

FOG-2 shares a similar protein structure with FOG-1, but has a divergent primary structure (Tevosian et al., 1999). FOG-2 is expressed in the heart, brain, testis, and ovaries and, like FOG-1, interacts with GATA factors via the N-terminal zinc finger. FOG-2 null mice die from defects in heart morphogenesis and coronary vascular development (Tevosian et al., 1999). Based upon expression, it has been shown that FOG-1 interacts mainly with GATA-1 and GATA-2, while FOG-2 interacts with GATA-4 and GATA-6. However, it is important to note that both FOG family members can interact with all GATA family members. When studying the transcriptional role of FOG proteins in gonadal cells, Robert el al used gene reporter assays in conjunction with transient overexpression of GATA-1, GATA-2, GATA-4, and GATA-6 along with either FOG-1 or FOG-2 to demonstrate that both FOG-1and FOG-2 repressed GATA activity at GATA dependent gonadal promoters (2002).

Additionally, there is a well known antagonistic relationship between GATA-1 and PU.1, GATA-2 and C/EBP$\alpha$, and GATA-3 and T-bet. In each case, the importance is centered on the development of hematopoietic cells and is actually concentration dependent. For example, in the case of GATA-1 and PU.1, overexpression of GATA-1 in myelomonocytic cells resulted in transformation into eyrthroid, megakaryocytic, and eosinophilic cells (Rekhtman et al., 2003). Furthermore, the ability to of PU.1 to negatively regulate GATA-1 activity was illustrated by Moreau-Gachelin et al, who showed that the transgenic overexpression of PU.1 in erythroid cells results in increased rates of erythroleukemia development (1996). The mechanism of this

inhibition has also been evaluated. PU.1 has been shown to physically interact with GATA-1 in mouse erythroleukemia cells (Burda et al., 2009). Amino acids in the C-terminal domain of PU.1 are required to bind to GATA-1, while the N-terminal domain is needed to block GATA-1 binding to DNA (P. Zhang et al., 2000). Although no such antagonistic relationship has been reported with the GATA 4, 5, or 6 family members, it would be interesting to investigate if such a phenomenon is occurring in the Hela or Panc-1 cell lines. It would also be interesting to determine if there is competition between a cofactors and GATA family member in cell lines which did not support UIRR function.

The ultimate goal of this work is to determine how to control HLA-E expression independently of other MHC molecules. We have previously shown that induction of HLA-E by interferon gamma is contingent upon activity at the IRR. Thus it is imperative to understand all components and characterize the proteins that interact with the IRR and the UIRR following interferon gamma stimulation. Although we have already demonstrated that STAT 1 alpha homodimers bind to the Interferon Response Region, there is evidence to suggest that other proteins may also be necessary for the full induction of HLA-E transcription. The Interferon Response Region is composed of an extended, imperfect inverted repeat consisting of two distinct half sites. However, neither the 5' half or the 3' half represent consensus STAT binding sites. These nonconsensus binding sites as well as the AT rich nature of the interferon response element led to the hypothesis that STAT 1 alpha was not acting alone at the HLA-E promoter. Since minor groove binding proteins interact with AT rich stretches of DNA and have the ability to change the architecture of DNA, we investigated the possibility of

minor groove binders such as HMGA1 or PSF as components of the IRR-AC. Based upon preliminary experimental results, both proteins seemed like putative candidates. HMGA1 was implicated via electrophoretic mobility shift assay via both oligonucleotide competition of the IRR-AC and by ablation of the complex that bound to the radiolabled IRR probe after incubation of stimulated U937 nuclear extracts with antibody specific for HMGA1. However, PSF, not HMGA1, was identified along with STAT 1 alpha in biochemically purified IRR binding complexes. However specific siRNA knockdown of both PSF and HMGA1 gene expression showed no effect on HLA-E induction. It is important to note that a subsequent purification attempt did show several small molecular weight bands that were not able to be identified by the mass spectrophotometry analysis. Thus, it is possible that another protein with minor groove binding capabilities can interact with the IRR.

In the future it will be necessary to purify the complex that binds to the Interferon Response Region in order to elucidate the components of the IRR-AC. Previous attempts to purify the complex consisted of a three-step purification strategy that utilized successive anion exchange columns followed by a DNA affinity column. It may prove useful to investigate a strategy that provides higher purification yields in order to identify any low abundant proteins that may be a part of the complex. This could be achieved by implementing a double affinity purification strategy. Briefly, a recombinant STAT 1 protein that is tagged with a V5 epitope can be generated and expressed in Hela S3 cells. Therefore the initial purification step will utilize the specificity of anti-V5 antibody, whereas the subsequent step will use DNA affinity chromatography.

The discovery of a component of the IRR-AC that is specific for HLA-E induction and is required for the HLA-E interferon gamma response in all cell types, as opposed to the cell type specific response mediated by GATA transcription family members is needed for specific HLA-E modulation. MATInpsector analysis of the IRR promoter region suggested that IRF-4 could interact with this portion of the HLA-E promoter. However, IRF-4 expression is restricted to T-cell and B-cell lineages. As we stated previously, the Interferon Regulatory Factor family share a conserved DNA binding domain. Therefore, the information from this program suggested that an interferon regulatory factor could potentially interact at the IRR. Examination of the literature revealed that IRF-9 has been shown to form a heterotrimer with a STAT 1α homodimer or with a STAT1/STAT2 heterodimer in response to interferon. Based upon these findings, we decided to evaluate the involvement of IRF-9 in the transactivation of the HLA-E gene. We used siRNA specific gene targeting to decrease IRF-9 expression and observed a concomitant decrease in HLA-E induction. This result is particularly intriguing because it shows that the same factor can be involved in the transcriptional regulation of both classical MHC and nonclassical MHC in response to different interferon types. As stated previously, IRF-9 interacts with STAT1 and STAT2 in response to IFNα/IFNβ signaling. A different family member, IRF-1, mediates the classical MHC response to IFN-γ. However in the case of HLA-E, we have demonstrated that IRF-9 helps to mediate the response to IFN-γ. Therefore in the context of IFN-γ signaling, the differential regulation of MHC could possibly be achieved by targeting IRF-9 expression. Further studies are required to ensure that a decrease in IRF-9 has no effect on the induction of Classical MHC in response to IFN-γ. Finally it would be interesting to

determine the functional consequence of controlling the expression of the HLA-E molecule on the surface of target cells and the subsequent ability of Natural Killer cells and CD8 + T cells to destroy the targets in cytolytic assays. Since HLA-E serves as the principal ligand for the NKG2A/CD94 receptor that is present on many natural killer cells, NK-T cells, and a subset of cytotoxic T lymphocytes, the ability to regulate this gene has the potential to have a major impact on adoptive immunotherapy and bone marrow transplantation.

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