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MyD88: A Key Regulator of Chemoresistance, Differentiation and Hypoxia Resistance in Cancer Stem Cells?

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Doctor of Philosophy

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Under the supervision of Professor John O'Leary

Declaration

I declare that this is my own work and has not been submitted previously for a PhD degree at this or any other university. I agree that the library may lend or copy this thesis on request.

Aoife Cooke

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Summary

Ovarian cancer, the leading cause of gynaecologic cancer deaths in the western world is characterised by high rates of chemoresistant recurrence. While in primary cases, differentiation status of the tumour is considered a primary prognostic indicator, in recurrent disease this is no longer the case. Recurrent disease could be explained by the cancer stem cell theory. Cancer stem cells (CSCs) are a minority population of cancer cells with stem like properties including enhanced proliferation. CSCs are considered a potential source of recurrent disease. The adaptor molecule myeloid differentiation-primary response gene (88) (MyD88), is a key constituent of several toll like receptor (TLR) pathways including TLR4. In normal circumstances TLR pathways mediate the body's inflammatory response to pathogens; however MyD88 has been recently suggested as a possible marker of cancer stemness in ovarian cancer. Previous data has shown a negative correlation between MyD88 expression and patient survival. Cell line experiments were carried out in two CSC lines: NTera2 and 2102Ep. NTera2 cells are pluripotent and readily differentiate in response to retinoic acid treatment while 2102Ep cells are nullipotent and resist differentiation via retinoic acid. The work presented here investigates the involvement of TLR4-MyD88 in the CSC response to chemotherapy, retinoic acid and hypoxia treatment.

Initial experiments assessing mRNA expression via qPCR analysis showed that MyD88 was involved in the CSC response to all three treatments in both a pluripotent and nullipotent cell line. Functional analysis was then carried out in both cell lines using siRNA gene knockdown and/or gene overexpression via plasmid insertion in each cell line as appropriate. This work has shown that knockdown of MyD88 expression in a nullipotent cell line causes cells to differentiate in response to stimulus, and to improve their survival in hypoxia. Concurrently overexpression of MyD88 in a pluripotent cell line removes their

ability to differentiate in response to stimulus but has no effect on cell survival in hypoxia. Affymetrix Gene Arrays, SOLiD second generation sequencing and chemokine/cytokine arrays were performed to assess the downstream effects of MyD88 knockdown and to obtain a secretion profile of treated cancer stem cells.

These data demonstrate the previously unrecognised importance of MyD88 expression to the characteristic differentiation responses of both pluripotent and nullipotent CSCs. As CSCs lose their tumorigenic potential upon differentiation, targeting of MyD88 in CSCs may represent a potential therapeutic target. Novel ncRNAs regulated by MyD88 in pluripotent hEC cells have been identified. It has been demonstrated that MyD88 expression influences the secreted cytokine and chemokines from the CSC population, which in turn utilise inflammatory pathways to affect the tumour microenvironment. This comprehensive secretory profile for both pluripotent and nullipotent cells will provide a legacy of potential targets.

List of Abbreviations

AP	Alkaline Phosphatase
BCA	Bicinchoninic Acid
BRCA	Breast Cancer Associate Gene
cDNA	complementary DNA
CNS	Central Nervous System
CSC	Cancer Stem Cell
CT	Threshold Cycle
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dsRNA	Double Stranded RNA
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
EOC	Epethelial Ovarian Cancer
FAM	6-carboxyfluorescein
FBS	Foetal Bovine Serum
FRET	Forster Resonance Energy Transfer
GAPDH	Glyceraldhyde 3-phosphate dehydrogenase
hEC	Human Embryonal Carcinoma
HIF	Hypoxia Indubcible Factor
IL	InterLeukin
IRF	Interferon Regulatory Factor
LPS	LipoPolySaccharide
miRNA	Micro RNA
mRNA	messenger RNA
MSCs	Mesenchymal Stem Cells

MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid Differentiation Primary Response Gene 88
ncRNA	non-coding RNA
NF-κB	Nuclear Factor kappa light polypeptide gene enhancer in B cells
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
piRNA	piwi-interacting RNA
PNS	Peripheral Nervous System
PRRs	Pattern Recognition Receptors
Q-PCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RISC	RNA-Induced Silencing Complex
rRNA	ribosomal RNA
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
snRNA	Small Nuclear RNA
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
tRNA	transfer RNA
TIR	Toll/Interleukin-1 Receptor
TLR	Toll Like Receptor
Tm	Melting Temperature
TNF	Tumour Necrosis Factor
VHL	Von Hippel-Lindau

Publications and Presentations

Cooke A., Gallagher M, O'Leary J. MyD88: A New Role as Gatekeeper of hEC differentiation? Manuscript in Preparation

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Cooke A., Gasch C., Gallagher M., O'Leary J. (2012) The Role of MyD88 in Chemoresistance, Differentiation and Hypoxia in Cancer Stem Cells, Poster presented at the Irish Association for Cancer Research Annual Meeting 1st – 3rd March 2012, Belfast, UK

Cooke A., Gallagher M., O'Leary J. (2011) Investigating the Effect of Differentiation Status on the CSC Response to Chemotherapy, Poster presented at ""Rediscovering Pluripotency: From Teratocarcinomas to Embryonic Stem Cells" conference, 10-12th October 2011, Cardiff, UK

Gallagher M., **Cooke A**., Ffrench B., O'Leary J. (2011) MyD88: A Retinoic Acid Differentiation Gatekeeper, Poster presented at "Rediscovering Pluripotency: From Teratocarcinomas to Embryonic Stem Cells" conference, 10-12th October 2011, Cardiff, UK

Cooke A., Gallagher M., O'Leary J. (2010) The effect of differentiation status on the response of cancer stem cells to chemotherapy Poster presented at the United States and Canada Academy of Pathology (USCAP) Annual Meeting, 20th-26th March 2010, Washington DC, USA

Chapter One

Introduction

1.1 Ovarian Cancer

Ovarian cancer is the fifth highest cause of cancer related death in women in the United States (Siegel et al., 2013) and the most common cause of gynaecologic cancer deaths worldwide (Schwartz 2002). It is estimated that in the UK, 125 new cases will be diagnosed every week (Cancer Research UK, 2011). In Ireland, it is the fourth most common cause of cancer death amongst women (National Cancer Registry of Ireland, 2011). There are a range of risk factors for ovarian cancer, including endometriosis (Aris, 2010), germ line mutations in *BRCA1* and *BRCA2* (Easton, Ford, Bishop, & Linkage, 1995; King, Marks, & Mandell, 2003), nulliparity and early menarche (Schorge et al., 2010).

Survival of ovarian cancer is dictated by two factors: age at diagnosis, and cancer stage at diagnosis with patients diagnosed at a late stage and older age facing a poorer prognosis. Five year survival rate for women stands at just 38% worldwide. In Ireland women aged 15-49 have a five year survival rate of 71.6%, compared with just 37.4% for women aged 50-69 (Women's Health Council). Women diagnosed at stages 0-2 have a 5 year survival rate of 80.4%, compared with 14.5% for women diagnosed at stages 3-4 (National Cancer Registry of Ireland, 2011). Unfortunately, ambiguous symptoms lead to late diagnosis, with 75% of patients being diagnosed at a late clinical stage (Lutz, Drescher, Ray, Cochran, & Urban, 2011). Recurrence of ovarian cancer is common, with long term survival dropping to between 15-20%. This low survival rate is believed to be caused by the development of chemoresistance, following recurrence. The rate of mortality in ovarian cancer has changed little in the past three decades (Barnholtz-Sloan, 2003). Chemoresistance remains the major therapeutic barrier (Agarwal & Kaye, 2003). As it stands the mechanism responsible for chemoresistance remains unknown.

In the case of women who do respond positively to treatment, many go on to experience poor quality of life outcomes. In a study carried out by Holzner et al, 32% of long term survivors of ovarian cancer were classified as suffering from fatigue: within this cohort there was an increase in anxiety and depression (Holzner et al., 2003). Furthermore, long term survivors of ovarian cancers may experience cognitive and neurological complications caused by systemic chemotherapy ((Schultz, Beck, Stava, & Vassilopoulou-Sellin, 2003). Additionally up to 20% of ovarian cancer survivors experience abdominal and gynaecological side effects and neurotoxication (Wenzel et al., 2002).

Standard primary chemotherapy treatment for ovarian cancer consists of a combination paclitaxel/platinum-based therapy regime. Paclitaxel is a natural derivative from the bark of the Pacific Yew Tree (*Taxus brevifolia*), first artificially synthesised for use in medicine in 1971 (Wani et al., 1971). Paclitaxel functions in cancer therapy by stabilising the microtubule polymer, protecting it from disassembly (Singla, Garg, & Aggarwal, 2002). This means that mitosis cannot proceed, resulting in either triggering of apoptosis or reversion to the G-phase of the cell cycle with no cell (Fuchs and Johnson, 1978). Paclitaxel is most commonly used in combination with a platinum-based chemotherapy: either cisplatin, carboplatin or oxaliplatin. The first discovered of these platinum based chemotherapy agents was cisplatin.

The anti-cancer properties of cisplatin (*cis*-diamminedichloroplatinum (II)) were first discovered in the mid 1960's when it was noted that electrolysis of platinum electrodes generated a soluble platinum complex which inhibited binary fission in *Escherichia coli* (Rosenberg et al 1965). This platinum complex was later described as cisplatin, and its anti-cancer potential was instantly recognised. It was licensed for use in ovarian and

testicular cancers in 1978 and has since become one of the key therapies for ovarian cancer treatment. Cisplatin is introduced to the body intra-venously and so is delivered to the tumour site via the blood. The high chloride concentration (~100mM) of the blood suppresses the exchange of the chloride leaving groups for water molecules until the cisplatin has entered the cell which has a much lower chloride concentration ~4mM. Once inside the cell; hydrolysis of the chloride takes place, leaving the platinum atom free to bind to the nucleic bases of the DNA, especially Guanine. Cisplatin is the primary platinum based chemotherapy used combination with paclitaxel in the treatment of patients with ovarian cancer in Ireland.

Resistance to cisplatin treatment is thought to be mediated by increased levels of cellular glutathione (Godwin et al., 1992). Glutathione may protect cells by binding to or reacting with drugs, by interacting with reactive oxygen moieties or with other radicals produced by radiation, by preventing damage to proteins or DNA, or by participating in repair processes. Other work has suggested that resistance to cisplatin is achieved by alterations in trafficking and localisation of the drug within the cell (Liang et al 2009). Data from a microarray study carried out on cisplatin resistant cells indicated that resistance can also be linked with enrichment of pathways associated with fatty acid metabolism and oxidation (Sherman-Baust, Becker, Wood, Zhang, & Morin, 2011).

1.2 The Cancer Stem Cell Theory

The Cancer Stem Cell (CSC) theory dates back to the late 19th Century with the work of Rudolf Virchow who proposed the "embryonal rest" hypothesis of tumour formation based on the histological similarities between tumours and embryonic tissues (reviewed in Huntly & Gilliland, 2005). However it was not until the middle of the century that experimental evidence was first published. The work of Kleinsmith and Pierce

demonstrated that single cells isolated from embryonal carcinomas, were multi-potential and could regenerate the somatic tissues of well-differentiated teratocarcinoma (Kleinsmith & Pierce, 1964).

The CSC theory suggests that there is a cellular hierarchy within tumours. Only a specific cell population within a tumour is thought to possess the ability to drive the growth and spread of the tumour (Figure 1.1). The wide variety of cellular types within a tumour is believed to be descended from a small population of cancer cells with stem like properties (reviewed in Lobo, Shimono, Qian, & Clarke, 2007). It is thought that the CSC population of a tumour operates in much the same way as the adult stem cell population of any organ; i.e. to drive (tumour) repair and growth (Clarke & Fuller, 2006; Reya, Morrison, Clarke, & Weissman, 2001a). Cancer stem cells were originally identified in the context of leukaemia (P. Fialkow, Gartler, & Yoshida, 1967; P. J. Fialkow et al., 1981) but have now been identified in a wide range of cancer types including breast (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Charafe-Jauffret et al., 2009), lung (Seo et al., 2007), and ovarian (Alvero, Chen, et al., 2009).

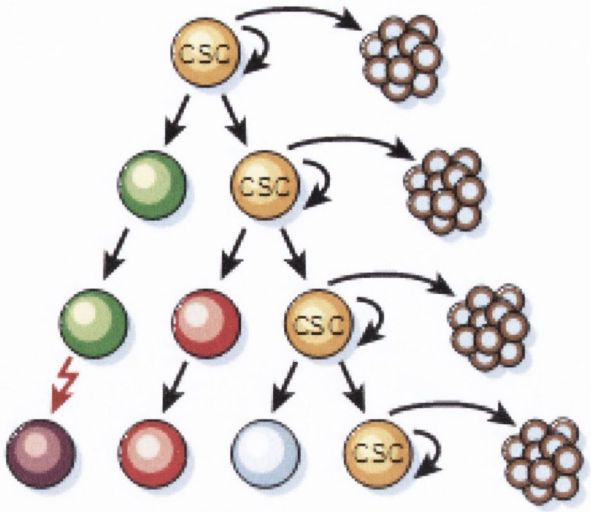

Figure 1.1: The cancer stem cell theory. The cancer stem cell theory holds that tumours are heterogeneous and only the cancer stem cell (CSC- yellow) component has the potential to proliferate and form new tumours (Adapted from Reya, Morrison, Clarke, & Weissman, 2001b)

CSCs display enhanced proliferation and tumorigenic capability as well as chemoresistance and resistance to hypoxia (Dean, Fojo, & Bates, 2005; Rich & Bao, 2007). Specific targeting of the cancer stem cell population has been trialled with some success in leukaemia (Foster et al., 2009). Since cancer stem cells are the driver of tumourigenisis and once differentiated they become less or non-tumourgenic, it is thought that by specifically targeting the stem cell population of a tumour, it will be possible to remove the self-renewal potential of the malignancy, leading to a decrease in recurrence. In the context of ovarian cancer, where high levels of recurrence present such a challenge, this idea is of particular interest.

NTera2 cells are a human teratocarcinoma derived cell line with a phenotype resembling committed central nervous system (CNS) neuronal precursor cells. Undifferentiated NTera2 cells form tumours containing differentiated neurons as well as residual cells when injected into nude mice (Andrews et al., 1984). Treatment of NTera2 cells with 10^{-5}M Retinoic Acid (RA) has been shown to induce cells to differentiate into post mitotic

neurons (Pleasure & Lee, 1993a).When treated with retinoic acid, most cells differentiate into neurons, but a residual population of undifferentiated cells is retained, which resemble untreated cells both in morphology, and proliferative potential. Retinoic acid achieves this by binding to cellular RA –binding protein, (RABP) that facilitates uptake of RA and transport to the nucleus were RA binds the RA receptor (RAR). The ternary complex of ligand-bound RAR with RXR (which binds the alternative isomer of RA) and a retinoic acid response element (RARE) regulates expression of RA target genes by altering the binding of co-repressors and co-activators. RA target genes include Hox family genes, Fgf8 and Pax6 (Duester, 2008). Differentiated NTera2 cells bear a strong resemblance to human CNS foetal neurons, expressing similar cytoskeletal polypeptides, cell - surface markers and synaptic proteins typical of CNS neurons. However, differentiated NTera2 cells, express no markers that are restricted to peripheral nervous system (PNS) neurons (Lee & Andrews, 1986). The 2102Ep cell line is derived from a primary human testicular teratocarcinoma. 2102Ep cells do not differentiate in response to retinoic acid treatment. When 2102Ep cells are xenografted into nude mice they form tumours entirely composed of embryonal carcinoma cells (Duran et al., 2001). Thus NTera2 cells can be said to be pluripotent, whereas 2102Ep cells are nullipotent. Germ cell tumours such as these can form in the ovary, and previous work from this group has demonstrated that hEC stemness genes are differentially expressed in ovarian cancer tumour samples (Gallagher, Flavin, Elbaruni, et al., 2009; Gallagher et al., 2012), making them an ideal model of ovarian cancer stem cells.

Downregulation of pluripotency markers Oct4, Sox2 and Nanog are used in this work to verify differentiation of hECs. Previous work has established that Oct4, Sox2 and Nanog positively regulate transcription of all pluripotency circuitry proteins in the leukaemia inhibitory factor (LIF) pathway (Niwa, Ogawa, Shimosato, & Adachi, 2009). Thus they are

considered markers of pluripotency (S. Lin, 2011). Knockdown of Nanog in embryonic stem cells has been demonstrated to cause their differentiation (T. Lin et al., 2005). Previous work from this group has demonstrated that knockdown of Sox2 in 2102Ep cells causes them to lose their nullipotency and differentiate (Vencken 2012).

Embryonal carcinoma cells can be thought of as the malignant counterpart to embryonic stem cells (Andrews et al., 2005). Work by Josephson et al demonstrated through microarray comparison of 2102Ep and NTera2 cells with BG01 Embryonic Stem Cells that the overall similarity between 2102Ep and NTera2 and human Embryonic Stem Cells (hESCs) approaches that between the most distant hESC lines. They also showed that both 2102Ep and NTera2 cells are easily maintained in a non-differentiated state (verified by constitutive expression of pluri- and nullipotent markers, respectively). (Josephson et al., 2007) These characteristics, along with their lack of ethical concerns make NTera2 and 2102Ep cell lines ideal models of embryonic stem cell biology. Recent work has also revealed a mechanism of resistance to chemotherapy, mediated by Mesenchymal Stem Cells (MSCS), activated by platinum-based chemotherapy (Roodhart et al., 2011).

Previous work has shown that degree of differentiation is the most important independent prognostic factor in primary epithelial ovarian cancer (Vergote et al., 2001) (Dembo et al., 1990). However in recurrent cases the degree of differentiation is no longer considered a prognostic factor (Colombo et al., 2010). This is further reflected in the differing genetic profiles seen between primary and recurrent disease in the same patient (Xu et al., 2010). This may suggest that in recurrent tumours, the differentiation status of cells has no bearing on their response to chemotherapy. Given the differing responses of NTera2 and 2102Ep cells to differentiation stimulus, they will provide an ideal model system in which to study the relationship between differentiation and response to chemotherapy.

1.3 Hypoxia and Stemness

Recent advances in cancer research have indicated that the enhanced expression and activation of hypoxia-inducible factors (HIFs) frequently occur in cancer cells during cancer progression and is associated with the acquisition of a more malignant behaviour (Jubb, Buffa, & Harris, 2010; Zhong et al., 1999). HIF transcription factors include HIF-1α, which is expressed in most tissues, and HIF-2α, which shows a more restricted tissue expression pattern in various locations, including kidneys, brain, lungs, liver, gastrointestinal tract, pancreas and heart (Wiesener & Jürgensen, 2003).

Hypoxia Inducible Factor-1 (HIF-1) is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits. While the expression and activation of the HIF-1α subunit is tightly regulated by cellular O_2 levels, the expression of HIF-1β subunit is constitutive (Semenza, 2002). The tumour suppressor gene von Hippel–Lindau (VHL) mediates degradation of HIF-1α in the presence of oxygen and loss of VHL results in HIF-1α accumulation (Figure 1.2). HIF-1α is a key regulator of cellular response to hypoxia and its presence or absence can be used to ensure hypoxic conditions (Iyer et al., 1998; Wenger, 2002). It can also be activated under normoxia in response to a variety of growth factors including insulin-like growth factors and pro-inflammatory cytokines such as IL-1β and TNFα (Fukuda et al., 2002; Hellwig-Bürgel & Rutkowski, 1999)

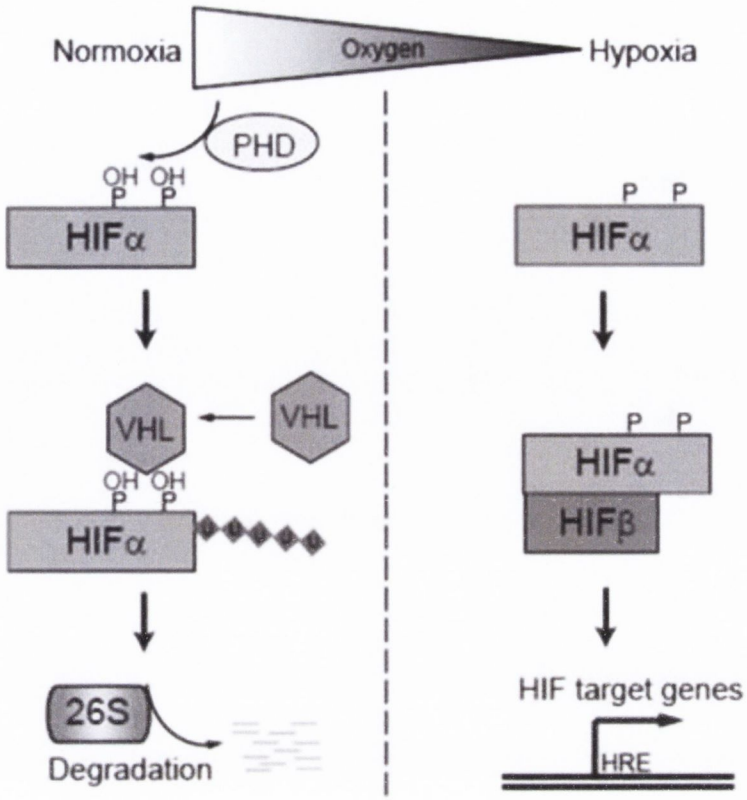

Figure 1.2 Cellular oxygen sensing. Transcriptional activity of HIF is regulated by proteasomal degradation of HIFα in the presence of oxygen. Prolylhydroxylases (PHD) use molecular oxygen to hydroxylate HIFα, allowing recognition by the von Hippel–Lindau (VHL) protein leading to ubiquitin mediated proteolysis of HIF-1α. (adapted from Cantley & Grey, 2010)

Genes regulated by angiogenesis, proliferation, immune evasion and metastasis can be induced, repressed or manipulated by the HIF transcription family (Keith, Johnson, & Simon, n.d.). In order to continue growing under hypoxic conditions, cancer cells adapt to the absence of exogenous mitogenic growth signals and become resistant to anti-proliferative signals (Harris, 2002). Previous work has demonstrated that over-expression of HIF1α represents a poor prognosis for cancer patients, alongside its direct negative effect on cancer therapy (Semenza, 2002).

Adult stem cells have long been acknowledged as the source of tissue regeneration within the body. Adult stem cells are known to experience improved proliferation in hypoxic conditions (Tsai, Yew, Yang, Huang, & Hung, 2012), and stem cells are known to be localised to hypoxic sites i.e. hematopoietic stem cells in bone marrow. In common with this, recent studies have concluded that the malignant re-programming of cancer and metastasis-initiating cells may occur within hypoxic intratumoral regions in primary neoplasms and "hypoxic niches" at distant metastatic sites, and that hypoxia plays a critical role in the acquisition of aggressive phenotypes and treatment resistance (Mimeault & Batra, 2013).

1.4 Inflammation, TLRs, MyD88 and Cancer

Inflammation is a physiological process involved in the immune response and tissue repair. Inflammatory signals released following invasion of a host stimulate both cell proliferation and neovascularisation, both of which are necessary for successful wound repair. Approximately 15% of the global cancer burden is thought to be directly attributable to infectious agents (Parkin et al., 1999). Under normal circumstances cell repair and proliferation processes are tightly regulated to ensure that they are only activated when needed. However in the case of chronic conditions, the long term presence of inflammatory signals is likely to have a proliferative effect on the cellular environment. This may be mediated by increase in interleukin-6 (IL6) secretions leading to activation of the JAK/STAT3 signalling pathway, known to increase cell proliferation (Ataie-Kachoie, Pourgholami, & Morris, 2013). Uncontrolled proliferation such as this is one of the hallmarks of cancer (Hanahan & Weinberg, 2011). The link between inflammation and cancer has long been recognised with inflammatory diseases such as Crohn's disease, chronic bronchitis and ovarian endometriosis increasing the risk of colon, lung and ovarian cancers respectively. Inflammatory responses can be mediated by toll like receptor (TLR)

pathways (Tsan, 2006). TLRs are pattern recognition receptors located in the cell surface membrane and are key constituents of the innate immune system. Stimulation of TLR pathways leads to NF-κB activation with the downstream effect of higher cell proliferation and neovascularisation via activation of JAK/STAT3 signalling. (Chow, Young, Golenbock, Christ, & Gusovsky, 1999; Ruslan Medzhitov, Preston-hurlburt, & Jr, 1997). The key signalling domain which is unique to TLRs is the Toll/interleukin-1 (IL-1) receptor (TIR) domain which is located in the cytosolic face of each of each TLR and also in TLR adaptors (O'Neill, Fitzgerald, & Bowie, 2003). Stimulation of the TLRs occurs via sensing of pathogen associated molecular patterns (PAMPS). TLRs occur as dimers, and are activated by homodimerization. Dimerization is triggered by ligand binding. A conformational change then occurs that brings the TIR domains closer together which creates the basis of the signalling complex which is necessary for adaptor recruitment (O'Neill and Bowie 2007).

LipoPolySaccharide (LPS), a cell wall component of gram negative bacteria is a ligand for TLR4. In normal circumstances, LPS binds to TLR4 causing homodimerization and eventual downstream activation of NF-κB. which ultimately leads to the synthesis and release of a number of pro-inflammatory mediators, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor-a (Chow et al., 1999). TLR4 can operate via two pathways, either MyD88 dependent or MyD88 independent pathways (Figure 1.3). Both pathways lead to eventual activation of NF-κB, however in the case of MyD88 Independent signalling, this is achieved at a kinetic delay (Kawai, Adachi, Ogawa, Takeda, & Akira, 1999; Lu, Yeh, & Ohashi, 2008).

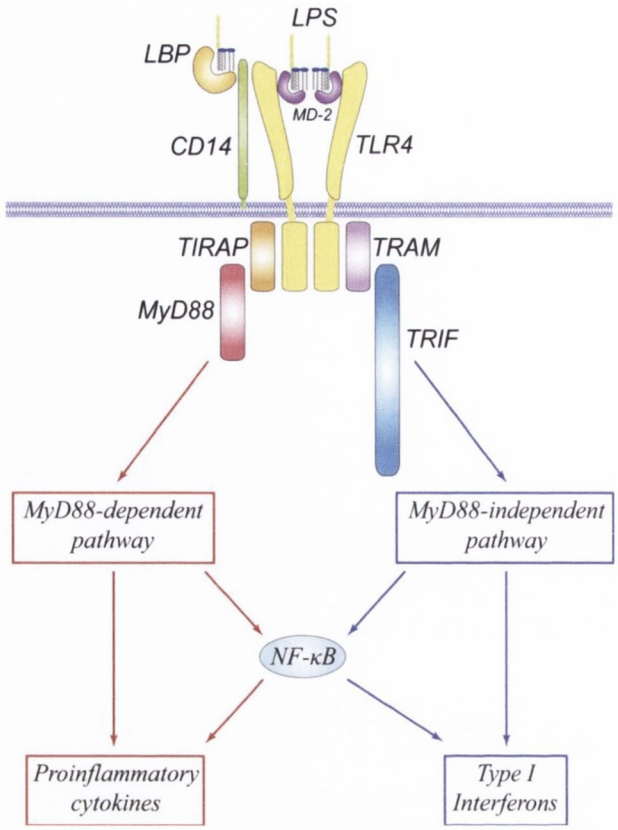

Figure 1.3: TLR4 signalling via MyD88 Dependent and MyD88 Independent Pathways. TLR4 can signal either via MyD88 leading to upregulation of NF-κB and pro-inflammatory cytokines or via MyD88 independent signalling (TRIF) leading to eventual upregulation of NF-κB and Type 1 Interferons. (adapted from Lu et al., 2008)

When TLR4 signals via the MyD88 dependent pathway, MyD88 recruits interleukin-1-receptor-associated-kinase-1 (IRAK-1) and interleukin-1-receptor-associated-kinase-4 (IRAK-4). IRAK-4 acts upstream of IRAK-1, activating it via phosphorylation. IRAK-1 in turn activates tumour necrosis factor (TNFR) associated factor-6 (TRAF6). Activation of TRAF6 causes disassociation of the IRAK-1/TRAF6 complex from the receptor and association with the transforming-growth-factor-β (TGFβ)-activated kinase-1 (TAK-1) and TAK-binding proteins TAB1 and TAB2. IRAK-1 stays in the membrane and is degraded, whereas the complex of TRAF6, TAK1, TAB1 and TAB2 moves to the cytoplasm where it forms a large complex with several other proteins, including the E2 ligases Ubc13 and Uev1A. The Ubc13 and Uev1A complex has been shown to catalyse the synthesis of a Lys

63-linked polyubiquitin chain of TRAF6, and thereby induce TRAF-6 mediated activation of TAK1 and finally of NF-κB.

Recently TLR4-MyD88 signalling has been used to classify epithelial ovarian cancer (EOC) tumours as either type I or type II EOC on the basis of their TLR4-MyD88 dependence or independence respectively (Chen, Alvero, Silasi, Steffensen, & Mor, 2008b; López, Valdez-Morales, Benítez-Bribiesca, Cerbón, & Carrancá, 2013). Their work demonstrated that $MyD88^{+}$ epithelial ovarian carcinoma cells formed spheroid bodies *in vitro*, and differentiated tumours *in vivo*. Type I EOC cells have a functional TLR4-MyD88 pathway, and display increased chemoresistance as well as continuous cytokine expression. In contrast TLR4-MyD88 signalling is absent in type II EOC cells, which are chemosensitive (Chen, Alvero, Silasi, Steffensen, & Mor, 2008a). It is now thought that type I EOC cells represent an ovarian cancer stem cell population, with high MyD88 expression levels seen as characteristic of this genotype (Alvero, Fu, et al., 2009). Previous work by Kelly et al has shown that ovarian cancer patients whose tumours tested negative for MyD88 expression had a statistically significantly improved progression free interval compared with patients whose tumours were MyD88 positive (Kelly et al., 2006). This observation has been confirmed in an Irish population by work from the O'Leary laboratory (d'Adhemar et al., in preparation). The standard chemotherapy for ovarian cancer is combination paclitaxel/cisplatin treatment. Paclitaxel is a known ligand for TLR4, suggesting that standard treatment could potentially cause upregulation of the TLR4-MyD88 response (Byrd-Leifer, Block, Takeda, Akira, & Ding, 2001; Wang, Su, Wu, Zhang, & Liu, 2008).

Previous work has shown a link between TLR4-MyD88 signalling and hypoxia (Ock et al., 2007). In a study carried out in culture microglia, hypoxia was found to upregulate TLR4

expression. Furthermore, hypoxia differentially regulated MyD88-dependent and independent pathways of TLR4 signalling. Hypoxia enhanced lipopolysaccharide (LPS)–induced interferon regulatory factor–3 (IRF-3) activation and the subsequent expression of IFNb (MyD88-independent pathway), whereas it suppressed LPS-induced NF-κB activation (MyD88-dependent pathway) (Ock et al., 2007).

MyD88 has been previously been implicated in a wide range of cancers. Mice that had MyD88 deleted specifically in keratinocytes demonstrated resistance to skin carcinogenesis in which indicates that MyD88 signalling in the epithelial cells contributes to tumour formation (Salcedo, Cataisson, Hasan, Yuspa, & Trinchieri, 2013). Data from the work of Kennedy et al, indicates that MyD88 dependent signalling contributes to a proliferative, anti-apoptotic phenotype in the context of gastric tumourigenesis (Kennedy et al., 2013). In hepatocellular carcinoma, MyD88 has been demonstrated to promote growth and metastasis (Liang et al., 2013). Thus the role of MyD88 has been well established in a broad range of cancer types, and is not limited to ovarian cancers.

1.5 Project Rationale

Previous work carried out in this group demonstrated that when gene expression between undifferentiated and differentiated embryonal carcinoma cell lines was compared via Affymetrix array, MyD88 was differentially expressed. (M. Gallagher, unpublished data). Given the significant role that MyD88 is known to play in a wide range of cancers, it was decided to further investigate the effect of MyD88 expression in ovarian cancer. MyD88 expression was investigated in clinical samples from ovarian cancer patients (D'Adhemar et al, in preparation). However, experiments described in this thesis focus on the role of MyD88 in an *in vitro* model of ovarian cancer stem cells i.e. the previously established embryonal carcinoma model (Gallagher, Flavin, Elbaruni, et al., 2009).

1.6 Project Overview

There is strong evidence indicating a link between MyD88 signalling and cancer stem cells, which are inherently capable of differentiation and resistant to chemotherapy and hypoxia treatments. Using two hEC cells lines as a model of ovarian cancer stem cells, their response to a range of challenges, alone and in combination was characterised in terms of TLR4-MyD88 expression. These challenges included cisplatin, retinoic acid and hypoxia, allowing for the assessment of hEC responses to conditions that would occur *in vivo* during the course of routine chemotherapy. This characterisation is described in Chapter 3. Following on from this characterisation, the functional effect of MyD88 alteration was assessed in all of these conditions. This was achieved by either knocking down or overexpressing MyD88 in both nullipotent and pluripotent cells, and challenging them with cisplatin, retinoic acid and hypoxia. This allowed for the determination of the function of MyD88 in the normal cellular response to these treatments. This characterisation is described in Chapter 4.

Downstream analysis of MyD88 knockdown cells was performed via Affymetrix Gene Array, SOLiD second generation sequencing and protein array. This allowed for the identification of novel downstream targets of MyD88 at both the gene and protein level. This characterisation is described in Chapter 5.

Having demonstrated that MyD88 signalling was affected by cisplatin, retinoic acid and hypoxia, media secreted by these cells was analysed for changes in secretory profile using protein arrays. This allowed for the identification of novel roles for chemokines and cytokines in the hEC response to all these three challenges.

1.7 Hypothesis and Aims

The initial hypothesis of this study was that TLR4-MyD88 signalling was involved in the response of hEC cells to cisplatin, differentiation and hypoxia. Furthermore it was hypothesised that pluripotent and nullipotent hEC cells would differ in this response.

When these responses had been established it was hypothesised that they were necessary and sufficient for hEC cell survival in these conditions. Furthermore it was hypothesised that these TLR4-MyD88 expression changes would have downstream effects on protein secretions.

These hypotheses led to a set of aims listed below:

- Investigate whether TLR4-MyD88 expression in hEC cell lines is affected by cisplatin, differentiation and hypoxia treatments.
- Treat both MyD88 knockdown and overexpression cells with a cisplatin, retinoic acid and hypoxia, alone and in combination, and determine the cell survival of altered cells in these conditions
- Determine and characterise the downstream effects of MyD88 alteration in hEC cells at the gene, miRNA and protein levels.
- Furthermore, determine the chemokine and cytokine profile of hEC cells in response to cisplatin, retinoic acid and hypoxia, alone and in combination.

Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 General Cell Culture Protocols

Two cancer stem cell lines were used for this work, 2102Ep and NTera2. NTera2 cells are a teratocarcinoma derived cell line with a phenotype resembling committed CNS neuronal precursor cells. Undifferentiated NTera2 cells form tumours containing differentiated neurons as well as residual cells when injected into nude mice (Andrews et al., 1984). Treatment with 10^{-5}M retinoic acid has been shown to induce NTera2 cells to differentiate into post mitotic neurons (Pleasure & Lee, 1993b). The 2102Ep cell line is also derived from a primary human testicular teratocarcinoma. 2102Ep cells do not differentiate in response to retinoic acid treatment. When 2102Ep cells are xenografted into nude mice they form tumours entirely composed of embryonic carcinoma cells (Andrews et al., 1984). The choice of these cell lines was based on a number of factors; 2102Ep and NTera2 maintain their undifferentiated state readily, without requiring supplementation of their media with growth factors. Furthermore, previous from this group has established them as a reliable model of ovarian cancer (Gallagher, Flavin, Elbaruni, et al., 2009; Gallagher et al., 2012).

All cells were maintained in a sterile environment in DMEM medium supplemented with 10% foetal bovine serum and 5% penicillin-streptomycin 100 I.U/ml, streptomycin (Lonza, Switzerland) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were passaged every three days via trypsinisation (2102Ep) or scraping (NTera2). All cell culture work was conducted under a laminar air flow hood, using aseptic technique.

Cells were harvested as follows. Old media was removed from the cell culture flask using a 10ml pipette, and then the cells were washed with 3-5mls phosphate buffered saline (PBS)

(Lonza, Switzerland). The cells were scraped in 5mls PBS, the flask was rinsed with a further 5mls of PBS before being transferred to a 15ml tube and centrifuged at 1,000 xG for five minutes. The supernatant was then removed, and the resulting cell pellet was stored at -80°C until required.

For long term storage of stocks, cells were either trypsinised or scraped as appropriate and resuspended in 1.5mls of freezer media (Gibco). 0.5ml aliquots of this were transferred into cryoproof vials and frozen at -80°C overnight before being transferred to a liquid nitrogen tank for long term storage. Cells brought back from storage were thawed rapidly to room temperature, washed with complete medium before being pelleted and resuspended in fresh medium. Cells were then transferred to a T25 cell culture flask containing 5mls of complete medium and passaged as appropriate.

2.1.2 Cell Counting

Cells were counted for appropriate seeding using a haemocytometer (Figure 2.1). Cells were resuspended in 4mls of media. 50 µl of this suspension was diluted in 50µl medium, and then added to 100µl of trypan blue stain. Trypan blue is a vital dye which contains a negatively charged chromopore and thus does not interact with cells unless the cell membrane is damaged. Exclusion of trypan indicates that the cellular membrane is intact and thus that the cell is viable. 10µl of this mix was added to each side of the haemocytometer. Viable cells were counted in each of the four corner squares of the haemocytometer on either side. An average for these two counts was obtained and then multiplied by 4 to allow for the dilution factor, and then by 2,500. This gives the number of cells/ml of the solution.

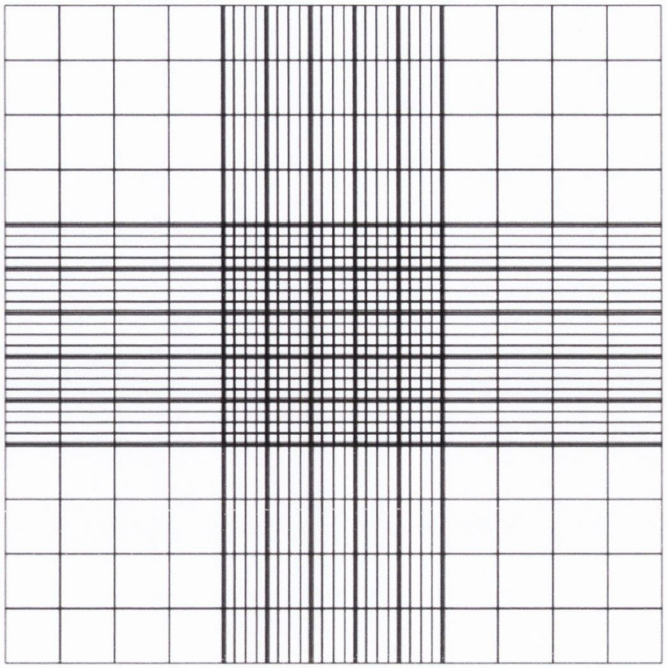

Figure 2.1: Grid layout of a Haemocytometer.

(Adapted from www.microbehunter.com).

2.2 Generation of a Cisplatin Dose Response Curve

The dose-response interaction between cisplatin and NTera2 cells and 2102Ep cells was assessed by incubating both cell lines in the presence of increasing doses of cisplatin and calculating cell viability.

Cell viability was assessed by measuring the mitochondrial activity of the cells by means of a MTT Assay Kit (Roche, UK). In an MTT assay, drug treated cells are incubated in the presence of MTT (3-[4, 5-dimethylthiazole-2-4]-2,5-diphenyl-2H-tetrazolium bromide). MTT is reduced by viable cells to a purple coloured, water-insoluble formazon salt. This is then dissolved in a solvent (DMSO) before absorbance of the cells is measured using an ELISA plate reader at 570nm. The cell viability is calculated as the absorbance of cells with drug divided by the absorbance of the control without drug addition. Having graphed the cell viability against drug concentration, the half maximal inhibitory concentration (IC_{50}) can be calculated as the midpoint of the graph.

Cells were seeded at a density of 6,000 cells/well in 100µl media in a 96 well plate and left to adhere overnight. Cisplatin was obtained from the pharmacy department, St James' Hospital as a 0.003M solution. Increasing concentrations of cisplatin were made up fresh in 50µl medium (10nm to 100µM range). 50µl of media was removed from each well, and replaced with 50µl media containing the appropriate concentrations of cisplatin. 50µl of media was removed from the negative control cells, and replaced with 50µl of fresh media. All cells were then left to incubate at 37°C and 5%CO_2 for 72 hours. Cell viability after the 72 hours was assessed by adding 10µl MTT solution to each well, leaving to incubate at 37°C, 5% CO_2 for four hours before removing the media and replacing with 100µl DMSO solution and incubating overnight at 37°C, 5% CO_2. Wells were then scanned the following day using an ELISA scanner at 595nm to measure absorbance of light through each well. Cell viability was calculated as absorbance of drug treated cells divided by absorbance of negative control cells. A graph was constructed of drug concentration against cell viability. The midpoint of this graph was taken as the IC_{50}.

2.3 Cisplatin Treatments

Following determination of the appropriate IC50 for cisplatin for each cell line, an experimental matrix was designed using combinations of retinoic acid and cisplatin to investigate the effect of chemotherapy on TLR4/MyD88 signalling and the influence of differentiation status on this effect (Table 2.2).

Cells were split at high confluence (NTera2 ~95%, 2102Ep~90%) to ensure a large cell population prior to drug treatment. Cells were counted and then seeded in 6 well plates in DMEM supplemented with FBS and pen/strep as described previously. Cells were incubated overnight at 37°C and 5% CO_2 to allow them to adhere. The following day

media was carefully removed from the wells before being replaced with media containing the appropriate concentration of the drug of interest. Media was also replaced on any untreated wells, to act as a negative control. Cells were incubated in the presence of the drug for three days, after which the cells were harvested in the usual way. For cells that were treated with retinoic acid, retinoic acid was added to the media in the plate before cells were seeded. Vehicle controls were prepared for both cell lines, comprising of a solution of 9% NaCl and 1% Mannitol in hEC media.

Table 2.1: Chemotherapy-Differentiation Drug Treatment Matrix

1st Treatment	Duration	2nd Treatment	Duration
Cisplatin	3 days	****	***
Retinoic Acid	3 days	****	***
Cisplatin	3 days	Retinoic Acid	3 days
Retinoic Acid	3 days	Cisplatin	3 days

***** indicates cells were treated with one treatment only*

2.4 Culture of CSCs in Hypoxic Conditions

Cells were split at high confluence and seeded in 6 well plates at 168,000 cells in 3mls of media per well. Cells were allowed to adhere overnight at 5% CO_2, 37°C. The following day cells were placed in a hypoxia chamber and incubated at 0.5% O_2, 37°C. Media was also left in the chamber overnight, in order to allow it to become hypoxic. The chamber was maintained at 0.5% O_2 under Nitrogen for the duration of all experiments. Media was changed and cells harvested within the chamber. Where necessary, medium was changed within the chamber; cells were harvested within it and all treatments (such as MTT assays) were carried out within it. Confirmation of hypoxia was achieved via western blot for Hif-1α.

2.5 Differentiation Stimulus via Retinoic Acid

Differentiation was stimulated via addition of Retinoic Acid. A stock solution of $1x10^{-3}$M Retinoic Acid (Sigma-Aldrich) was made up in DMSO and stored at -20°C. To stimulate differentiation a sufficient volume of retinoic acid was added to the cell culture media at the time of cell plating to give a final concentration of $1x10^{-5}$M retinoic acid.

2.6 Total RNA extraction

RNA was extracted using a mirVana miRNA Extraction Kit (Ambion). The kit uses an organic extraction followed by immobilization of RNA on glass-fibre filters to purify either total RNA or RNA enriched for small species from cells or tissue samples.

Historically RNA has been purified in one of two ways, either by chemical extraction or via solid-phase extraction. Chemical extraction methods use highly concentrated chaotropic salts in conjunction with either acidic phenol or phenol-chloroform solutions to inactivate RNases and purify RNA from other biomolecules. This method provides very pure preparations of RNA, however the RNA recovered must be desalted and concentrated with an alcohol precipitation step. Solid phase extraction relies on high salt or salt and alcohol to decrease the affinity of RNA for water and increase its affinity for the solid support used. The mirVana miRNA extraction kit combines the advantages of chemical extraction and solid-phase extraction while avoiding the disadvantages of both.

During the protocol the sample is first lysed in a denaturing lysis solution which stabilizes RNA and inactivates RNases. The lysate is then extracted once with Acid-Phenol: Chloroform which removes most of the other cellular components, leaving a semi-pure RNA sample. This is further purified over a glass-fibre filter by one of two procedures to yield either total RNA or a size fraction enriched in miRNAs.

For all experiments described here the procedure to yield total RNA was used. Protocols were followed as per manufacturer's instructions with no modifications. RNA was eluted from sample columns in RNase free H_2O.

2.7 RNA Quantification

RNA was quantified using a Nanodrop-2000 (Thermo Scientific). The Nanodrop is a full spectrum UV-Vis micro-volume spectrophotometer that is used to assess the purity and concentration of DNA, RNA and protein. The Nanodrop measures the absorbance of an RNA sample at 260 and 280nm. For this work RNA concentration was calculated using the pre-set formula on the Nanodrop. RNA quality was assessed via the ratio of the absorbance at 260nm and 280nm. Pure RNA has an A260/A280 of 2.1. For these experiments a ratio greater than 1.8 was deemed to indicate sufficient quality to proceed.

2.8 TaqMan PCR

2.8.1 Overview of TaqMan PCR

A TaqMan PCR-based system was selected for mRNA quantification in this study, due to its requirement for relatively small amounts of input material and its high specificity and sensitivity. TaqMan PCR is a quantitative real-time (RT) PCR technique, which exploits the dual 5' polymerisation and exonuclease functionality of certain DNA polymerases. It detects the amplification of PCR product in real time by hybridisation and cleavage of a dual labelled fluoregenic probe. The TaqMan probe is composed of a short oligonucleotide sequence, 20-25 bases in length, a 5' and 3' fluorescent molecule and a 3' blocking phosphate that prevents nucleotide extension. The probe is designed to hybridise with the target sequence of the forward and reverse primers. While the probe is intact the proximity of the 5' end fluorescent molecule, known as the reporter dye, to the 3' end fluorescent

molecule, known as the quencher dye, results in energy transfer between the two molecules which suppresses fluorescent emission from the reporter dye. This phenomenon is known as Forster resonance energy transfer (FRET; Forster, 1948, Lakowicz, 1983). On binding of the primers to the target sequence, sequence elongation occurs resulting in cleavage of the probe due to the 5'-3' exonuclease activity of the polymerase used. Consequently there is a separation of the reporter and quencher molecules, which results in an increased fluorescent emission from the reporter molecule. Therefore the accumulation of PCR product is directly proportional to the increase in the fluorescent emission from the reporter dye, which can be monitored in real time using the 7500HT sequence detection system (Applied Biosystems, USA).

The most commonly used enzyme for TaqMan PCR is Taq polymerase, which is isolated from the thermophilic bacteria *Thermus aquaticus*. This is used due to its ability to function in temperatures over 70°C and its 5' exonuclease activity. The exonuclease activity of this enzyme is double strand specific and thus will only act when the probe is hybridised to the target molecule. Detection of target molecule amplification relies on probe binding and subsequent release of the reporter molecule. Therefore hybridisation of the probe prior to primer binding and elongation is critical. For this reason, the melting temperature (Tm) of the probe is approximately 10°C higher than that of the primers which ensures the probes remain bound to the target molecule (Kenneth J Livak & Schmittgen, 2001) and prevents the generation of PCR products without fluorescence. In addition, TaqMan probes are designed as minor groove binding probes. This consists of a probe conjugated to a minor groove binder at the 5' end, which can be a naturally occurring antibiotic like distamycin A or synthetic molecules. The incorporation of a minor groove binding (MGB) molecule allows the formation of extremely stable hybrids with

complementary DNA. MGB probes also allow for higher melting temperatures as they bind more tightly to their targets.

All primers and probes used in this study including gene targets of interest and endogenous controls, used throughout this study were commercial pre-designed primer and probe mixes (20X) obtained from Applied Biosystems (USA).

All TaqMan PCR experiments were performed using a two step method where messenger RNA (mRNA) is converted firstly to complementary DNA (cDNA) and this is then used as the template in the TaqMan PCR reaction.

2.8.2 Synthesis of cDNA

Generation of cDNA was performed using the High Capacity cDNA Archive kit (Applied Biosystems, USA) according to the manufacturer's protocol. Each cDNA reaction contained 1X reverse transcription buffer, 25mM deoxynucleotide triphosphate (dNTP) mix, 1X random primers and 2.5U multiscribe reverse transcription enzyme. Multiscribe reverse transcriptase is a recombinant Moloney murine leukaemia virus reverse transcriptase and is an RNA dependant DNA polymerase which uses single stranded RNA in the presence of primer to generate cDNA. 650ng of template RNA was used in each 100µl cDNA reaction. The cDNA reaction was performed on the 9600 Thermal Cycler (Applied Biosystems, USA) as follows 25°C for 10 mins, 37°C for 120 mins, 85°C for 5 seconds to deactivate, and cooling to 4°C.

2.8.3 Quantitative PCR (qPCR)

TaqMan PCR was performed using Universal PCR Master Mix (Applied Biosystems, USA), which contains AmpliTaq Gold DNA polymerase, dNTPs with dUTP and passive

reference 1 which is an internal control that correct for inter well signal variation. RT-PCR reactions were performed in triplicate and per 20µl contained: 4µl cDNA, 1X Universal Master Mix and 1X predesigned primers and probe mix. The RT-PCR was performed on the 7500HT (Applied Biosystems, USA) under the following thermal cycling conditions: 50°C for 2min, 95°C for 10mins, and 50 cycles of 95°C for 15secs and 60°C for 1mins.

A no template control was included in each run for each primer and probe set. The endogenous control used in this study was a FAM labelled Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan primer and probe. This was assayed in a separate TaqMan PCR reaction to the target gene using the same PCR conditions and in the same run. Primers and probes were used as listed in Table 2.2, all were ordered from Applied Biosystems.

Table 2.2: qPCR Primers and Probes

Gene Name	Assay Number
GAPDH	4326317E
TLR4	Hs00152939_m1
MyD88	Hs00182082_m1
Oct4	Hs01654807_s1
Sox2	Hs00415716_m1
Nanog	Hs02387400_g1

2.8.4 Data Analysis of TaqMan PCR

TaqMan RT-PCR is a real time PCR technique, thus data is collected throughout the PCR process, rather than at the end of the PCR. Consequently reactions are characterised by the point in time during cycling when amplification of a target is first significantly detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

In the analysis, the initial cycles of PCR, where there is little change in the fluorescent signal, are used to define the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated target and a fixed fluorescence threshold is set above this baseline. The threshold is set at the point where a statistically significant increase in the fluorescent signal, i.e. the PCR product, is first detected. The cycle at which the sample crosses this threshold is determined by the analytical software (Applied Biosystems RQ Manager V1.2). This is known as the threshold cycle (CT) and is used to calculate the relative expression levels. Relative quantification relates the CT of a target transcript to that of a control transcript, known as the calibrator. This allows the expression of the data as a fold change of expression levels and can be achieved either using the standard curve method or the comparative CT method. In this study the comparative CT method was utilised.

Relative quantification of the target genes using the comparative CT method was first described by Livak and Schmittgen in 2001. The comparative CT method calculates relative gene expression using the following equation:

$$\text{Relative Quantity} = 2^{-\Delta\Delta Ct}$$

Initially, the mean CT value is calculated for each sample along with the standard deviation (StDev; all StDev were below .25). The ΔCT is then calculated by normalising the CT of the target sample with the CT of the endogenous control (CT target – CT endogenous control). Next the ΔΔCT is calculated by subtracting the ΔCT for the calibrator sample from the ΔCT for the test sample (K J Livak & Schmittgen, 2001). In this study the ΔCT for the calibrator sample represented an average value of at least three biological replicates, the StDev between these ΔCT values was below .25. Finally the relative levels of the target gene expression expressed as a fold change can be calculated

with the above formula. This fold change was then multiplied by 100 to give the percentage expression values used in all graphs here.

2.9 Protein Extraction

Directly before use, radioimmunoprecipitation (RIPA) buffer was prepared as follows: 100µl of protease inhibitor cocktail (Sigma-Aldrich, USA), 100µl of phosphatase inhibitor cocktail (Sigma-Aldrich, USA), 100µl of phenylethanesulponylfluoride (PMSF) (final concentration 2mM) was added to 350µl of 10X RIPA Buffer, containing a final concentration of 0.05M Tris-HCL, 0.15M NaCl, 0.25% deoxycholic acid, 1.0%Np-40, 1.0mM EDTA. The solution was made up to 10ml with H_2O.

Cells were seeded in a 6 well plate and when treatments were complete media was removed from the cells. Cells were immediately placed on ice and then washed twice with ice cold PBS (1ml). RIPA buffer was then added to each well. Cells were scraped and placed into pre-chilled eppendorf tubes which were shaken for 30 min on ice. Samples were centrifuged at 4°C at 1,000 XG for 15mins to collect debris, and supernatants transferred to fresh pre-chilled eppendorf tubes which were stored at -20°C. These samples were further sonicated at amplitude 50 in 10 second cycles until the solution was no longer viscous.

2.10 Protein Quantification

Protein content of BSA standards and cell extracts were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, USA). The Thermo Scientific Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantisation of total protein. The assay involves a two step process: one, the chelation of copper with protein in an alkaline environment, which results in the reduction of copper (Cu2+) to cuprous cat-ion (Cu1+), and the formation of a light

blue complex and two, the chelation of the cuprous cat-ion (Cu1+) from step one with BCA producing an intense purple colour. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

Initial set up involved diluting protein extracts samples 1:5 with H_2O and preparing BSA standards with H_2O as per manufacturer's instructions. An additional sample included was RIPA buffer diluted 1:5 with H_2O which was used as a blank for the protein extracts. The BCA working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part BCA reagent B. Each of the standards and extracts (10µl) were pipetted into the wells of a 96-well plate in triplicate and mixed with BCA™ working reagent (200µl). The plate was incubated at 37°C for 30mins. The plate was cooled to room temperature prior to absorbance being measured using the Sunrise TECAN microplate reader at 562nm. Protein standards were used to construct a standard curve, which was subsequently used to determine protein concentration of the cell extracts.

2.11 Protein Analysis by Western Blot

2.11.1 SDS polyacrylamide gel electrophoresis

SDS-PAGE was conducted according to the method of Laemmli (Laemmli, 1970), as modified by Studier (Studier, 1973). Protein extract samples (30µg) and appropriate pre-stained (Lonza Group Ltd, Switzerland) and biotinylated (Bio-Rad, USA) protein markers were loaded into separate wells in a sample buffer. This sample buffer consisted of 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH adjusted to 6.8). Gel electrophoresis was performed at a constant current of 120mV. Samples were first run through an upper gel, known as the stacking gel (1.33ml 30 % bisacrylamide mix, 2.5ml 1M Tris pH 6.8, 100µl of 10% sodium dodecyl sulphate (SDS), 50µl 10 % ammonium persulphate (APS) and 10µl TEMED, 6.1ml H_2O), which

condenses the proteins to form a thin, sharply defined bands. Then the samples were resolved by size using 12% polyacrylamide gels (4.0 30% bisacrylamide mix, 2.5ml 1.5M Tris pH8.8, 100µl of 10% SDS, 50µl 10% APS, 5µl TEMED and 3.35ml H_2O).

2.11.2 Transfer of Proteins to Membrane

The resolved proteins were transferred to Immobilon polyvinylidene diflouride (PVDF) membrane (Millipore, USA) using a wet transfer system, with all components soaked beforehand in cold transfer buffer (25mM Tris-HCl pH8.0, 0.2M glycine, 20 % methanol). The gel was placed on a layer of filter paper and sponge overlaid with the membrane. A second piece of filter paper was placed on top followed by a second sponge. The entire assembly was placed in a cassette, the chamber filled with transfer buffer and a constant current of 100mV was applied for 1hr.

2.11.3 Antibody Blotting

Prior to antibody blotting, the membranes were blocked in blocking buffer, either 5% w/v Bovine Serum Albumin (BSA) for MyD88 blots or 5 % w/v non-fat dried milk for all other blots in 1 % (v/v) Tris Buffered Saline (TBS)-Tween) for 1hr at room temperature to remove non-specific binding. Primary antibodies were prepared with blocking buffer using a 1:250000 – 1:100 dilution as appropriate. The membranes were incubated with shaking in the primary antibody overnight at 4°C. The following day membranes were washed in 1% TBSTween for 5 minutes on a rocking platform at room temperature three times. The secondary antibodies were prepared in blocking buffer using 1:1000 dilutions, membranes were left shaking in the secondary antibody solution for 1hr at room temperature. The membranes were then washed in 1% TBS-Tween for 5 minutes on a rocking platform at room temperature three times. Blots were developed by enhanced chemiluminesence

(ECL). The membranes were covered in chemiluminesence reagent and the membranes were covered with acetate. Membranes were imaged using a LAS-3000 Imaging System, (Fujifilm), exposing blots to light for various time periods as appropriate.

2.12 Knockdown of MyD88 in 2102Ep Cells

Knockdown of MyD88 in 2102Ep was achieved via a siRNA transfection protocol which had previously been optimised in the lab (Vencken, 2012). Silencer select MyD88 siRNA (Applied Biosystems Cat # 4390824) was transfected into adherent cells using Lipofectamine 2000 (Invitrogen) in OptiMEM reduced serum medium (Gibco) as a transfection medium. Silencer Negative Control #1 (Applied Biosystems Cat #AM4611) was used as a negative control for all experiments. All Applied Biosystems siRNAs are supplied as a dry powder and reconstituted with RNase and DNase free water to give a final concentration of 5μM. It was decided to further optimise the transfection protocol to suit MyD88 specifically, so a range of siRNA concentrations were tested: 10nM, 1nM and 0.1nM. Volumes given here are for 10nM siRNA, lower concentrations were prepared by diluting siRNA 1 in 10 or 1 in 100 as appropriate.

Cells were seeded in 6-well plates at a density of 160,000 cells per well and allowed to adhere overnight. Enough wells were seeded to allow for MyD88 siRNA, negative siRNA control, mock control and non-transfected control (NTC) treatments. These treatments were performed in biological triplicate. Cells that were to be treated as NTC cells had their medium removed on the same day as transfected cells but this was replaced with fresh medium. For each of the remaining treatments, two tubes were prepared; Mix A and Mix B. Volumes shown are for 3 wells of a six well plate, allowing 15% overage.

Table 2.3 2102Ep siRNA transfection mix A

Treatment	Addition (µl)	Opti-MEM (µl)	Total Volume (µl)
MyD88 siRNA	20 MyD88siRNA*	846	310.17
Negative Control	20 Neg Control siRNA	846	310.17
Mock Control	20 Opti-MEM	846	310.17

** at appropriate concentration*

Table 2.4 2102Ep siRNA transfection mix B

Treatment	Lipofectamine 2000 (µl)	Opti-MEM (µl)	Total Volume (µl)
MyD88 siRNA	37.9	846	324.229
Negative Control	37.9	846	324.229
Mock Control	37.8	846	324.229

Mix B was allowed to incubate for 5 minutes at room temperature before being added to the corresponding tube of mix A, to make the transfection medium. The transfection medium was gently agitated using a pipette before being allowed to incubate at room temperature for a maximum of twenty minutes.

During this incubation, the medium was removed from the seeded cells and replaced with 2.5ml of Opti-MEM. After the transfection medium had been allowed to incubate for twenty minutes 500µl was added to the appropriate wells. The cells were then allowed to incubate for four hours at 37°C, before the transfection medium was removed and replaced with hEC medium.

2.13 Overexpression of MyD88 in NTera2 Cells

The overexpression protocol for NTera2 cells via an overexpression plasmid had previously been optimised in the lab (Elbaruni, 2011). A full open reading frame (ORF)

clone for MyD88 was obtained from Imagene (Germany) (Clone number IOH9958-pDEST26). This was transfected in NTera2 cells using Lipofectamine RNAiMax (Invitrogen) in Opti-MEM (Gibco) as transfection medium.

Cells were seeded in 6-well plates at a density of 160,000 cells per well and allowed to adhere overnight. Enough wells were seeded to allow for MyD88 overexpression, mock control and non-transfected control (NTC) treatments. These treatments were performed in biological triplicate. Cells that were to be treated as NTC cells had their medium removed on the same day as transfected cells but this was replaced with fresh medium only. For each of the remaining treatments, a mix was prepared as outlined in Table 2.5. Volumes shown are for three wells of a six well plate allowing 15% overage. This was allowed to incubate at room temperature for 15 minutes. Meanwhile the old medium was removed from the previously seeded cells and replaced with 2.5ml of Opti-MEM. After the incubation time had elapsed 500µl of mix was added to the appropriate wells. These were then allowed to incubate at 37°C for four hours before removing the Opti-MEM and replacing with hEC medium.

Table 2.5 MyD88 Overexpression Mix for NTera2 cells

Treatment	Addition(µl)	Lipofectamine RNAiMax(µl)	Opti-MEM(µl)
MyD88 Overexpression	8.75 (600ng/µl) MyD88 overexpression plasmid	18.03	1,750
Mock	8.75 Opti-MEM	18.03	1,750

2.14 Functional Experimental Protocol

For functional experiments it was considered important to maintain a clear correlation between the extent of the knockdown and the functional effect observed. To this end, all

knockdown and overexpression treated cells were split into thirds after three days of knock-down/over-expression. One third of the cells was used to verify the extent of the knockdown or overexpression via TaqMan analysis, while the remaining thirds were split between treatment (cisplatin, hypoxia or retinoic acid) and control. In this way it was possible to directly compare the functional effects observed, to the extent of the knockdown or overexpression achieved.

2.15 Affymetrix Arrays

2.15.1 Affymetrix Array Overview

In order to study the downstream effect of siRNA knockdown of MyD88 in 2102Ep cells the human Affymetrix GeneChip Gene 1.0ST array system was utilised. This array system is designed to measure the gene expression of well-annotated genes, using a single probe set per gene comprised of multiple probes that are distributed along the entire length of the genomic locus. The current system utilises a new target preparation protocol, the Whole Transcript (WT) Assay which generates labelled targets across the entire transcript, which provides the opportunity for exon level analysis of splice variants.

Affymetrix arrays use a process combining photolithograph and combinatorial chemistry. The arrays are composed of a quartz wafer which is naturally hydroxylate. A set of photolithographic masks are manufactured that allow the sequential addition of specific nucleotides to particular location on the chip. When ultraviolet light is shone over the mask in the first step of synthesis the exposed linkers become deprotected and are available for nucleotide coupling. The single type nucleotide solution is then washed over the wafers surface and attaches to the activated linkers. In the next step another mask is placed over the wafer for the next round of deprotection and coupling. The process is sequentially repeated until the probes reach their full length. The GeneChip Human Gene 1.0 ST Array

interrogates 28,869 well-annotated genes with 764,885 distinct probes. The design of this array was based on the March 2006 human genome sequence assembly with comprehensive coverage of RefSeq, Ensembl and putative complete CDS GenBank transcripts.

On the GeneChip 1.0 ST array individual genes are represented using a series of different 25-mer perfect match (PM) oligonucleotides. The probe sets are designed to be distributed across the transcribed region of each gene. The Gene 1.0 ST Array System uses a PM-only design with probes that hybridize to sense targets. Background is estimated using a set of approximately 17,000 generic background probes (BGP). This is a collection of probes that were selected based on the fact that they are not present in the human genome and are not expected to cross-hybridize to transcribed human sequences. Background is calculated by subtracting the mean BGP intensity of the BGP probes with the same GC content as the PM probe.

2.15.2 Affymetrix Array Protocol

Sample preparation using the Affymetrix WT assay starts with synthesis of doublestranded cDNA from mRNA by reverse transcription with random oligo d(T) primers engineered to contain a T7 RNA promoter site. The double stranded cDNA is subsequently used as a template by T7 RNA polymerase producing many copies of antisense cRNA. In the second cycle of cDNA synthesis, random primers are used for reverse transcription of the cRNA to produce single stranded DNA in the sense orientation. During this process dUTP is incorporated into the DNA. This singlestranded DNA sample is then treated with a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) that specifically recognises the unnatural dUTP residues and breaks the DNA strand. DNA is labelled by terminal deoxynucleotidyl transferase (TdT) with the Affymetrix proprietary DNA Labelling Reagent that is covalently linked to biotin. The

frragmented labelled DNA sample is then hybridised onto the GeneChip Gene 1.0 ST array. Two commercially available kits were used for this process: The Ambion WT Expression Kit (Applied Biosystems) and the GeneChip WT Terminal Labeling and Hybrisation Kit (Affymetrix). All kits were used as per manufacturer's instructions with no modifications. Figure 2.1 outlines the procedure involved.

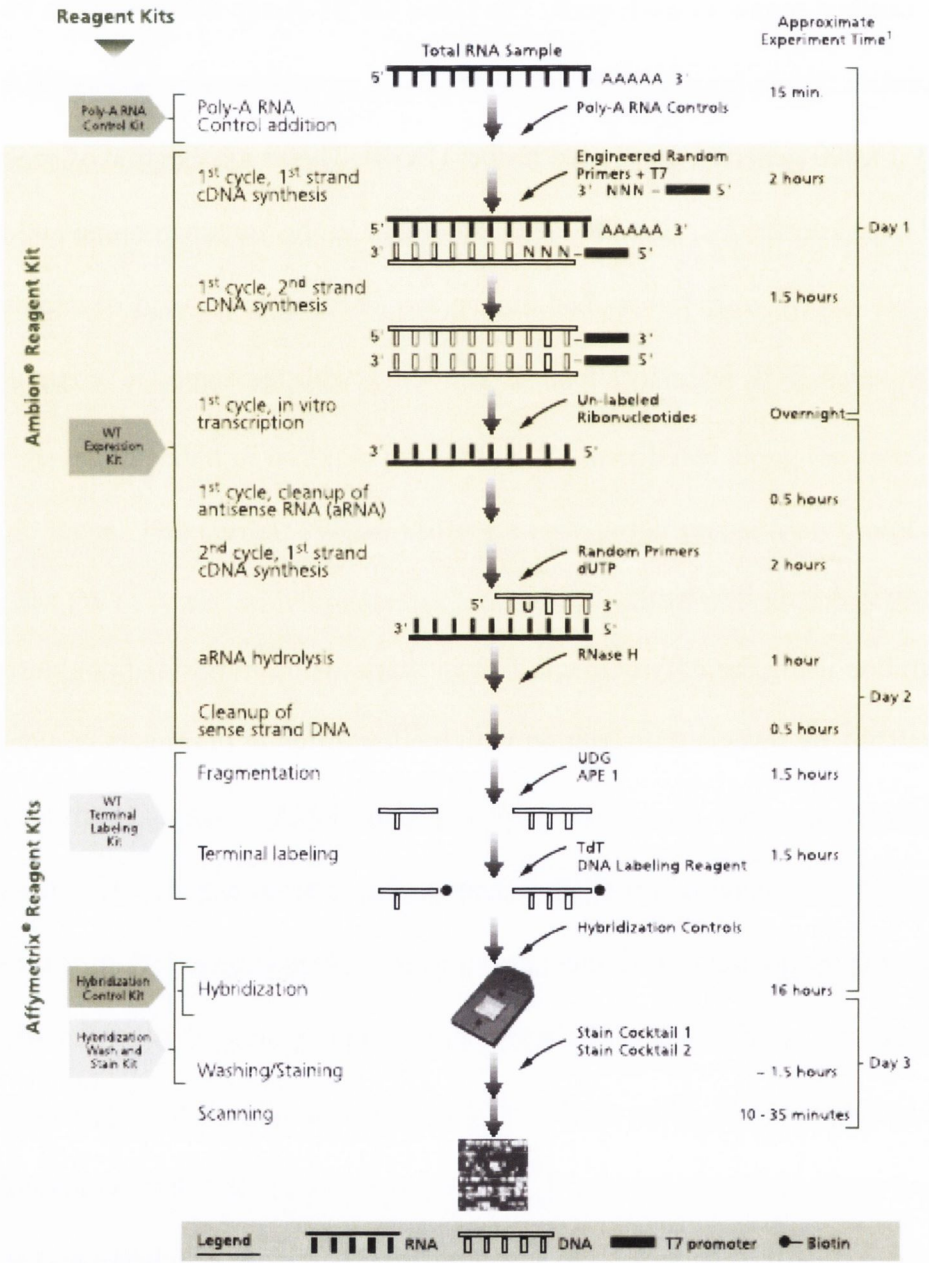

Figure 2.2 Affymetrix GeneChip 1.0 ST Array overview. (Adapted from Affymetrix.com)

2.15.3 Affymetrix Array Quality Control

Quality control of Affymetrix arrays was performed using Affymetrix Expression Console (EC) software. The objective of this is to identify any outlying microarrays. Initial analysis involves a visual inspection of the arrays images for hybridization artefacts. Subsequent quality control analysis depends on the generation of a set of quality control metrics using the EC software. Some are based on probe intensities and others on probset intensitites. Probe intensities were summarised into probset intensitites using the Robust Multiplechip Analysis (RMA) summarisation method. A summary of the different quality control metrics, their meanings and functions is given below.

1. **Probe Level Metrics:** This first set of quality assessment metrics is based on probe level data.

   pm_mean is the mean of the raw intensity for all of the Perfect Match (PM) probes on the array prior to any intensity transformations or background correction. The value of this field can be used to ascertain whether array chips are unusually dim or bright. Dimmer or brighter chips may warrant closer inspection to check if a problem results.

   bgrd_mean is the mean of the raw intensity for the probes used to calculate background prior to any intensity transformations.

   2. Probeset Summarisation Metrics: the majority of these metrics are available for different groupings or probesets i.e. hybridization or "bac spike" controls.

pos_vs_neg_auc is the area under the curve (AUC) for a plot comparing signal values for the positive controls to the negative controls. The curve is generated by evaluating how well the signals separate the positive controls from the negative controls, with the assumption that the negative controls are a measure of false positives and the positive controls are a measure of true positives. An AUC of 1 indicates perfect separation, whereas, an AUC value of 0.5 would reflect no separation. The expected value for this metric is tissue type specific and may be sensitive to the quality of the RNA sample; values between 0.80 and 0.90 are typical.

x_mean is the mean signal value for all the probesets analyzed from category "X".

x_mad_residual_mean is the mean of the absolute deviation of the residuals from the median, for all probesets analysed from category "X". Different probes return different intensities when hybridised to a common target. To account for these relative differences in intensity, the RMA algorithm creates a model for individual probe responses. The difference between the actual signal intensity value and the predicted value is the residual. If the residual for a probe on any given array is very different from the median, it means that it is a poorer fit to the model. Thus, calculating the mean of the absolute value of all the deviations produces a measure of how well or poor all of the probes on a given array fit the model. An unusually high mean absolute deviation of the residuals from the median suggests problematic data for that array.

x_rle_mean is the mean absolute relative log expression (RLE) for all the probesets analyzed from category "X". This metric is generated by taking the signal estimate for a given probeset on a given array and calculating the difference in log base 2 from the median signal value of that probeset over all the chips. The mean is then computed from the absolute RLE for all the probe sets analyzed from category "X". When only replicates from a tissue or cell line are analysed together, the mean absolute RLE should be consistently low, reflecting the low biological variability of the replicates.

3. **Probeset signals as quality metrics**: These sets of quality assessment metrics are individual probe set signal values for various controls. These include the bacterial spike and polyA spike probesets. The main use in looking at specific probeset values is to determine if expected behaviours for these probesets are observed, i.e. constant expression levels for housekeeping genes, rank order of signal values between spike probe sets.

As is apparent from the above descriptions, many of the quality assessment metrics are reported not just for all the probe sets analyzed, but also for particular subsets of probesets. The group specific metrics are particularly useful when troubleshooting a poorer performing sample. The different categories of probesets are described below.

1. all_probeset is all the probe sets analyzed. In most cases, this category is the bulk of probesets that will be carried into downstream statistical analysis. Thus the metrics reported for this category will be the most representative of the quality of the data being used downstream.

2. bac_spike is the set of probesets which hybridise to the pre-labelled bacterialspike controls (BioB, BioC, BioD, and Cre). This category is useful in identifying problems with the hybridisation and/or array. Metrics in this category have more variability than other categories (i.e. positive controls, all probesets) due to the limited number of spikes and probesets for this category.

3. polya_spike is the set of polyadenylated RNA spikes (Lys, Phe, Thr, and Dap). This category is useful in identifying problems with the target preparation. As with the bacterial spike controls, metrics in this category have more variability than other categories due to the limited number of spikes and probesets for this category.

4. neg_control is the set of putative intron-based probesets from putative housekeeping genes. Multiple species-specific probesets were selected against putative intronic regions in genes that were previously shown to have constitutive expression over a large number of samples. Thus in any given sample, some (or many) of these putative intronic regions may be transcribed and retained. These probesets form a moderately large collection which generally has very low signal values. They are used to estimate the false positive rate for the pos_vs_neg_auc metric.

5. pos_control is the set of putative exon-based probe sets from putative housekeeping genes. Multiple species-specific probesets were selected against the putative exonic regions in these genes as they were previously shown to have constitutive expression over a large number of samples. These probesets form a moderately large collection of probesets with target present which generally have

moderate to high signal values. These probesets are used to estimate the true positive rate for the pos_vs_neg_auc metric.

2.15.4 Affymetrix Samples Analysed

2102Ep cells that were transfected with either MyD88 or Negative control siRNA (three biological replicates of each) for three days were harvested, and RNA was isolated. TaqMan analysis was used to confirm that knockdown of more than 85% had occurred. Affymetrix array analysis was then performed using the Ambion WT Expression Kit (Applied Biosystems) and the GeneChip WT Terminal Labeling and Hybridization Kit (Affymetrix) as per manufacturer's instructions. Following successful calculation of quality control metrics, data was analysed using the XRAY version 3.99 software from Biotique Systems Inc. (Reno, NV, USA).

2.16 Sequencing by Oligonucleotide Ligation and Detection (SOLiD)

SOLiD was used to determine the downstream effects of MyD88 knockdown on ncRNAs in 2102Ep cells. SOLiD is a new 'Second Generation Sequencing' technology that can sequence an entire human genome in a single instrument run. Briefly, a library of RNA fragments is prepared from the sample to be sequenced and these are used to prepare clonal magnetic bead populations. Each fragment attached to the magnetic beads will have a universal P1 adapter sequence attached so that the starting sequence of every fragment is both known and identical. Emulsion PCR takes place in microreactors containing all the necessary reagents. The resulting PCR products are then covalently bound to a glass slide. A set of four fluorescently labelled di-base probes compete for ligation to the sequencing primer. Specificity of the di-base probe is achieved by interrogating every 1^{st} and 2^{nd} base in each ligation reaction. Multiple cycles of ligation, detection and cleavage are performed with the number of cycles determining the eventual read length. Following a series of ligation cycles the extension product is removed and the template is reset with a primer

complementary to the n-1 position for a second round of ligation cycles. Five rounds of primer rest are complete for each sequence tag.

2102Ep cells that were transfected with either MyD88 or Negative control siRNA (three biological replicates of each) for three days were harvested, and RNA was isolated. TaqMan analysis was used to confirm that knockdown of more than 85% had occurred. Samples were prepared run in the Central Pathology Laboratory of St James's Hospital. Bioinformatics was performed by an in-house bioinformatician.

2.17 TNF-alpha ELISA

In order to test that the protein concentration in hEC media samples was within a range appropriate for protein assays, a human Tumour Necrosis Factor-alpha (TNF-α) quantikine Enzyme-linked Immunsorbent Assay (ELISA) kit was purchased from R&D Systems. The TNF-α ELISA kit consists of a microplate precoated with an anti-bdy for TNF-α. Samples are added and any TNF-α within the sample is bound by the antibody, unbound materials are washed away. A second Horse Radish Peroxidase (HRP) detection antibody is added and binds to the captured TNF-α. Any unbound detection antibody is washed away. Tetramethylbenzadine (TMB) substrate is added to the wells of the microplate and a blue colour develops to the proportion of TNF-α present in each sample. Colour development in each well is stopped turning the final colour to yellow. The absorbance of each well is measured at 450nm.

The TNF-α kit was used as per manufacturer's instructions. Data analysis was performed by creating a standard curve using absorbance readings for the standards provided and all sample concentrations were calculated from this.

2.18 Concentrating Media Samples

In order to increase the concentration of chemokines and cytokines in the biobanked media to a level detectable by the protein arrays, all samples were spun through Amicon Ultracel 3kDa spin filters, purchased from Millipore. 500µl of each sample was added to a filter and spun at 14,000x G for 30 seconds. The filter was removed and placed in reverse in a fresh sample tube. This was then spun at 1,000 x G for 10 seconds. In this way the concentration of proteins within each sample was increased by a factor of 20.

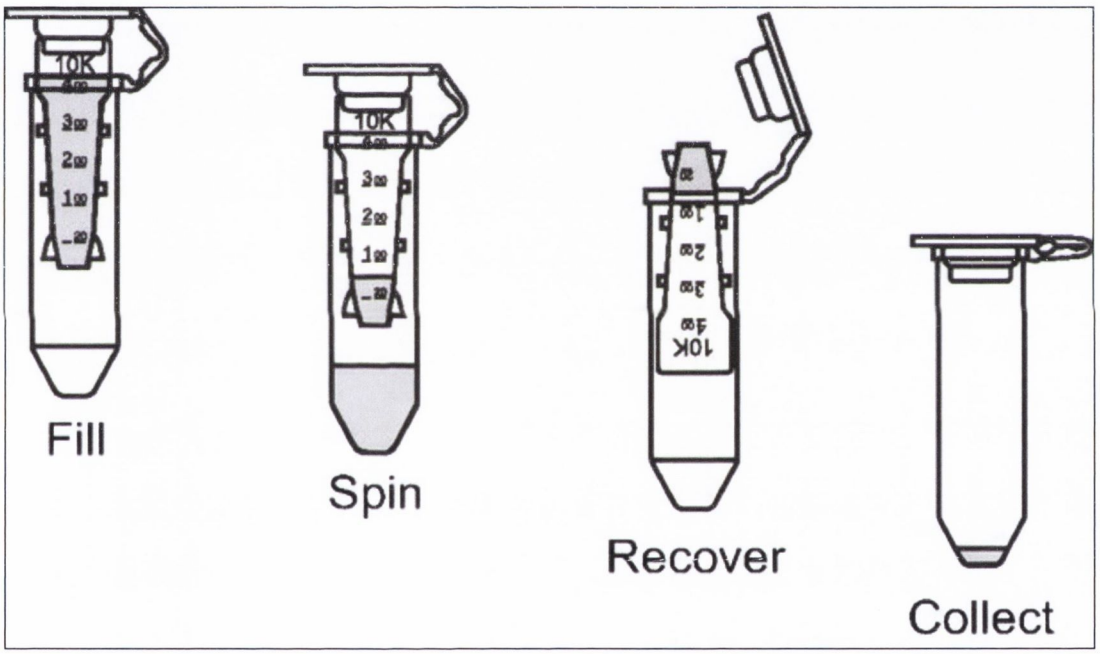

Figure 2.3 Amicon Ultra Filters Overview. (Adapted from Millipore.com)

2.19 RayBioTech Quantikine Protein Arrays

Quantikine protein arrays were purchased from RayBioTech. The Quantibody system is an array-based multiplex ELISA system for simultaneous quantitative measurement of multiple cytokines, growth factors, proteases, soluble receptors and other proteins in a wide variety of sample types. Quantibody combines the high specificity and sensitivity of ELISA with the high throughput of a glass-chip based array.

Similar to the traditional ELISA described in Section 2.17, Quantibody uses a matched pair of antibodies for target protein detection. A panel of capture antibodies is printed in multiple identical arrays on a standard slide. After a blocking step, samples are incubated with the arrays. Nonspecific proteins are washed off and the arrays are incubated with a cocktail of biotinylated detection antibodies, followed by a streptavidin-conjugated fluor. Signals are then visualized using a fluorescence laser scanner.

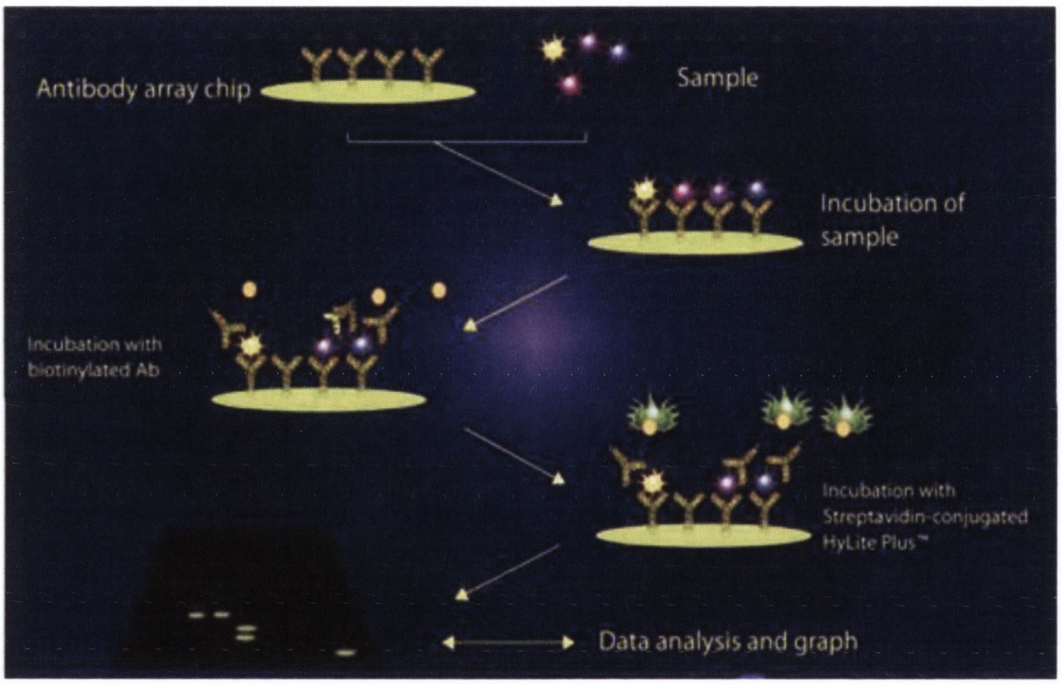

Figure 2.4 RayBioTech Quantibody Array System Overview. (Adapted from http://www.raybiotech.com/quantibody-en-2.html)

In order to quantify target protein concentrations, array specific protein standards, whose concentrations have been pre-determined are provided to generate an 8 point standard curve of each target protein. By comparing signals from unknown samples to the standard curve, the unknown cytokine concentration in the samples can be determined.

The Quantibody Human Chemokine Array and Cytokine Arrays 4 and 5 (QAH-GF-1, QAH-CYT-4 and QAH-CYT-5) were obtained from RayBiotech. These allowed for the measurement of 120 different chemokines and cytokines in each sample. Media samples used on the Quantibody arrays are listed in Table 2.6. Triplicate biological replicates were arrayed for each treatment. Data was analysed using the software provided by Ray Biotech. Quantibody arrays allow for the quantitative analysis of chemokine and cytokines within a media sample. However, the data analysis package provided does not yet allow for normalisation within biological replicates. For this reason quantitative values obtained included a range of values for each treatment. Thus, data presented here is qualitative rather than quantitative.

Table 2.6: List of Media Samples tested on each array

Treatment
2102Ep Untreated
NTera2 Untreated
2102Ep in Retinoic Acid
NTera2 in Retinoic Acid
2102Ep in Cisplatin
NTera2 in Cisplatin
2102Ep in Hypoxia
NTera2 in Hypoxia
2102Ep PreTreated with Retinoic Acid in Cisplatin
NTera2 Pre-Treated with Retinoic Acid in Cisplatin
2102Ep MyD88 siRNA
2102Ep Negative Control siRNA
2102Ep MyD88 siRNA 6 day in Retinoic Acid
2102Ep MyD88 siRNA 6 day
2102Ep Negative Control siRNA 6 day in Retinoic Acid
2102Ep Negative Control siRNA 6 day

2.20 Statistical Analysis and Bioinformatics

All gene expression and cell survival comparisons were tested or significance using a student's t-test, with $p < 0.5$, in order for a result to be deemed statistically significant. Gene expression levels can vary naturally, so in order to account for these levels of biological variation, a cut off of 2-fold change in over/under expression was deemed necessary to be considered biologically significant. All Affymetrix and SOLiD bioinformatics analysis was carried out in-house by Dr Gordon Blackshields. Protein array analysis was carried out using the software provided by Ray Biotech. This software did not contain an endogenous control that could normalise between biological samples (i.e. as GAPDH would be used in q-PCR analysis), so the data is presented here as qualitative rather than quantitative.

Chapter Three

Characterisation of TLR4-MyD88 signalling in the response of hEC cells to cisplatin, differentiation and hypoxia treatments.

3.1 Introduction

The standard first-line chemotherapy for ovarian cancer is a platinum-taxane combination regimen. This usually is achieved via treatment with both paclitaxel and cisplatin simultaneously. Paclitaxel is a known ligand for TLR4, suggesting that standard treatment could potentially cause upregulation of the TLR4-MyD88 response (Byrd-Leifer et al., 2001). However, prior to this study there was no known connection between cisplatin resistance and TLR4-MyD88 expression.

TLRs are pattern recognition receptors (PRRs) located on the cell surface membrane and are key constituents of the innate immune system. TLR pathways mediate the body's response to pathogens via recognition of pathogen associated molecular patterns (PAMPs) (R Medzhitov & Janeway, 1997). Stimulation of TLR pathways leads to NF-κB activation, with the downstream effect of increased cell proliferation and neovascularisation (Chow et al., 1999; Ruslan Medzhitov et al., 1997). TLRs share a common signalling domain: the Toll/Interleukin-1 (IL-1) receptor (TIR) domain, which allows them to share a common adaptor molecule – myeloid differentiation primary response gene 88 (MyD88) (O'Neill et al., 2003). Most TLRs signal via MyD88. However, both TLR4 and TLR3 utilise an alternate adaptor molecule named TIR-domain containing adaptor inducing IFN-β (TRIF) (Yamamoto *et al* 2002). In the case of TLR4, signalling via TRIF activates IFN- β preferentially, although NF-kB is eventually activated via TRIF also. Previous work by Kelly and Zhu has shown that ovarian cancer patients whose tumours tested negative for MyD88 expression had a statistically significant improved progression free interval compared with patients whose tumours were MyD88 positive(Kelly et al., 2006; Zhu, Huang, Zhang, Zha, & Deng, 2012). This work has been corroborated within an Irish cohort by work from the O'Leary laboratory (d'Adhemar et al, in progress).

This group has worked extensively with both 2102Ep and NTera2 cells, with a particular focus on their ability to resist or respond to differentiation via retinoic acid. Of particular interest, are methods by which differentiation resistance could potentially be altered, thereby allowing for force differentiation. Previous research efforts have focused on markers of pluripotency, one of which: Sox2, has been demonstrated to cause force differentiation of 2102Ep cells when overexpressed (Vencken et al, in preparation). Previous Affymetrix array data from these studies, that when 2102Ep and NTera2 cells are treated with retinoic acid, MyD88 was one of the most significantly altered genes. Furthermore, alterations in MyD88 associated gene signalling including SIGRR, TIMP3, IFI16 and ZHX1 were also observed. This led to the conclusion that MyD88 may play a significant role in the differentiation potential of hEC cells.

Previous work has shown that degree of differentiation is the most important independent prognostic factor in primary epithelial ovarian cancer (Vergote et al., 2001) Dembo et al., 1990). However, in recurrent cases degree of differentiation is no longer considered a prognostic factor (Colombo et al., 2010). This suggests that in recurrent tumours, the differentiation status of cells has no bearing on their response to chemotherapy. Pluripotent NTera2 cells and nullipotent 2102Ep cells are therefore an ideal model system in which to study the relationship between differentiation and response to chemotherapy.

3.2 Aims and Hypotheses

Cancer stem cells possess three properties that contribute to their tumourgenicity, namely a) resistance to chemotherapy, b) altered differentiation capacity and c) resistance to hypoxia. Several studies have shown an association between TLR4-MyD88 expression and resistance to chemotherapy drug Paclitaxel. The TLR4-MyD88 response to cisplatin has not been studied previously. Additionally previous array data generated within our group showed alteration of MyD88 signalling in response to retinoic acid treatment. A relationship between TLR4-MyD88 signalling and hypoxia resistance in CSCs has not been established.

Studies to date have focussed on the effect of individual treatments. However, *in vivo* cells will be exposed to a constant range of simultaneous stimuli. To model this, we included combination treatments in our experimental plan.

We hypothesised that TLR4-MyD88 signalling was involved in the response of hEC cells to cisplatin, differentiation and hypoxia. We further hypothesised that pluripotent and nullipotent hEC cells would differ in this respect. Finally, we hypothesised that the response of cells to a treatment, would affect their response to subsequent treatments.

Testing these hypotheses in this chapter we aimed to:

- Investigate whether TLR4-MyD88 expression in hEC cell lines is affected by cisplatin, differentiation and hypoxia treatments.
- Investigate whether any demonstrated change in TLR4-MyD88 expression is altered by pre-treatment with cisplatin and retinoic acid.

3.3 Results

3.3.1 Establishment of chemotherapy drug cisplatin half maximal inhibitory concentration (IC_{50}) for human embryonal carcinoma (hEC) cells.

Several experiments in this chapter involve treatment of hEC cells with the chemotherapy drug cisplatin. The accepted scientific approach is to treat cells with the appropriate "IC_{50}" for the specific drug. The IC_{50} is the concentration at which half of the cells survive. This is a standard method of comparing the efficacy of a drug across cell lines (Blumenthal & Goldenberg, 2007). Therefore, cisplatin IC_{50}s were calculated for each hEC cell type in this section. Three days was selected as the time point of interest, this was based on previous work on retinoic acid signalling within this group, which demonstrated that a time point of three days allowed for the determination of early gene expression changes. Choosing the same time point for cisplatin studies allowed for direct comparison between treatments.

Cells were seeded at 6,000 cells per well in 96 well plates and allowed to adhere overnight. Half the media was removed and replaced with a range of concentrations of cisplatin diluted in media to the appropriate final concentration. After three days cell viability was measured using an MTT cell viability assay. Percentage cell viability was calculated relative to a vehicle control which was set to 100% viability. Both cell lines displayed an inversely proportional relationship between cell survival and cisplatin concentration, as expected (Figure 3.1-3.2). The IC_{50} was calculated as 3.2μM and 1.2μM for 2102Ep and NTera2 cells respectively. This demonstrates that 2102Ep cells are more cisplatin resistant than NTera2 cells. 1.2μM, since it was the lower IC50 value was used as the standard treatment for both cell lines, to allow direct comparison between them.

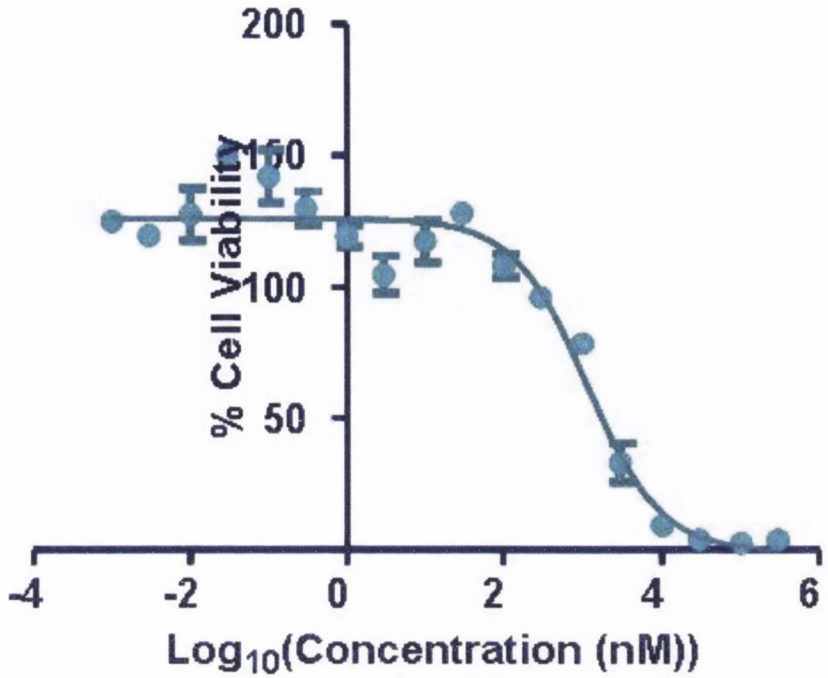

Figure 3.1 Establishment of cisplatin IC_{50} for 2102Ep cells.

Graph shows the relationship between percentage cell survival of 2102Ep cells and increasing concentrations (log_{10} (nM)) of chemotherapy drug cisplatin. Cells were treated with increasing concentrations of cisplatin for three days. Cell survival was then assessed via an MTT cell proliferation assay. Cell viability data for each treatment is presented as a percentage of vehicle control. GraphPad was used to calculate the half maximal inhibitory concentration (IC_{50}), which was used for subsequent cisplatin experiments. The IC_{50} was calculated as 3.2μM, indicating higher cisplatin tolerance relative to NTera2 cells.

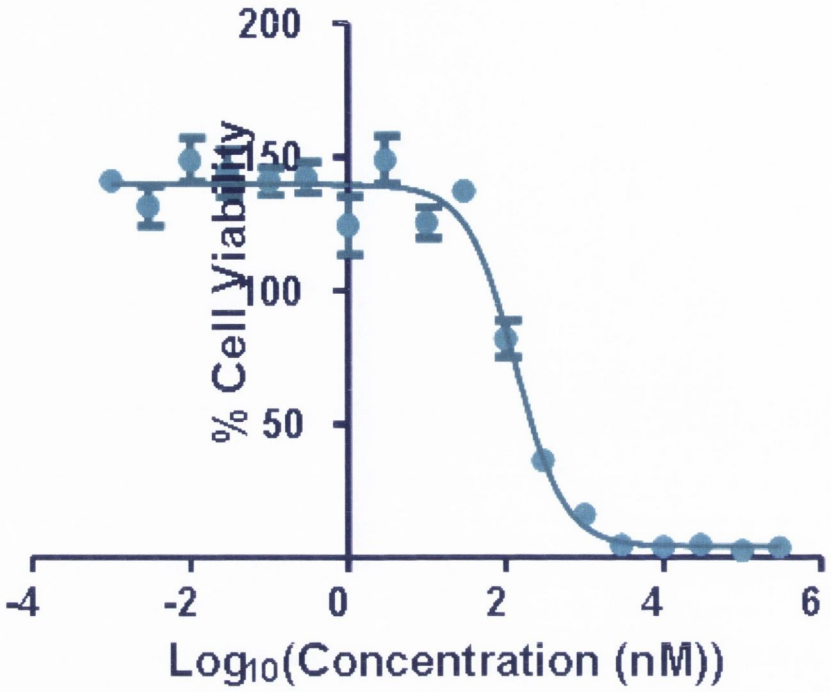

Figure 3.2 Establishment of cisplatin IC_{50} for NTera2 cells.

Graph shows the relationship between percentage cell survival of NTera2 cells and increasing concentrations (log_{10} (nM)) of chemotherapy drug cisplatin. Cells were treated with increasing concentrations of cisplatin for three days. Cell survival was then assessed via an MTT cell proliferation assay. Cell viability data for each treatment is presented as a percentage of vehicle control. GraphPad was used to calculate the half maximal inhibitory concentration (IC_{50}), which was used for subsequent cisplatin experiments. The IC_{50} was calculated as 1.2μM, indicating lower cisplatin tolerance relative to 2102Ep cells.

3.3.2 Cisplatin treatment causes TLR4-MyD88 gene expression changes in hEC cells

In order to investigate the extent of the role of the TLR4-MyD88 signalling mechanism in cisplatin resistance, it was first necessary to determine whether cisplatin treatment affected expression of TLR$ and MyD88. In order to do this, both NTera2 and 2102Ep cells were treated with an appropriate concentration of cisplatin and changes in TLR4-MyD88 expression levels were assessed. These data show that TLR4 and MyD88 signalling is altered in both cell lines in response to cisplatin treatment.

Cells were seeded at 168,000 cells per well in 6-well plates and allowed to adhere overnight. The following day media was removed and replaced with media containing 1.2μM cisplatin as determined by the IC_{50} experiment (Section 3.3.1). This value corresponds with the IC_{50} for NTera2 cells, and was used to enable direct comparison of gene expression changes between cell lines. After three days cells were harvested, RNA was isolated and assessed for quality. cDNA was synthesised and Q-PCR was performed using probes for TLR4, MyD88 and GAPDH as an endogenous control. Fold change in gene expression relative to untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. The cut-offs for biological significance were set at <50% and >200% expression. These are equivalent to the conventional +/- 2-fold limit, within which it is assumed that changes of expression are due to biological variation. Changes in gene expression were tested for statistical significance using a student's t-test. 2102Ep cells showed no significant change in expression of TLR4 but a significant reduction in MyD88 expression in response to cisplatin treatment (Figure 3.3 A, B). NTera2 cells responded to cisplatin by upregulating both TLR4 and MyD88 (Figure 3.3 C, D). However, the large error bars observed in these figures, demonstrate that

although TLR4 and MyD88 were consistently upregulated, the level of upregulation varied considerably.

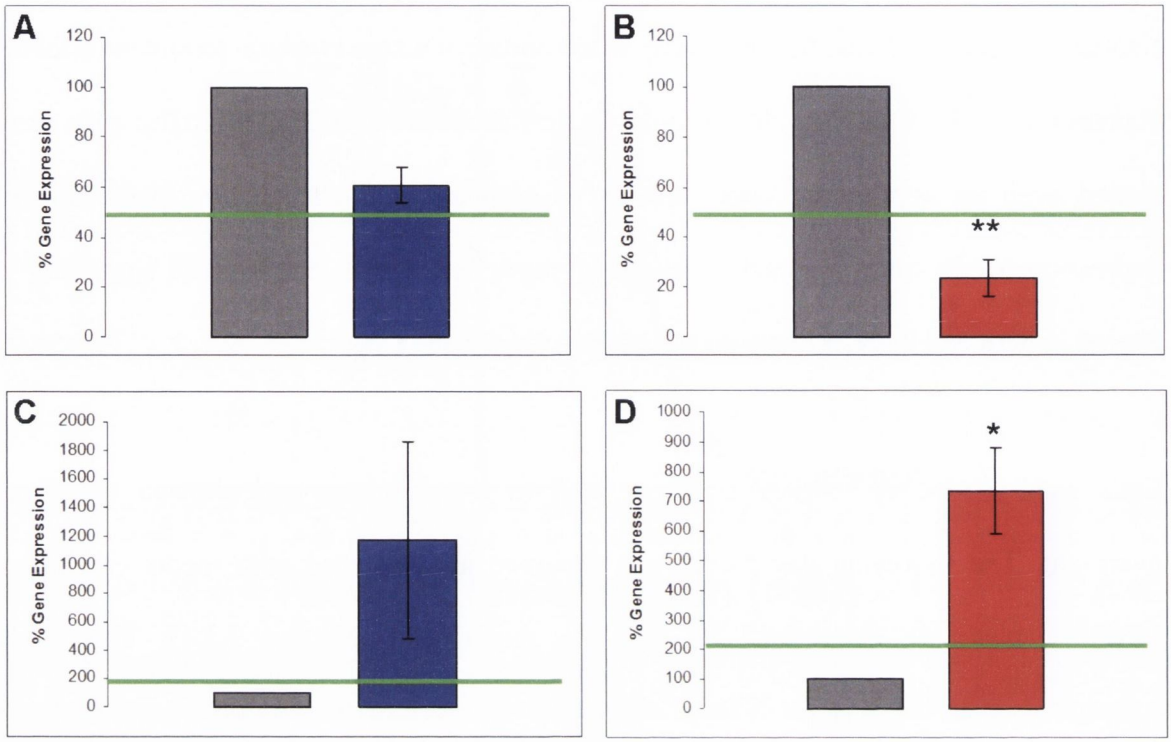

Figure 3.3 TLR4-MyD88 expression in cisplatin treated hEC cells.

Cells were incubated in the presence of 1.2μM cisplatin for three days. RNA was isolated and interrogated for TLR4 (blue) and MyD88 (red) expression via Q-PCR compared to untreated control (grey). Data shown are from a minimum of n=3, displayed as percentage expression relative to untreated cells. Green lines denote the limits of biological variation. A) 2102Ep cells displayed no change in TLR4 expression. B) 2102Ep cells displayed statistically significant MyD88 downregulation. C) + D) NTera2 cells substantially upregulated both TLR4 and MyD88. *P<0.05, ** P<0.01

3.3.3 Confirmation that NTera2 cells are pluripotent whereas 2102Ep cells are nullipotent.

Differentiation status of a tumour is considered one of the key indicators of prognosis for primary ovarian cancer. 2102Ep and NTera2 are highly similar cell lines, with the exception of their response to differentiation stimulus. While NTera2 cells are pluripotent and so differentiate in response to retinoic acid treatment, 2102Ep cells are nullipotent and resist differentiation. In this section these differing responses were confirmed, which was important for subsequent analysis, and the role of TLR-MyD88 signalling is investigated.

2102Ep and NTera2 cells were grown in the presence of $1x10^{-5}$M retinoic acid for seven days. The concentration of retinoic acid used ($1x10^{-5}$M) is an established standard concentration. Cells were then stained using a stain for alkaline phosphatase (AP), a marker of differentiation. Undifferentiated cells stain red whereas differentiated cells remain unstained. NTera2 cells lost their red staining in response to retinoic acid and so can be said to differentiate (Figure 3.4 A, B) 2102Ep cells maintain their red staining when treated with retinoic acid and so can be said to be resistant to differentiation via retinoic acid. (Figure 3.4 C, D). Thus the AP technique was now available to confirm pluripotency or nullipotency during later experiments.

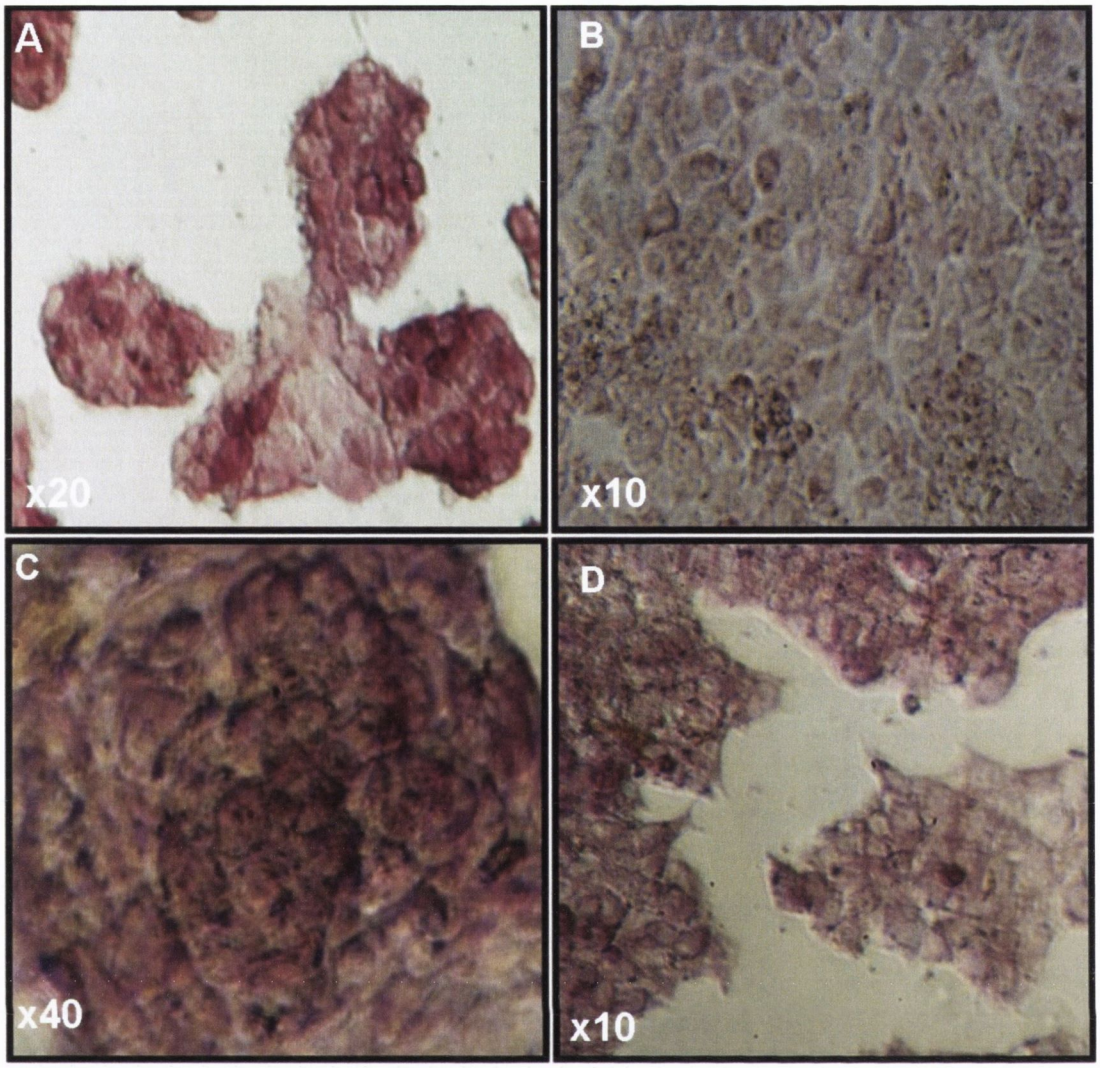

Figure 3.4: Alkaline Phosphatase (AP) staining of undifferentiated and retinoic acid treated hEC cells. 2102Ep and NTera2 cells were grown in media containing retinoic acid for seven days. Cells were then stained for AP expression (red), a marker of pluripotent cells. A) Untreated NTera2 cells maintained strong red staining. B) NTera2 cells grown in retinoic acid media displayed considerably less red staining. C) 2102Ep cells stained red. D) 2102Ep cells grown in retinoic acid media maintained red staining, confirming nullipotency.

The AP stain was found to be less suitable to earlier differentiation time points such as three days. As such, qPCR of pluripotency marker genes Oct4, Sox2 and Nanog was investigated as a potential indicator of three day differentiated cells. Cells were seeded at 168,000 cells per well in 6-well plates in media containing 1x 10^{-5}M retinoic acid. After three days cells were harvested, RNA was isolated and assessed for quality. cDNA was synthesised and Q-PCR was performed using probes for Oct4, Nanog, Sox2 and GAPDH

as an endogenous control. Fold change in gene expression relative to untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. The cut-offs for biological significance were set at <50% and >200% expression. These are equivalent to the conventional +/- 2-fold limit, within which it is assumed that changes of expression are due to biological variation. When treated with retinoic acid for just three days, 2102Ep cells showed no change in Oct4 or Nanog expression, but some downregulation of Sox2. In contrast, NTera2 cells downregulated all three genes to a considerable extent (Figure 3.5). As such, qPCR of Oct4, Sox2 and Nanog were suitable measures of nullipotency (high levels maintained) and pluripotency (decreased levels) for use in subsequent analysis. As previously, a three day time point was selected to allow for the detection of early changes in gene expression. The large error bars seen in Figure 3.5 indicate that these cells are in the process of differentiating and are not fully differentiated, so gene expression levels are not as consistent as fully differentiated cells would be.

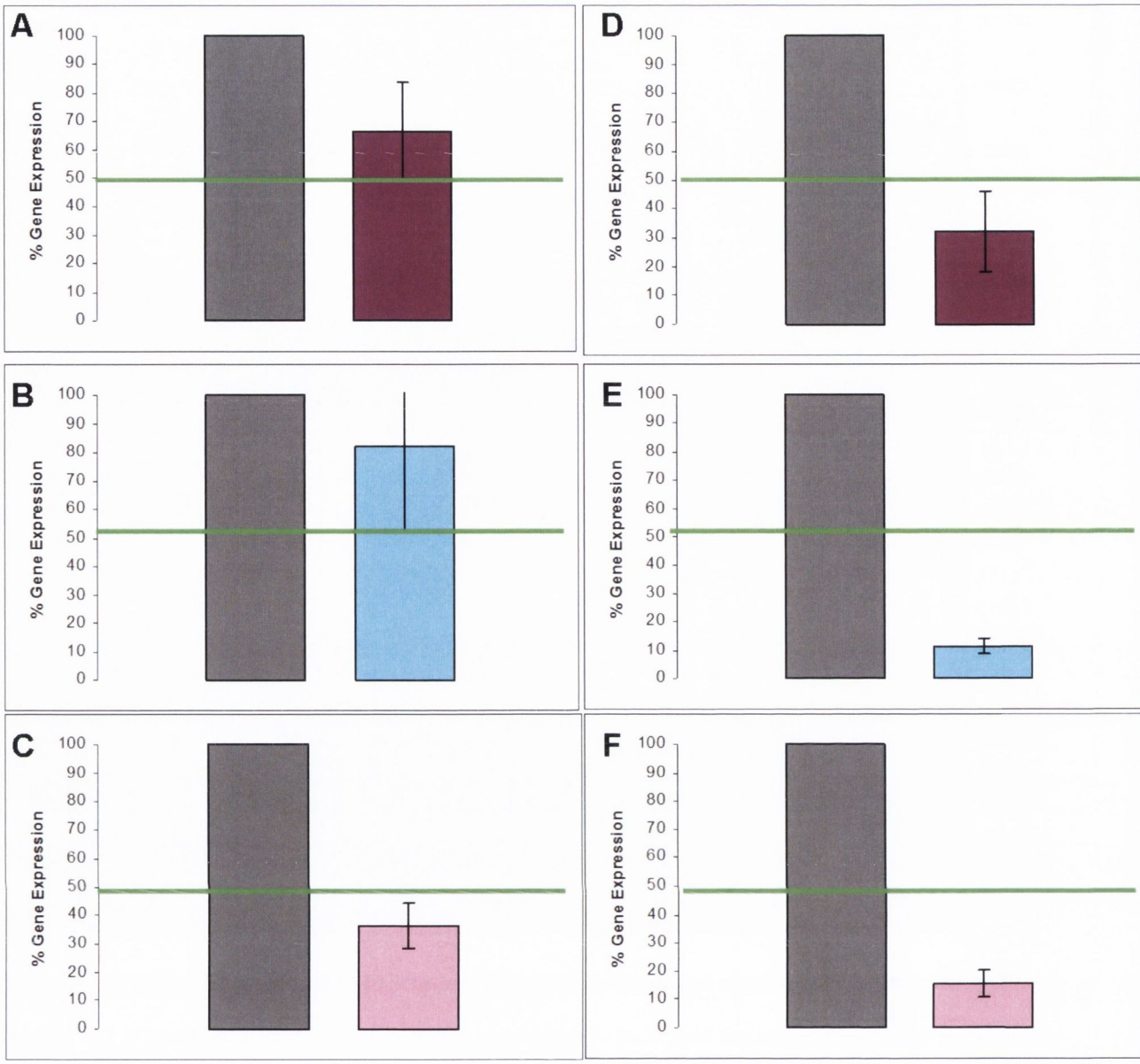

Figure 3.5: **Pluripotency marker expression in retinoic acid treated hEC cell lines.**

2102Ep and NTera2 cells were grown in media containing retinoic acid for three days. RNA was isolated and interrogated for Oct4 (purple), Nanog (blue) and Sox2 (pink) expression via Q-PCR compared to untreated control (grey). Data shown are from a minimum of n=3, displayed as percentage expression relative to untreated cells. Green lines denote the limits of biological variation. A-C) 2102Ep cells showed no change in Oct4 or Nanog expression but some limited downregulation of Sox2 .D-F) NTera2 cells showed downregulation of all three markers following retinoic acid treatment.

3.3.4 TLR4–MyD88 is involved in the hEC response to differentiation stimulus.

Previous work in the lab had shown via an Affymetrix gene array that when 2102Ep and NTera2 cells were treated with retinoic acid, MyD88 signalling was significantly altered (M Gallagher, unpublished data). Further to this, altered expression of other genes activated by MyD88 was also detected. This led to the belief that MyD88 potentially played a role in differentiation and/or differentiation avoidance in hEC cells. The concentration of retinoic acid used ($1x10^{-5}M$) is an established standard concentration. Three days was selected as the time point of interest since it allowed for the determination of early changes in gene expression.

Cells were seeded at 168,000 cells per well in 6-well plates in media containing $1x\ 10^{-5}M$ retinoic acid. After three days cells were harvested, RNA was isolated and assessed for quality. cDNA was synthesised and Q-PCR was performed using probes for TLR4, MyD88 and GAPDH as an endogenous control. Fold change in gene expression relative to untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. The cut-offs for biological significance were set at <50% and >200% expression. These are equivalent to the conventional +/- 2-fold limit, within which it is assumed that changes of expression are due to biological variation. Changes in gene expression were tested for statistical significance using a student's t-test. 2102Ep cells showed downregulation of both TLR4 and MyD88 in response to retinoic acid treatment. (Figure 3.6 A, B). NTera2 cells responded to retinoic acid treatment by downregulating both TLR4 and MyD88. (Figure 3.6 C, D). This indicates that TLR-MyD88 signalling is altered in hEC cells in response to retinoic acid treatment.

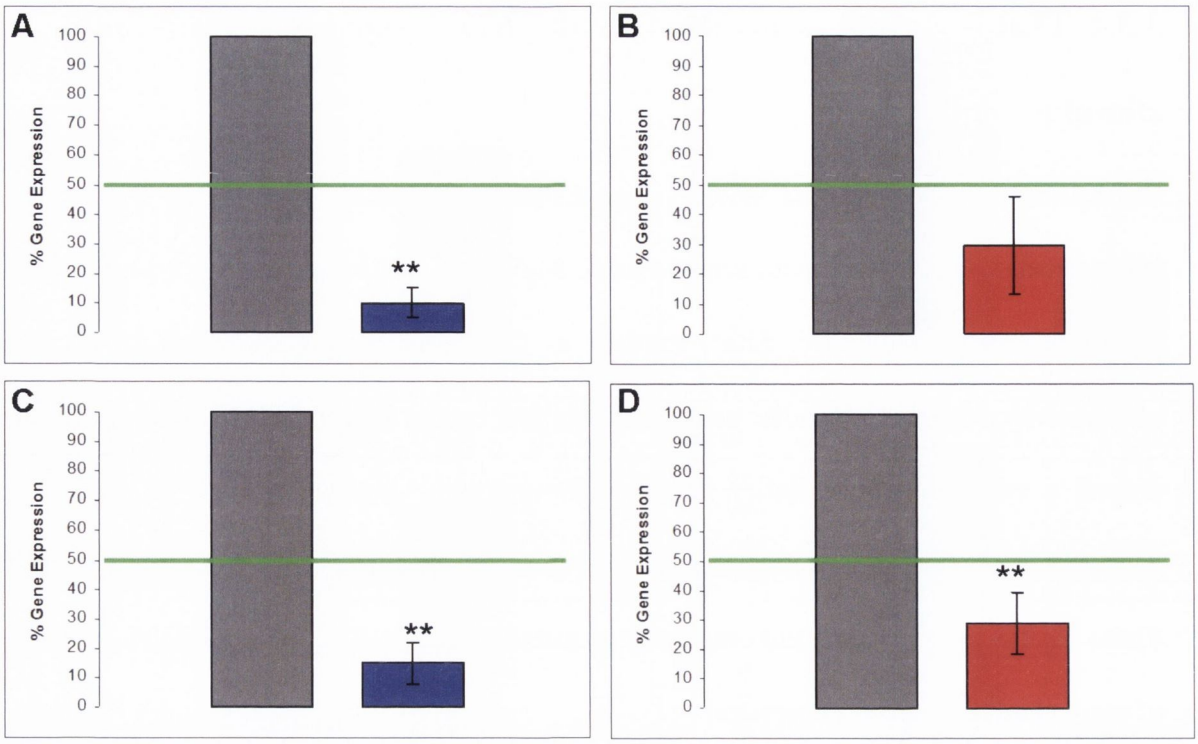

Figure 3.6: TLR4-MyD88 expression in retinoic acid treated hEC cells.

Cells were incubated in the presence of 1x 10^{-5}M retinoic acid for three days. RNA was isolated and interrogated for TLR4 (blue) and MyD88 (red) expression via Q-PCR compared to untreated control (grey). Data shown are from a minimum of n=3, displayed as percentage expression relative to untreated cells. Green lines denote the limits of biological variation. A) +B) 2102Ep cells downregulated both TLR4 and MyD88. C) +D) NTera2 cells down regulated both TLR4 and MyD88. ** $P<0.01$

3.3.5 hEC cell survival in Hypoxia

As cancer stem cell lines, both NTera2 and 2102Ep cells should in theory show resistance to hypoxia, since stem cells are thought to originate in hypoxic environments. Cells respond to hypoxic conditions by maintaining high cellular levels of Hif-1α. In this set of experiments we verified that hEC cells were hypoxic, established hEC cell survival in hypoxic conditions and investigated whether TLR4-MyD88 signalling was involved in the hEC response to hypoxia

In order to confirm that chamber was effective and that cells within were experiencing hypoxic conditions a western blot was performed to interrogate for Hif-1α expression. Briefly, 2102Ep cells were seeded overnight at 160,000 cells per well in six well plates. The following day they were moved to a hypoxic chamber at 0.5% oxygen for 24 hours. 0.5% oxygen is considered a standard oxygen concentration used in hypoxia experiments. Cells grown in normal atmospheric conditions were used as a normoxic control. Protein was harvested within the chamber as per the protocols described in Chapter 2. 4 A western blot was performed using the protocol outlined in Chapter 2.11 (Figure 3.7). This confirmed the presence of Hif-1α in these cells, verifying that they were hypoxic.

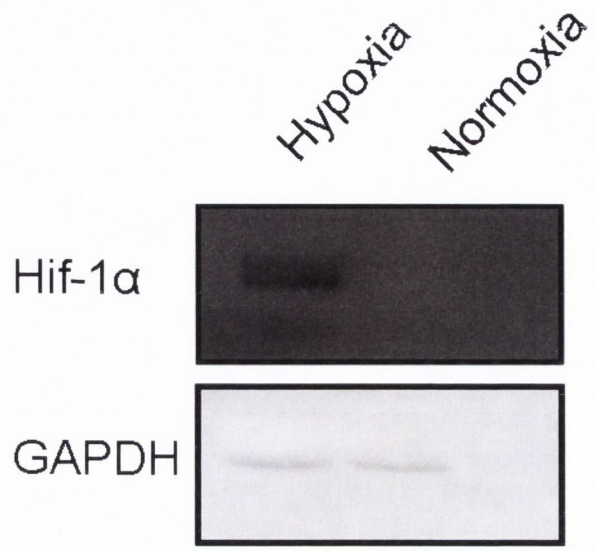

Figure 3.7: Verification of Hypoxic conditions in 2102Ep cells. Cells were seeded overnight and transferred to hypoxia for 24 hours. Protein was isolated and interrogated for Hif-1α via Western Blot. Blots were stripped and subsequently interrogated for GAPDH, as an endogenous control and to confirm equal loading. Hif-1α is present in the cells grown in hypoxic conditions but not in cells grown in normoxia.

Cells were seeded at 6,000 cells per well in 96 well plates and allowed to adhere overnight. Cells were then placed in a hypoxic chamber at 0.5% oxygen for three days. Cell viability was then measured using an MTT cell viability assay. Percentage cell viability was calculated relative to cells grown simultaneously in normoxic conditions, which was set to 100% viability. T-tests were carried out to test for statistical significance. Both cell lines showed no change in cell survival in hypoxic conditions (Figure 3.8).

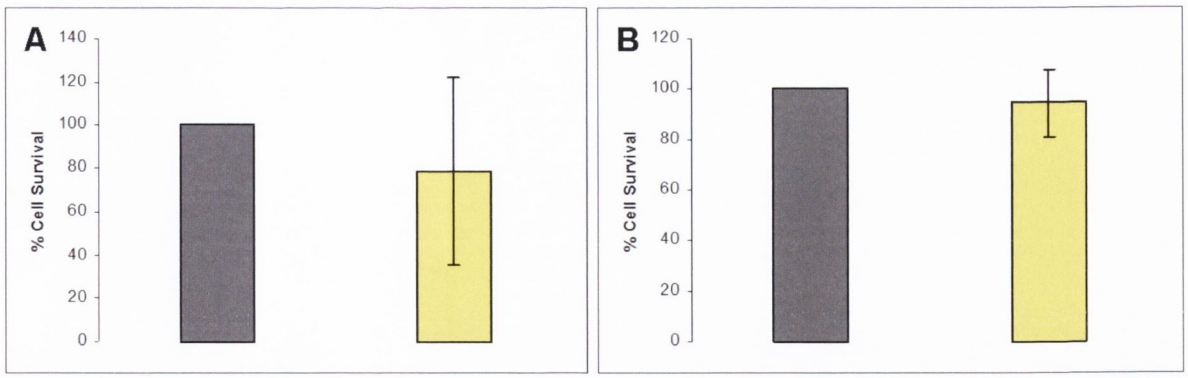

Figure 3.8: hEC cell survival in hypoxia. Cells were grown in 0.5% O_2 for three days (yellow). Cell survival was then assessed using an MTT cell proliferation kit. Percentage cell survival data shown here was calculated relative to cells grown in normal oxygen (grey). A) 2102Ep cells show no significant change in cell survival in hypoxic conditions. B) NTera2 cells treated in hypoxia show no significant change in cell survival.

3.3.6 TLR4 –MyD88 are involved in the hEC response to hypoxia

Following establishment of hypoxia tolerance, hEC cells were assessed for alterations in Tlr4-MyD88 in hypoxic conditions. Cells were seeded at 168,000 cells per well in 6-well plates and allowed to adhere overnight. The following day cells were placed in a hypoxic chamber at 0.5% O_2 for three days. After three days cells were harvested, RNA was isolated and assessed for quality. cDNA was synthesised and Q-PCR was performed using probes for TLR4, MyD88 and GAPDH as an endogenous control. Fold change in gene expression relative to untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. The cut-offs for biological significance were set at <50% and >200% expression. These are equivalent to the conventional +/- 2-fold limit, within which it is assumed that changes of expression are due to biological variation. T-tests were carried out to test for statistical significance Three days was chosen as the time point of interest to allow for the detection of early response change in gene expression and to allow direct comparison between treatments. 2102Ep cells showed downregulation of both TLR4 and MyD88 in response to hypoxia. (Figure 3.9 A, B). NTera2 cells showed no change in TLR4 or MyD88 expression in response to

hypoxia. (Figure 3.9 C, D). This suggested a differential involvement of TLR4-MyD88 in hypoxia tolerance in these cells. NTera2 cells showed a high variability in the level of expression of MyD88 (Figure 3.9D), this may suggest that gene expression levels are state of flux at the three day timepoint, if this is the case, then at a later timepoint a decrease in MyD88 would be expected.

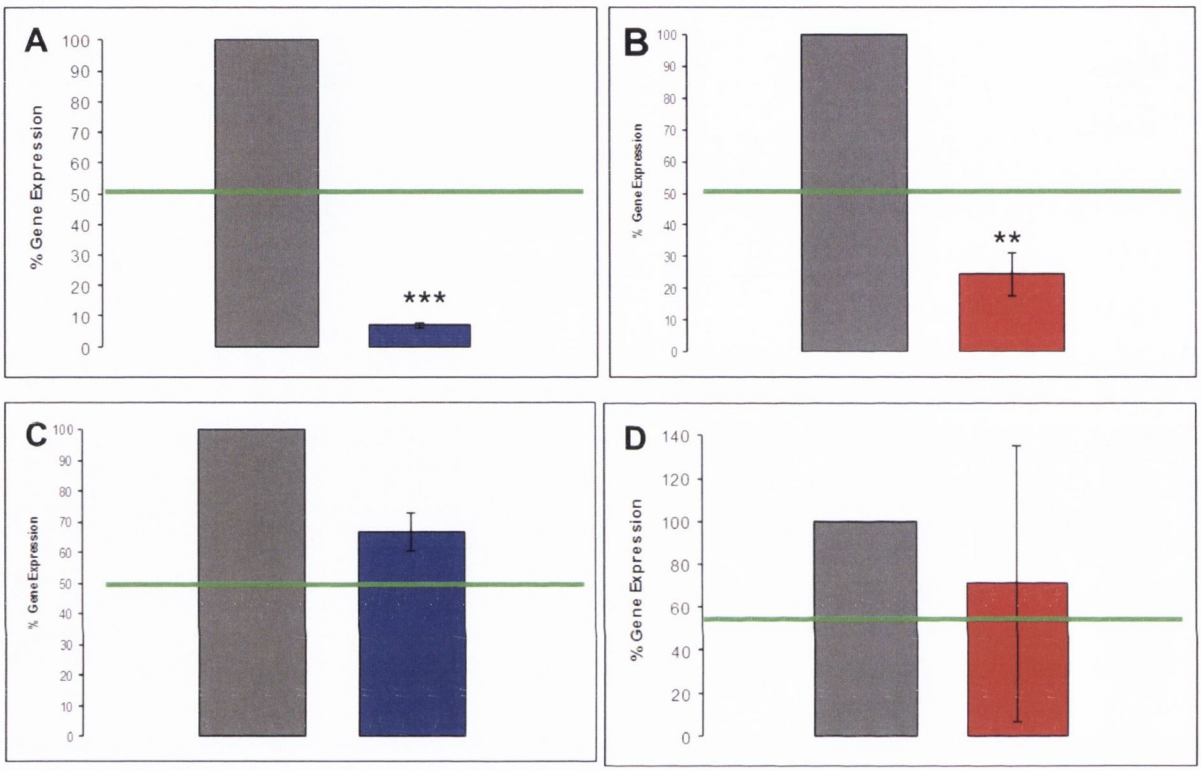

Figure 3.9: TLR4-MyD88 expression in hEC cells grown in hypoxia.

Cells were grown in 0.5% oxygen for three days. RNA was isolated and interrogated for TLR4 (blue) and MyD88 (red) expression via Q-PCR relative to untreated control (grey). Data shown are from a minimum of n=3, displayed as percentage expression relative to untreated cells. Green lines denote the limits of biological variation A) +B) 2102Ep cells substantially downregulated both TLR4 and MyD88. C) + D) NTera2 cells displayed no change in TLR4 or MyD88 expression. ** $P<0.01$ *** $P<0.001$

3.3.7 Retinoic acid pre-treatment affects the TLR4-MyD88 response of hEC cells to cisplatin

In the normal cellular environment cells will be constantly exposed to a range of signals from their environment, which will include differentiation signalling. To investigate whether differentiation status had an effect on hEC cells TLR4-MyD88 response to cisplatin, cells were treated with retinoic acid for three days, so as to initiate the differentiation process, and then treated with cisplatin. This pre-treatment did in fact alter the TLR4-MyD88 response of both cell lines to cisplatin treatment.

Cells were seeded at a density of 168,000 cells per well in media containing $1x10^{-5}M$ retinoic acid for three days. Media was then removed from the plate and replaced with media containing 1.2μM cisplatin for three days. Cells were then harvested, RNA was isolated, cDNA was synthesised and Q-PCR was performed using probes for TLR4, MyD88 and GAPDH as an endogenous control. Fold change in gene expression relative to untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. The cut-offs for biological significance were set at <50% and >200% expression. These are equivalent to the conventional +/- 2-fold limit, within which it is assumed that changes of expression are due to biological variation. T-tests were carried out to test for statistical significance. 2102Ep cells showed no final change in either TLR4 or MyD88 expression following predifferentiation followed by cisplatin treatment (Figure 3.10 A, B) NTera2 cells that had been predifferentiated before cisplatin treatment also showed no change in TLR4 or MyD88 expression (3.10 C, D).

When analysing combination treatment data, it is important to consider the results from corresponding single treatments. Previous work (Figure 3.6) had shown a specific response in both cell lines to retinoic acid treatment alone. However we see here that when this

treatment is followed with cisplatin, these results are altered. This demonstrates that the cell retains its ability to respond to stimulus, even when previously stimulated. Furthermore when we compare these results to the data from cisplatin treatment alone (Figure 3.4), it is clearly demonstrated that the pre-treatment has altered the ability of both cell lines to respond to cisplatin.

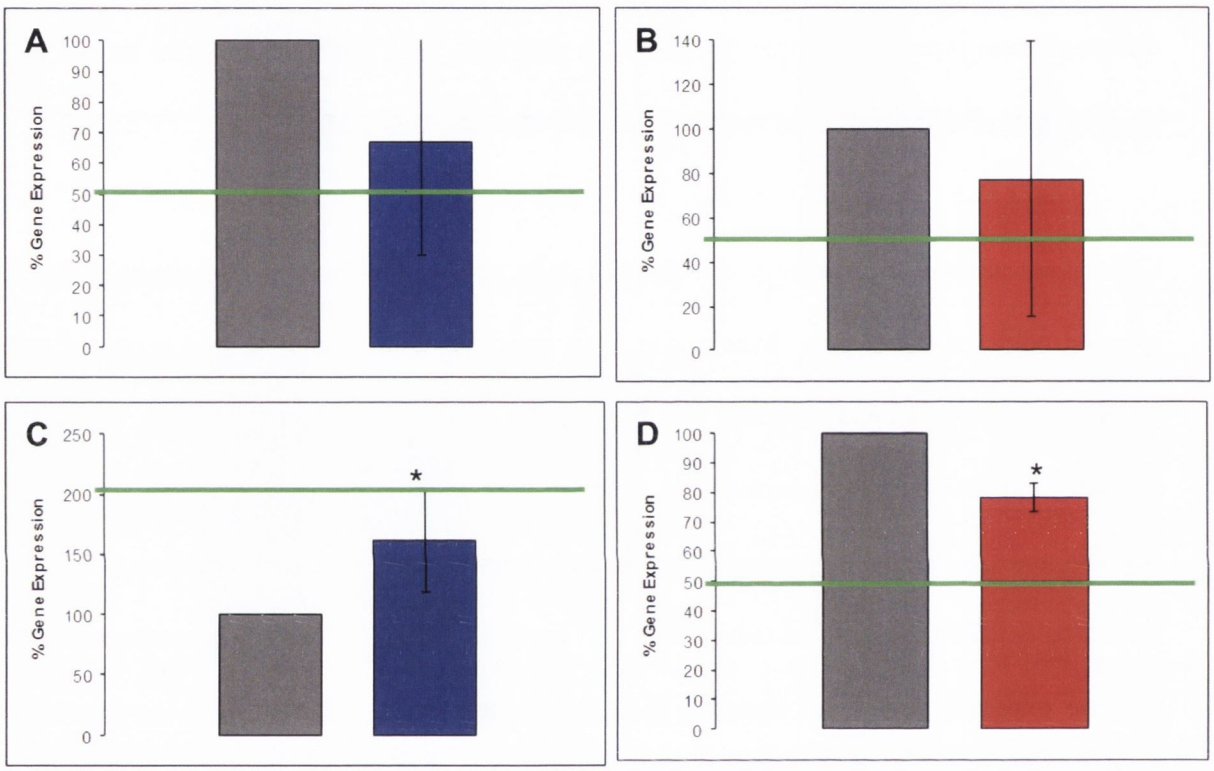

Figure 3.10: The effect of pre-differentiation on the expression of TLR4-MyD88 in response to cisplatin.

Cells were grown in retinoic acid for three days followed by cisplatin treatment for three days. RNA was isolated and interrogated for TLR4 (blue) and MyD88 (red) expression via Q-PCR relative to untreated control (grey). Data shown are from a minimum of n=3, displayed as percentage expression. Green lines denote the limits of biological variation. A) +B) 2102Ep cells showed no change in TLR4 or MyD88 expression. C) + D) NTera2 cells displayed no change in TLR4 or MyD88 expression. *$P<0.05$

3.3.8 Cisplatin pre-treatment affects the hEC response to differentiation

Further to our retinoic acid pre-treatment experiments we were also interested in whether treatment with chemotherapy altered the hEC response to differentiation stimulus. To ascertain this, we pre-treated both cell lines with cisplatin before treating with retinoic acid, and assessed their TLR4-MyD88 response. These data show that treatment with chemotherapy does affect the ability of hEC cells to respond to differentiation stimulus.

Cells were seeded at a density of 168,000 cells per well in a 6-well plate and allowed to adhere overnight. Media was then removed from the plate and replaced with media containing 1.2μM cisplatin for three days. This media was then removed and replaced with media containing $1x10^{-5}$M retinoic acid for three days. Cells were then harvested, RNA was isolated, cDNA was synthesised and Q-PCR was performed using probes for TLR4, MyD88 and GAPDH as an endogenous control. Fold change in gene expression relative to untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. The cut-offs for biological significance were set at <50% and >200% expression. These are equivalent to the conventional +/- 2-fold limit, within which it is assumed that changes of expression are due to biological variation.

2102Ep cells showed no change in either TLR4 or MyD88 expression when treated with cisplatin prior to retinoic acid treatment (Figure3.11 A, B). In contrast, NTera2 cells displayed dramatic upregulation of TLR4 along with limited upregulation of MyD88 (Figure 3.11C, D). Therefore both cell lines responded differently to either single treatment in terms of TLR4-MyD88 expression. Regulation of TLR4-MyD88 is more important to the NTera2 response to cisplatin treatment.

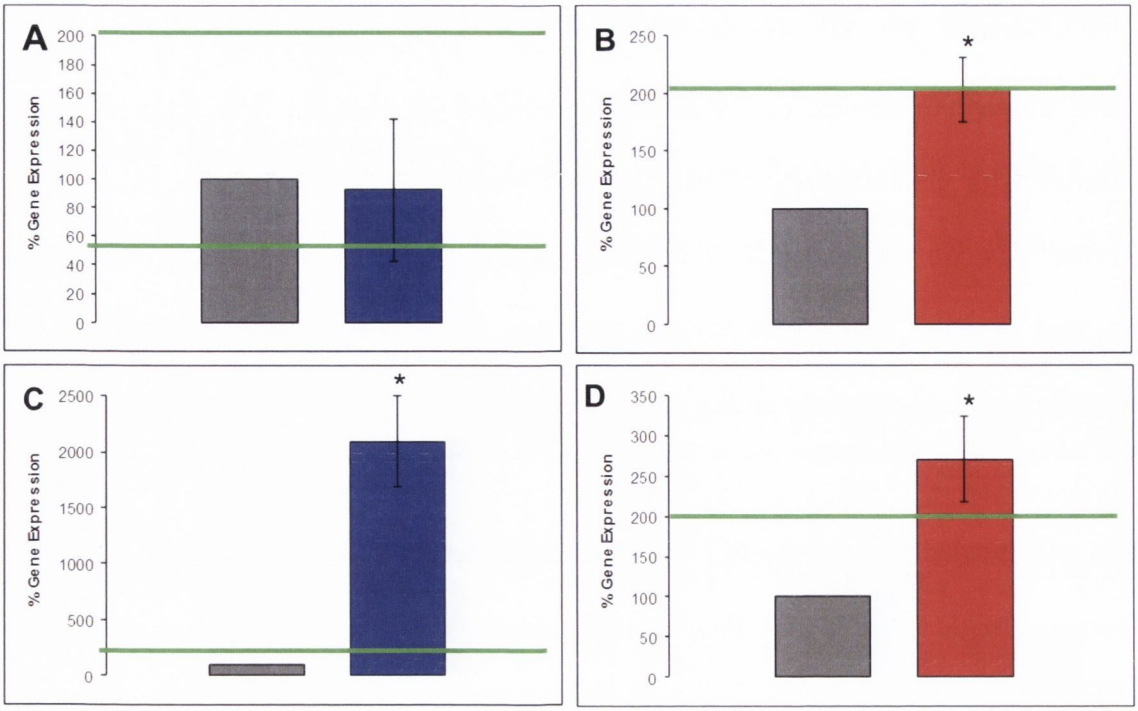

Figure 3.11: The effect of cisplatin pre-treatment on the expression of TLR4-MyD88 in response to differentiation. Cells were grown in cisplatin for three days followed by retinoic acid treatment for three days. RNA was isolated and interrogated for TLR4 (blue) and MyD88 (red) expression via Q-PCR relative to untreated control (grey). Data shown are from a minimum of n=3, displayed as percentage expression. Green lines denote the limits of biological variation. A) +B) 2102Ep cells showed no change in TLR4 or MyD88 expression. C) + D) NTera2 cells displayed substantial upregulation of TLR4 alongside limited upregulation of MyD88. *P<0.05,

3.3.9 Pre differentiation affects the hEC response to hypoxia

Given the constant presence of molecular signals in the cellular environment, and the strong association between CSCs and hypoxia we were interested in exploring whether molecular signals such as differentiation could affect the response of hEC to hypoxia. To achieve this we pre-treated both hEC cell lines with retinoic acid for three days before placing them in hypoxia and monitored the cells TLR4-MyD88 response.

Cells were seeded at a density of 168,000 cells per well in media containing 1x10-5M retinoic acid for three days. Media was then removed from the plate and replaced with normal media before placing the plate in a hypoxic chamber for 24 hours. Cells were then harvested, RNA was isolated, cDNA was synthesised and Q-PCR was performed using probes for TLR4, MyD88 and GAPDH as an endogenous control. Fold change in gene expression relative to untreated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. The cut-offs for biological significance were set at <50% and >200% expression. These are equivalent to the conventional +/- 2-fold limit, within which it is assumed that changes of expression are due to biological variation. 2102Ep cells showed dramatic downregulation of TLR4 alongside limited downregulation of MyD88 (Figure 3.12 A, B) TLR4 signalling in NTera2 cells that had been predifferentiated before hypoxia treatment showed no change in TLR4 but limited downregulation of MyD88 (Figure 3.12 C, D)

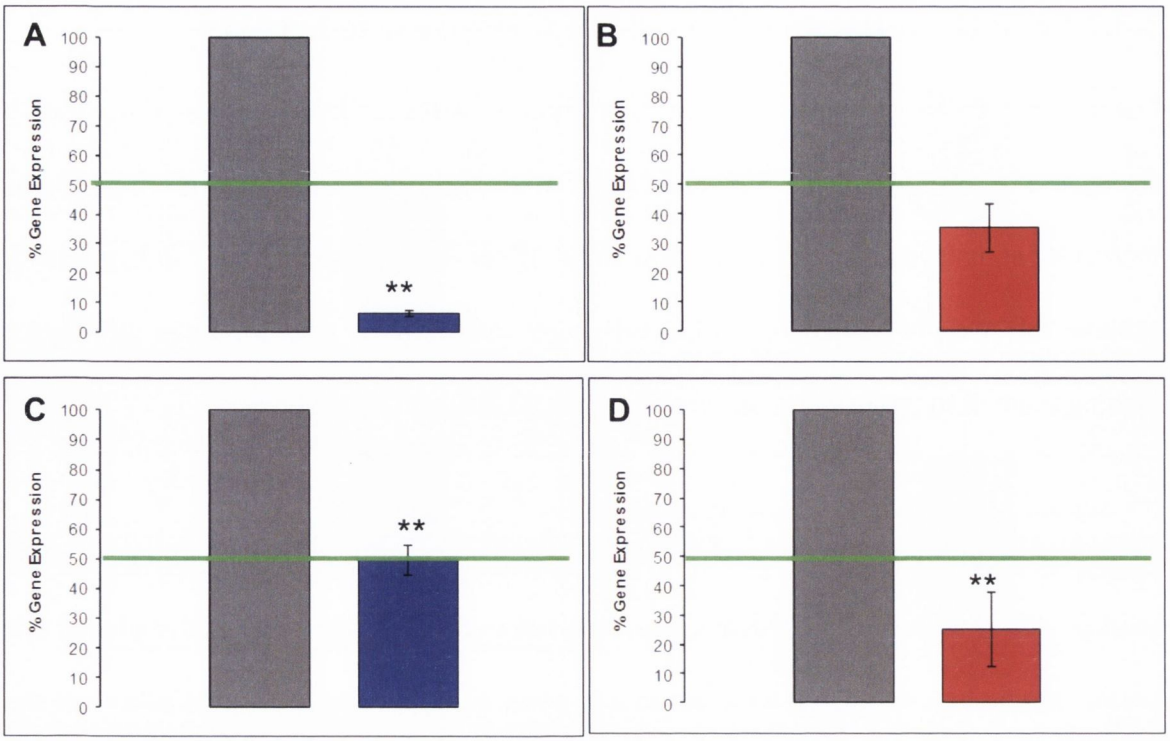

Figure 3.12: The effect of pre-differentiation on the expression of TLR4-MyD88 in response to hypoxia. Cells were grown in retinoic acid for three days followed by hypoxia for 24 hours. RNA was isolated and interrogated for TLR4 (blue) and MyD88 (red) expression via Q-PCR relative to untreated control (grey). Data shown are from a minimum of n=3, displayed as percentage expression. Green lines denote the limits of biological variation. A) +B) 2102Ep cells showed no change in TLR4 or MyD88 expression. C) + D) NTera2 cells displayed substantial upregulation of TLR4 alongside limited upregulation of MyD88. ** $P<0.01$, *** $P<0.001$

3.4 Discussion

The experiments outlined here aimed to investigate the extent of TLR4-MyD88 signalling involvement in the response of 2102Ep and NTera2 cells to cisplatin, retinoic acid and hypoxia treatment. We further aimed to investigate the potential effects of pre-treatments on these responses. Table 3.1 summarises these gene expression changes.

	Cisplatin Fig 3.3		**Retinoic Acid Fig 3.6**		**Hypoxia Fig3.9**		**Retinoic Acid Pre-Treatment, Cisplatin Fig 3.10**		**Cisplatin Pre-Treatment, Retinoic Acid Fig 3.11**		**Retinoic Acid Pre-Treatment, Hypoxia Fig 3.12**	
	TLR4	MyD88	TLR4	MyD88	TLR4	MyD88	TLR4	MyD88	TLR4	MyD88	TLR4	MyD88
2102Ep	No Change	Down	Down	Down	Down	Down	No Change	No Change	No Change	No Change	Down	Down
NTera2	Up	Up	Down	Down	No Change	No Change	No Change	No Change	Up	Up	No Change	Down

Previous work in the Andrew's lab in Sheffield has demonstrated that NTera2 and 2102Ep cell lines are virtually identical with the exception of their different responses to retinoic acid. NTera2 cells are pluripotent and respond to retinoic acid by differentiating towards a neural phenotype, 2102Ep cells are nullipotent and avoid differentiation. Few additional differences have been described between these cell types. The cisplatin dose response curves here demonstrate another difference in behaviour i.e. 2102Ep cells have a higher tolerance for cisplatin than NTera2 cells. This has led us to suspect that these two processes might be related: that the nullipotency of the 2102Ep cells is responsible for their increased chemoresistance. If this were the case then if we could alter the 2102Ep cells to make them pluripotent, then it could be possible to increase their chemosensitivity. The hEC TLR4-MyD88 response to cisplatin (Figure 3.3) combined with the IC_{50} data for both cell lines suggest that downregulation of MyD88 is associated with increased chemoresistance. Overexpression of MyD88 in this context then may present a method to decrease resistance artificially.

The differing responses of 2102Ep and NTera2 cell lines to retinoic acid were confirmed by the alkaline phosphatase staining data. From this it is clearly apparent that 2102Ep cells are nullipotent whereas NTera2 cells are pluripotent. Previous Affymetrix array data from our lab has demonstrated alteration of MyD88 signalling in the hEC response to differentiation stimulus. Small but consistent alterations in MyD88 signalling were observed between 2102Ep and NTera2 cells treated with retinoic acid. Furthermore, alterations in MyD88 associated gene signalling including SIGIRR, TIMP3 and IFI16 have also previously been recorded by this group (M Gallagher, Unpublished data). All of these genes are known to play a role associated with innate immunity. SIGIRR is a negative regulator of TLR-IL-1R receptor signalling, while TIMP3 is a specific inhibitor of TACE,

which interacts with TNF-α and plays an established role in the immune response (Fréour et al., 2009; Garlanda, Riva, Bonavita, Gentile, & Mantovani, 2013). IFI16 is an interferon-inducible myeloid differentiation transcriptional activator known to interact with MyD88 (Conrady, Zheng, Fitzgerald, Liu, & Carr, 2012; Unterholzner et al., 2010).These connections have led us to believe that MyD88 plays a role in the response of both cell lines to differentiation, i.e. that MyD88 is involved in the CSC ability to differentiate, and to avoid differentiation. 2102Ep cells displayed downregulation of both TLR4 and MyD88 in response to differentiation treatment, a response which mimics their response to cisplatin, although in cisplatin treatment this decrease was non-significant (Figure 3.6, Figure 3.3). This suggests that downregulation of TLR4 and MyD88 may be a stress response common to all stressors in 2102Ep cells. These data show that both lines downregulate TLR4 and MyD88 in response to retinoic acid treatment, despite their different potencies. If we assume that 2102Ep cells are essentially NTera2 cells with a lesion in their differentiation mechanism then we can say that the TLR4-MyD88 response to retinoic acid treatment is upstream of such a lesion. This would suggest that MyD88 activation is a key process of differentiation. TLR4 involvement in the response to retinol (a metabolite of retinoic acid) has only recently been demonstrated in microphages (S. Y. Kim, Koo, Song, & Lee, 2012). However, this work is the first time such a relationship has been shown in either a cancer or stem cell context.

The data presented here on 2102Ep and NTera2 cell survival in hypoxia demonstrate very clearly that both cell lines are unaffected by hypoxic conditions, in terms of cellular proliferation and TLR4-MyD88 signalling (Figure 3.7). The hypoxic chamber used for these experiments was set to 0.5% O_2, a severe depletion of oxygen compared to normal atmospheric conditions. Thus we can say that both hEC lines are strongly resistant to hypoxic conditions. Considering the known resistance of stem cells to hypoxia (Rich &

Bao, 2007), this is consistent with their cancer stemness. Data shown relating to TLR4-MyD88 expression in hypoxia treated cell lines demonstrated that whereas 2102Ep cells again downregulate both TLR4 and MyD88 expression, NTera2 cells show no alteration in TLR4-MyD88 signalling. The large error bar seen in Figure 3.8 was caused by the wide range of expression values obtained over a number of repetitions. Despite the fact that both cell lines survive well in hypoxic conditions the qPCR data suggests that this is achieved by different mechanisms or that the basal level of TLR4-MyD88 is sufficient for this process. 2102Ep cells are again downregulating TLR4 and MyD88, which further supports our theory that this is a stress response, common to all stressors of 2102Ep cells. However, this cannot be fully determined without performing functional analysis of the effects of MyD88 knockdown/overexpression.

Pre-treating the cells with retinoic acid dramatically altered their reactions to cisplatin treatment in terms of TLR4 and MyD88 expression. Whereas 2102Ep cells downregulated TLR4 and MyD88 in response to all individual stresses, in RA pre-treated cells there was no alteration in TLR4-MyD88 signalling. This suggests that TLR4 and MyD88 signalling has been "turned on" again following the cells initial TLR4-MyD88 decrease in response to retinoic acid treatment. NTera2 cells also show an altered response to cisplatin following pre treatment with differentiation stimulus.

Cisplatin pre-treatment also affected hEC cells. 2102Ep cells which had been pre-treated with cisplatin showed a distinct change in the responsiveness of the cells in terms of TLR4-MyD88. NTera2 cells were also affected by the cisplatin pre-treatment. These data suggest that once cells have been exposed to a stressor, they maintain their ability to respond to further stressors.

Investigations on the role of MyD88 in chemoresistance in other forms of cancer have yielded varied results. The work of Kfoury et al demonstrated that MyD88 plays an important role in promoting the optimal activation of the Ras/ERK survival pathway which is required for efficient DNA repair in colon cancer cells. They suggest that MyD88 plays a substantial role in survival signalling, suggesting its potential as a therapeutic target in Ras-dependent cancer cells (Kfoury et al., 2013). In the case of mammary carcinoma, previous work has shown that decrease in MyD88 expression is associated with decreased tumour growth and metastasis (Chalmers et al., 2013). While these results correlate with reduced patient survival in MyD88 positive ovarian cancer patients, observed by collaborators in St James' Hospital (d'Adhemar et al, in preparation), the gene expression data described in this chapter suggest a complex mechanism.

In order to determine the effect of alteration in MyD88 expression on this model system, functional analysis of MyD88 knockdown was performed in both cell lines in Chapter Four.

Chapter Four

Functional Analysis of MyD88 in Cancer Stem Cells

4.1 Introduction

The characterisation experiments carried out in Chapter 3 demonstrated very clearly that alteration of MyD88 expression occurred in response to challenging both 2102Ep and NTera2 cells with cisplatin, hypoxia and retinoic acid. In order to determine the significance of this alteration, functional experiments were performed. In these functional experiments, MyD88 protein expression was compromised to highlight the role(s) played by MyD88 in hEC CSCs.

Small interfering RNA (siRNA) is a class of double-stranded RNA molecules 20-25 base pairs in length. First discovered in 1999 (Hamilton, 1999), they have many roles but the most significant in this context is in the RNA interference (RNAi) pathway. This pathway provides a mechanism whereby a siRNA can prevent expression of specific genes with complimentary nucleotide sequence (S M Elbashir et al., 2001; Sayda M Elbashir, Lendeckel, & Tuschl, 2001). In brief, double stranded RNA (dsRNA) is cleaved into smaller siRNA pieces by the enzyme Dicer. These siRNA fragments are incorporated into the multi-component nuclease known as RISC (RNA-Induced Silencing Complex) siRNA is unwound in this process and guides the RISC complex's substrate selection by mRNA match (Figure 4.1) Thus cleavage of mRNA by the RISC complex is dependent on perfect complimentarity (Carmell, Xuan, Zhang, & Hannon, 2002). This function can be used to force downregulation of a gene of interest in a laboratory environment. Knockdown of a gene by siRNA transfection is detectable from 24 hours after transfection to approximately 7 days after transfection, depending on cell line and siRNA concentration.

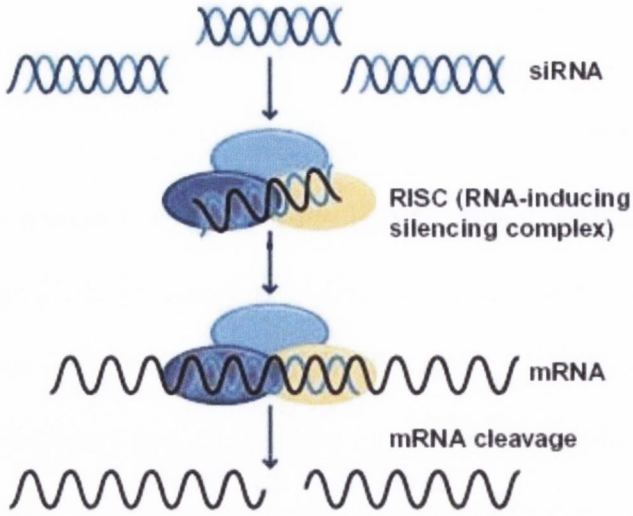

Figure 4.1: siRNA Pathway Overview (adapted from www.abcam.com)

A more long term knockdown can be achieved via use of short hairpin RNA (shRNA). shRNA is transfected into a cell via a plasmid or bacterial or viral vectors. Cells transfected in this way incorporate the shRNA into their genome and so the target gene is knocked down in all progenies of that cell.

siRNA and overexpression plasmids are transfected into cells by opening transient "pores" in the cell wall. This can be achieved via several methods including elecroporation or treatment with calcium phosphate; however the most commonly used method is by mixing the siRNA with a cationic lipid to produce liposomes which fuse with the cell membrane allowing the siRNA to pass through. Commercially available transfection reagents such as those used here have made this a standard method used in molecular biology laboratories. For the functional experiments described here it was decided to use siRNA transfection to knockdown MyD88, since the siRNA knockdown protocol had previously been optimised in the lab for both cell lines.

Both siRNA and shRNA knockdown have been widely utilised in the literature, however never before in the in the context of these cell lines and their response to these challenges.

The key role that MyD88 signalling plays in innate immunity has meant that the vast majority of studies focus on the implications of knockdown for immune signalling. In recent years, siRNA interference has been used to demonstrate the role of MyD88 in Interleukin-32α production in epithelial cells (Ko et al., 2011), to confirm that MyD88 independent signalling is responsible for β2-Adrenergic receptor activation in response to LPS stimulation (Kizaki et al., 2009), and to demonstrate the roll of early growth response 1 in formation of foam cells (J.-S. Kim, Park, Lee, Kim, & Baek, 2009),. Similarly shRNA knockdown of MyD88 have been used to demonstrate a diverse range of results. These include demonstrating the role of MyD88 in rhinovirus infection (Stokes et al., 2011), clarifying the role of TLRs in porcine innate immunity (Alves et al., 2007), and TLR activation in response to porin-incorporated liposomes in a mouse model (Banerjee, Biswas, & Biswas, 2008). From this it is evident that MyD88 plays a role in several different pathways in a range of model systems. Despite this diversity of research, it appears that MyD88 knockdown has never been investigated in the context of cancer stem cells, and rarely in the context of cancer itself.

Based on the characterisation work outlined in Chapter 3, it was also decided to investigate the effect of overexpression of MyD88 in NTera2 cells to their response to cisplatin, hypoxia, and retinoic acid. This was achieved via transfection of NTera2 cells with an overexpression plasmid for MyD88. Up until recently, production of overexpression plasmids was a time consuming and complicated endeavour that was off-putting for many due to the time and cost involved. In recent years however, custom cloning of plasmids became commercially available. This has made their use considerably easier. In addition to this, these commercially available plasmids contain constitutive promoters, unlike previous drug-selected plasmids that required the addition of a drug to cells. Despite this, MyD88 overexpression studies remain rare with only two published to date. The first investigates

the role of MyD88 in binding Protein Kinase Cϵ to Toll like receptors (Faisal, Saurin, Gregory, Foxwell, & Parker, 2008). Later work describes a mechanism of NF-κB inhibition in response to immune stimulation (Randall, Jokela, & Shisler, 2012). Aside from these two papers there is no existing literature on the effects of MyD88 overexpression.

2102Ep and NTera2 cell lines were the cell lines of choice for this work, since they had been used in the characterisation studies. However there were further benefits of their use. Unlike other stem cell lines, both 2102Ep and NTera2 cells have no requirement for a feeder layer of cells and grow well as adherent cells in normal hEC medium. They are also resilient enough to allow functional experiments to be performed, where most other stem cell lines would 'force differentiate' in response to any trauma. The differences in phenotype between the cell lines allow for the investigation of the effects of these functional experiments on cells of different potencies.

4.2 Aims and Hypotheses

Having shown that MyD88 expression was altered in both 2102Ep and NTera2 cells in response to treatment with hypoxia, cisplatin and retinoic acid in Chapter 3, it was hypothesised that these changes in alteration were necessary and sufficient for cell survival in those treatments.

Testing this hypothesis in this chapter, it was aimed to:

- Knockdown expression of MyD88 in 2102Ep cells.
- Overexpress MyD88 in NTera2 cells.
- Treat both MyD88 knockdown and overexpression cells with a cisplatin, retinoic acid and hypoxia, alone and in combination, and determine the cellular response of altered cells in these conditions.

4.3 Results

4.3.1 Optimisation of MyD88 knockdown in 2102Ep Cells.

In order to perform functional experiments it was first necessary to reliably knockdown MyD88 in our model. In order to ensure this, optimisation of the standard knockdown protocol described in Section 2.12 was carried out. In brief, cells were transfected with decreasing concentrations of siRNA for three days before cells were harvested; RNA was isolated and assessed for quality. cDNA was synthesised and interrogated for MyD88 expression relative to Negative siRNA treated cells via Q-PCR using GAPDH as an endogenous control. Fold change in gene expression relative to Negative siRNA treated cells was calculated using the $2^{-\Delta\Delta Ct}$ method, and from this the percentage change in gene expression was calculated. Substantial knockdowns were achieved at all concentrations (Figure 4.2). Since a MyD88 siRNA concentration of 1nM gave the highest consistent knockdown combined with the tightest error bars, this was selected as the standard siRNA concentration for all functional work. To confirm that this knockdown at the gene level was reflected at the protein level a western blot was carried out according to the protocols described in Chapter 2.11 (Figure 4.3). This confirmed that knockdown of MyD88 at the protein level had occurred.

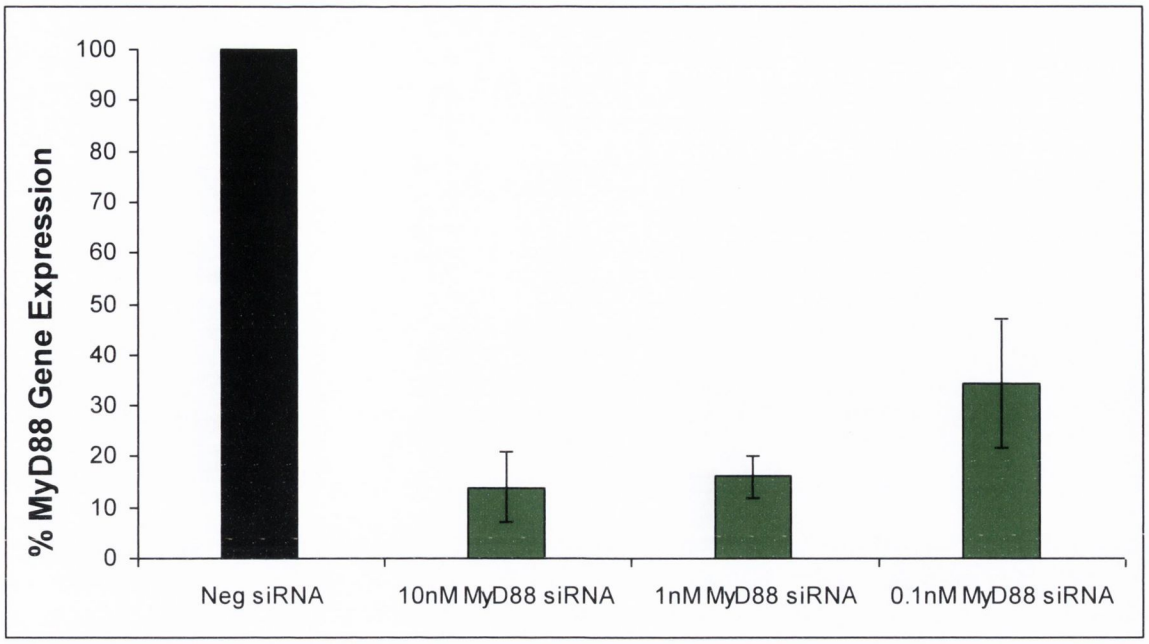

Figure 4.2: Knockdown of MyD88 in 2102Ep cells. Cells were seeded overnight and transfected with decreasing concentrations of MyD88 siRNA or Negative siRNA for three days. RNA was isolated and interrogated for MyD88 (green) expression via Q-PCR relative to Negative siRNA treated cells (black). Data shown are n=3, displayed as percentage expression. Since 1nM MyD88 siRNA gave consistent substantial knockdown, this concentration was chosen for our experimental protocol.

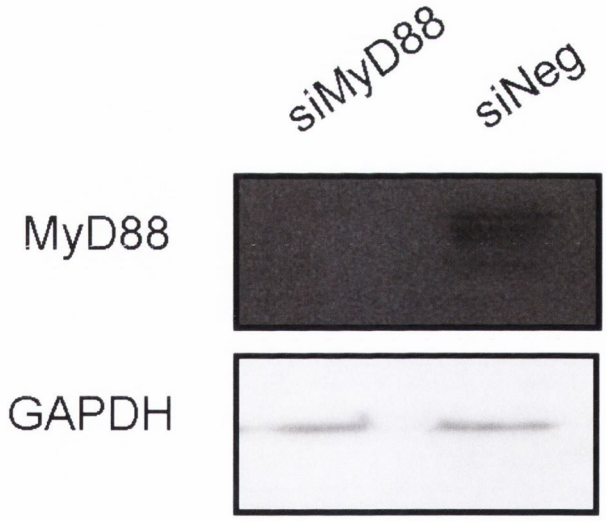

Figure 4.3: Knockdown of MyD88 in 2102Ep cells. Cells were seeded overnight and transfected with the appropriate concentration of MyD88 siRNA or Negative siRNA for three days. Protein was isolated and interrogated for MyD88 via Western Blot. Blots were stripped and subsequently interrogated for GAPDH, as an endogenous control and to confirm equal loading. MyD88 was knocked down at the protein level in siMyD88 treated 2102Ep cells, however MyD88 was expressed in siNeg treated 2102Ep cells (upper band).

4.3.2 Survival of 2102Ep MyD88 knockdown cells in hypoxia.

2102Ep cells decreased expression of MyD88 in response to hypoxia (Chapter 3.3.6). Thus we would expect that artificial knockdown of MyD88 would increase cell survival in hypoxic conditions. To investigate this MyD88 expression was knocked down in 2102Ep cells via transfection with MyD88 siRNA, before placing the cells in a hypoxic chamber for 72 hours.

2102Ep cells were transfected with an optimal concentration of either MyD88 siRNA or a negative control as determined in section 4.4.1 for three days. In order to achieve an accurate reading of the effect of the knockdown on cell survival it was decided to arrange the experiment so that the extent of the knockdown for each set of results was known. This was achieved by dividing each biological replicate into thirds. One third was used for Q-PCR analysis to determine the extent of the knockdown. The other two thirds were placed in either hypoxic (0.5% O_2) or normoxic conditions for 72 hours. An MTT assay was used to compare cell survival in MyD88 knockdown and negative control cells in hypoxia relative to normoxia. Thus the degree of knockdown and response to hypoxia and normoxia for each individual biological replicate could be correlated. Data shown here depicts the highest level of knockdown achieved from this set of experiments, and its correlating survival data.

Figure 4.4 illustrates cellular proliferation of 2102Ep cells treated with siMyD88 or siNegative in hypoxia treatments compared to normoxia. In 2102Ep cells where MyD88 was knocked down by 66%, survival was higher in hypoxia than in normoxia, however this was just outside the limits of statistical significance P=0.0536. In negative control cells survival remained unchanged.

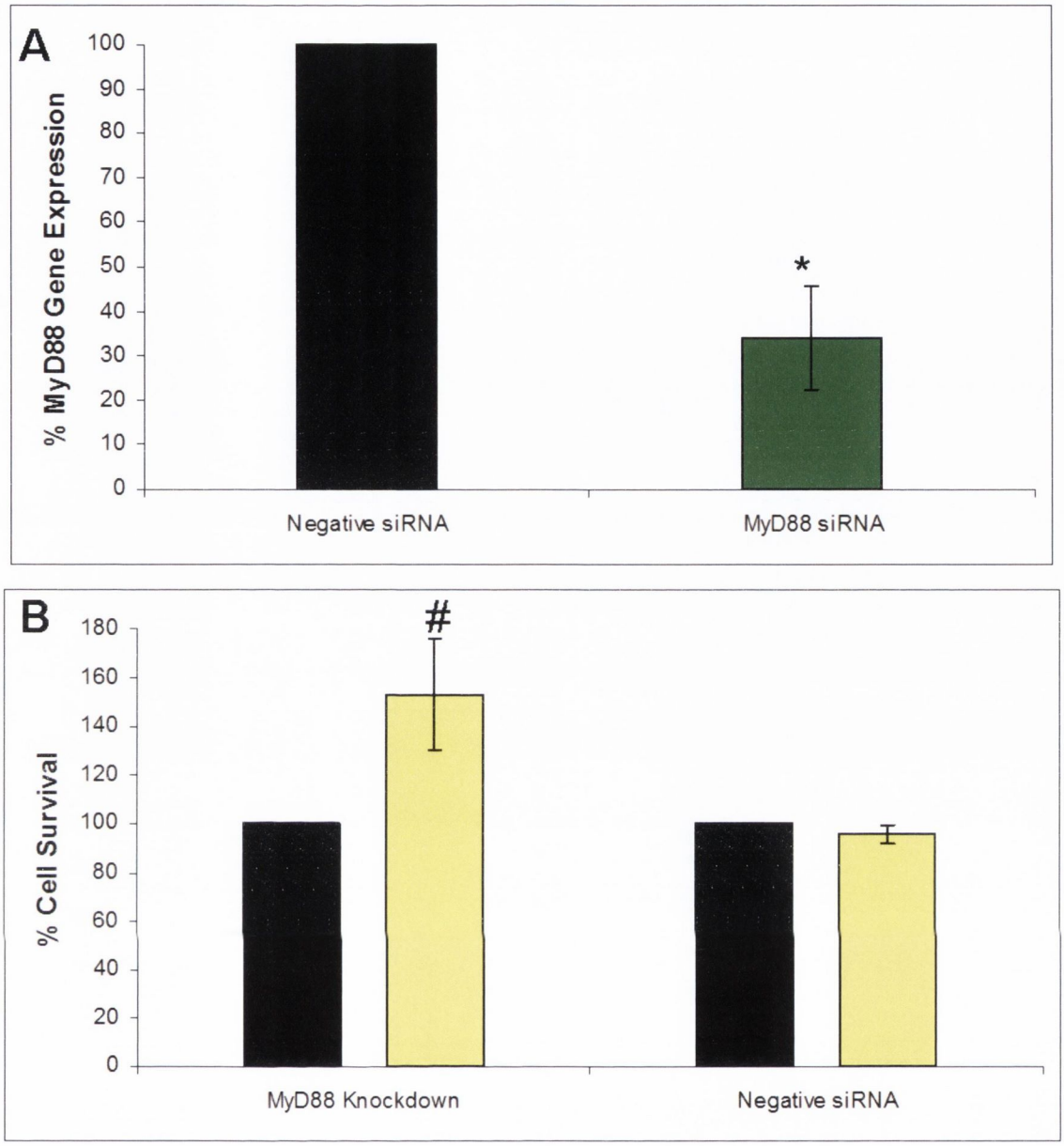

Figure 4.4: Knockdown of MyD88 in 2102Ep cells and subsequent cell survival in hypoxia.

Cells were seeded overnight and transfected with MyD88 siRNA or Negative siRNA for three days. A) RNA was isolated from a third of the cells and interrogated for MyD88 (green) expression via Q-PCR relative to Negative siRNA treated cells (black). B) The other two thirds of the cells were divided between two plates and seeded overnight. One of these plates was transferred to hypoxia for three days while one plate remained in normoxic conditions, before assessing cell survival using an MTT kit. Data is displayed as cell survival in hypoxia (yellow) relative to normoxic cells (black). * $P<0.05$, #$P=0.0536$

4.3.3 Survival of 2102Ep MyD88 knockdown cells in cisplatin.

2102Ep cells responded to being challenged with cisplatin by a limited decrease of their expression of MyD88 (Chapter 3.3.2). In order to determine if this decrease was necessary and or sufficient to cell survival of cisplatin MyD88 expression was knocked down via siRNA transfection, before treating the cells with a range of cisplatin concentrations and assessing cell survival.

As in the prior section, a correlation between knockdown and cell survival was maintained by dividing each biological replicate into thirds. RNA was isolated from one third and used to determine the extent of the knockdown. The remaining thirds were divided between two plates and treated with either a range of doses of cisplatin or a vehicle control. Cell survival was then determined using an MTT assay, comparing survival of knockdown cells in cisplatin to survival in vehicle control and doing the same for negative control transfected cells (Figure 4.5). There was no significant difference in cell survival in cisplatin between MyD88 siRNA and negative control siRNA cells. As such, MyD88 is not a functional component of 2102Ep cisplatin tolerance.

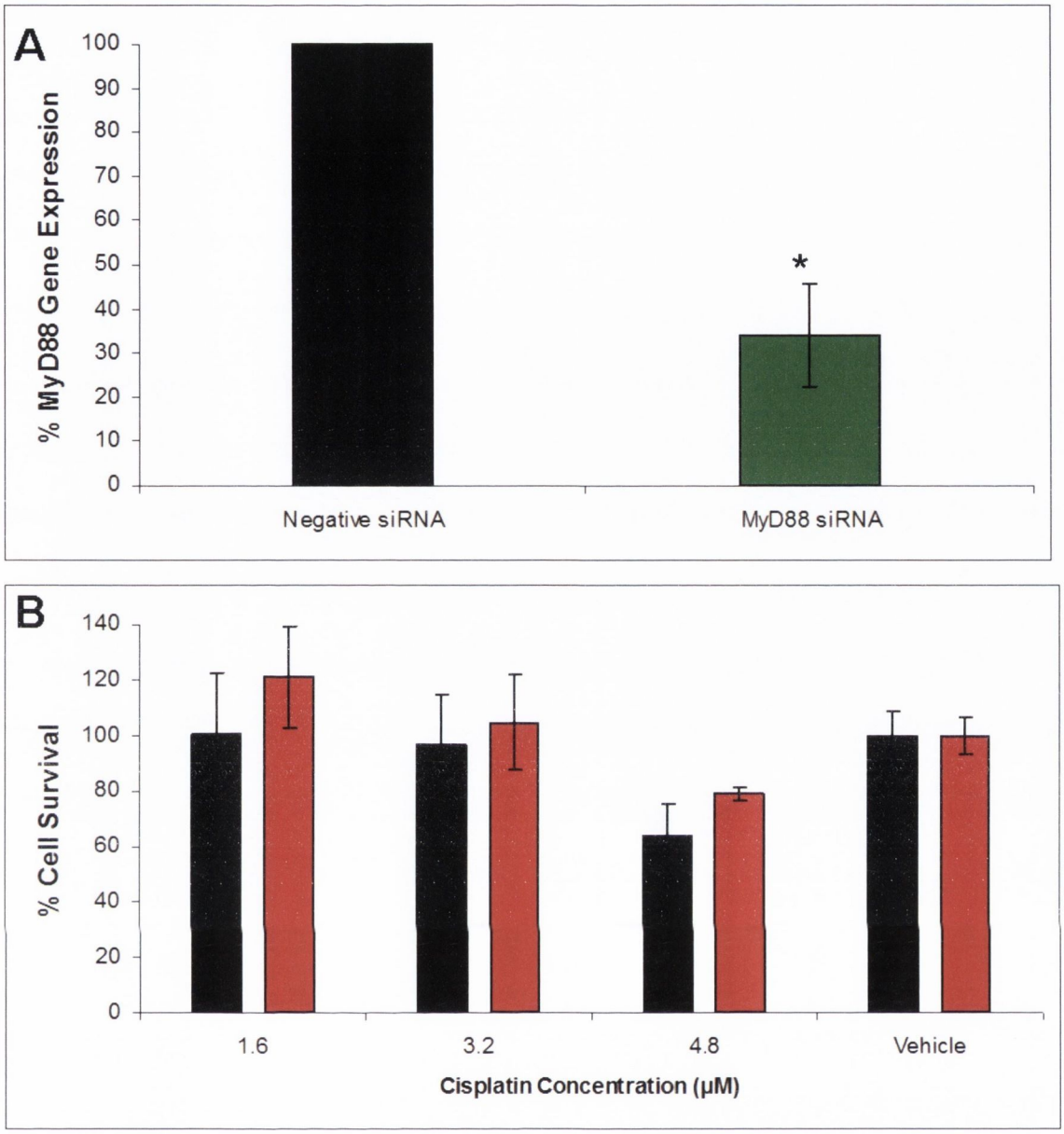

Figure 4.5: Knockdown of MyD88 in 2102Ep cells and subsequent cell survival in cisplatin.

Cells were seeded overnight and transfected with MyD88 siRNA or Negative siRNA for three days. A) RNA was isolated from half the cells and interrogated for MyD88 (green) expression via Q-PCR relative to Negative siRNA treated cells (black). B) The other half of the cells were seeded overnight, and then treated with increasing concentrations of cisplatin for three days, before assessing cell survival using an MTT assay. Data is displayed as cell survival relative to negative siRNA cells (black) in vehicle control. MyD88 knockdown cells are shown in red. * $P<0.05$

4.3.4 Survival of 2102Ep MyD88 knockdown cells in cisplatin and hypoxia

Previous characterisation of the 2102Ep response to cisplatin and hypoxia had shown a similar alteration in MyD88 expression in response to separate treatments (Chapter 3.3.2, Chapter 3.3.6). Functional work presented previous to this shows that while MyD88 expression is a functional determinant of 2102Ep cell survival in hypoxia (Chapter 4.3.1) this is not the case for cisplatin (Chapter 4.3.2). In order to investigate this anomaly further, a functional experiment investigating 2102Ep cell survival in cisplatin and hypoxia in combination was performed.

This was achieved as previously by knocking down expression of MyD88 via transfection with MyD88 siRNA. Transfected cells were then challenged with hypoxia and cisplatin in combination before cell survival was assessed using an MTT assay. Cell survival between MyD88 knockdown and negative control cells was then compared in order to determine any difference caused by the knockdown (Figure 4.6). In this case there was no significant difference in cell survival between cells that had been transfected with MyD88 siRNA and those that had been transfected with a negative control. Thus, MyD88 is not a functional component of 2102Ep tolerance of cisplatin and hypoxia in combination.

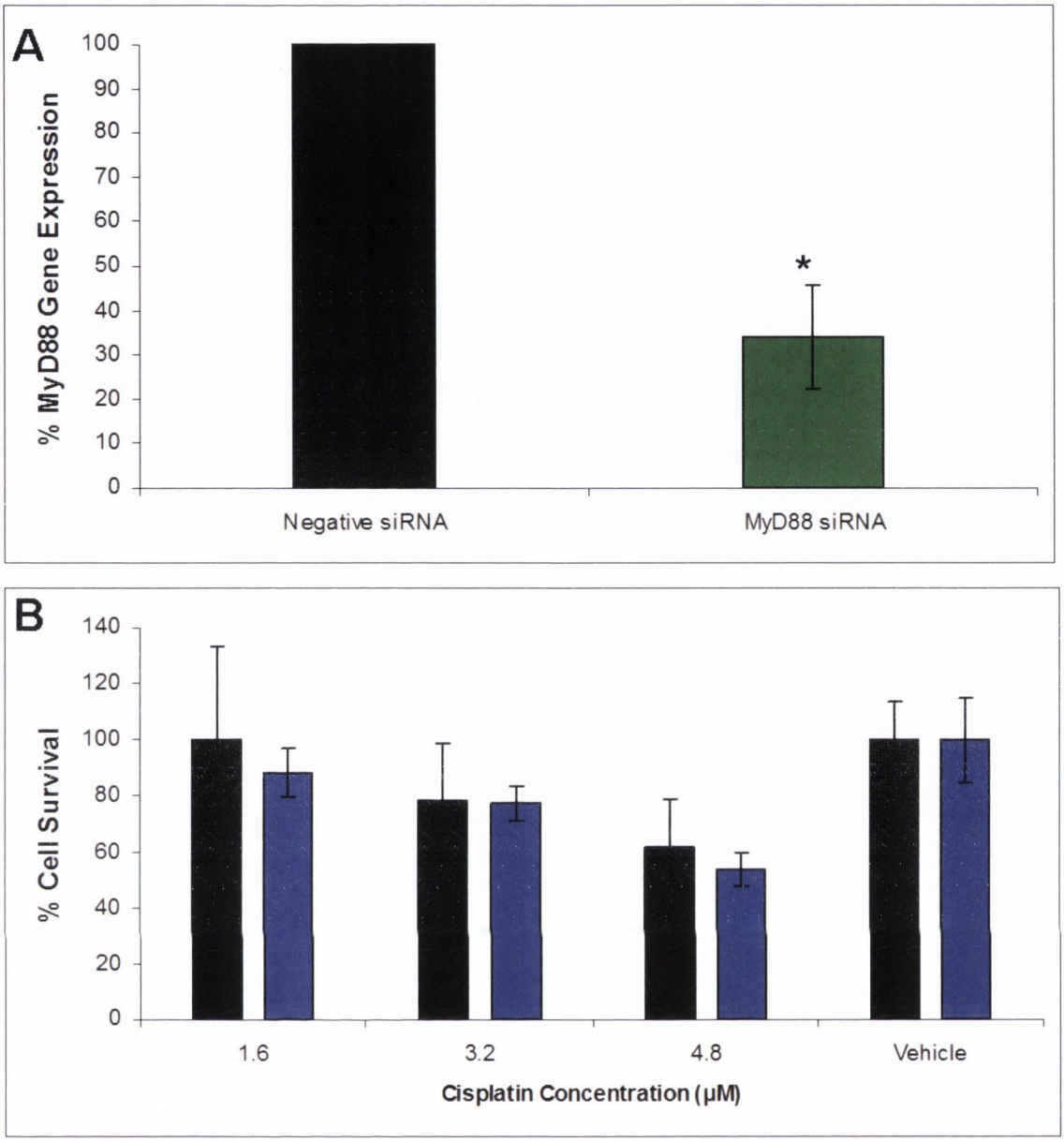

Figure 4.6: Knockdown of MyD88 in 2102Ep cells and subsequent cell survival in cisplatin and hypoxia

Cells were seeded overnight and transfected with MyD88 siRNA or Negative siRNA for three days. A) RNA was isolated from half the cells and interrogated for MyD88 (green) expression via Q-PCR relative to Negative siRNA treated cells (black). B) The other half of the cells were seeded overnight, and then treated with increasing concentrations of cisplatin for three days in a hypoxic chamber, before assessing cell survival using an MTT assay. Data is displayed as cell survival relative to negative siRNA cells (black) in vehicle control. MyD88 knockdown cells are shown in blue. * $P<0.05$

4.3.5 Differentiation Capacity of 2102Ep MyD88 knockdown cells in retinoic acid.

Characterisation work in Chapter 3.3.4 had shown that 2102Ep cells decrease expression of MyD88 in response to retinoic acid treatment. To further investigate this MyD88 siRNA was knocked down in 2102Ep cells before placing them in media containing retinoic acid for three days. Following retinoic acid treatment the cells were transferred to a 96 well plate before being treated with an alkaline phosphatase (AP) stain. Alkaline phosphate is a cell surface protein that is lost when cells differentiate (O'Connor et al 2008). The stain used here stains red when AP is present on the cells, therefore differentiated cells show no staining. All data shown was obtained from cells with 85% or more knockdown of MyD88.

2102Ep cells transfected with MyD88 siRNA showed no loss of AP staining after three days in control media (Figure 4.7C). However MyD88 knockdown cells transferred to media containing retinoic acid were not stained red, indicating that the retinoic acid had caused them to differentiate (Figure 4.7F). Notably, 2102Ep cells retained nullipotency in siNegative cells treated with RA. Thus, MyD88 is necessary for 2102Ep nullipotency. However, once MyD88 is lost, differentiation is not spontaneous, which is the case in most stem cells experiments of this type. The requirement of RA addition to stimulate differentiation in siMyD88 treated 2102Ep cells suggests a novel mechanism, as will be discussed in Section 4.5.

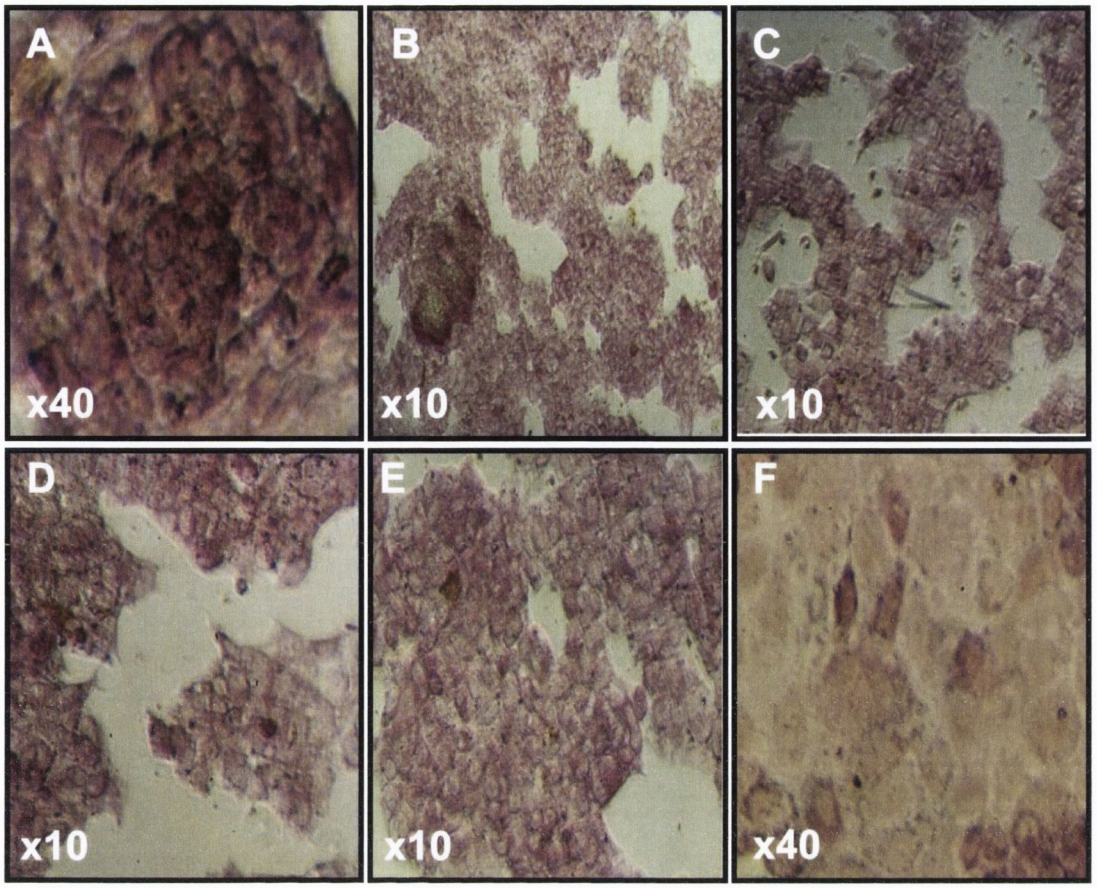

Figure 4.7: Differentiation potential of 2102Ep MyD88 knockdown cells. Cells were seeded overnight and either grown for three days (A,D) or transfected with Negative siRNA (B,E) or MD88 siRNA (C,F) or for three days. Media was then changed on the cells and replaced with fresh media (A-C) or media containing retinoic acid (D-F). Cells were then reseeded in a 96 well plate and stained with alkaline phosphate (AP) stain. 2102Ep cells treated with MyD88 siRNA (F) stain less red in retinoic acid medium compared to both NTC (D) and Negative siRNA (E) controls. There is no difference in AP staining between MyD88 siRNA cells in normal media (C) compared to NTC (A) and Negative siRNA controls (B).

4.3.6 Confirmation of MyD88 overexpression in NTera2 cells by Western Blot.

The protocol for overexpression of MyD88 in NTera2 cells had previously been optimised in the lab (Elbaruni 2011). In order to confirm that overexpression of MyD88 at the gene level resulted in overexpression of MyD88 at the protein level, a western blot was performed. Cells were treated with overexpression plasmid as outlined in Chapter 2.13 for

three days, protein was harvested and a western blot was performed as described in Chapters 2.9-2.11 This confirmed that overexpression of MyD88 had occurred at the protein level (Figure 4.8).

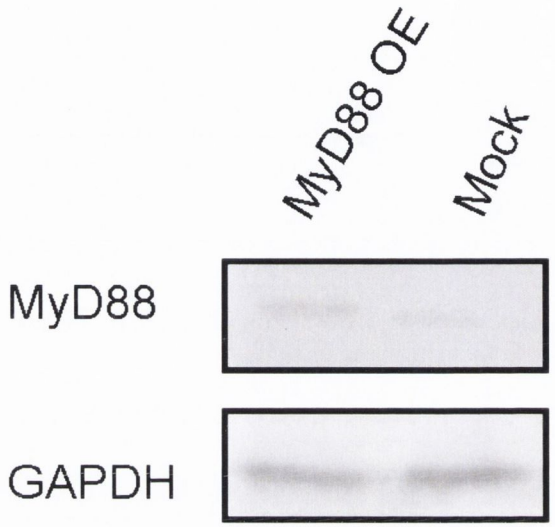

Figure 4.8: OverExpression of MyD88 in NTera2 cells. Cells were seeded overnight and transfected with the appropriate concentration of MyD88 overexpression plasmid or mock treatment for three days. Protein was isolated and interrogated for MyD88 via Western Blot. . Blots were stripped and subsequently interrogated for GAPDH, as an endogenous control and to confirm equal loading. MyD88 was overexpressed at the protein level in NTera2 cells.

4.3.7 Survival of NTera2 MyD88 overexpression cells in hypoxia.

Previous characterisation work carried out had shown that NTera2 cells did not change their expression of MyD88 in response to hypoxic conditions (Chapter 3.3.6). Based on functional work performed in Chapter 4.3.2 that demonstrated increased survival of 2102Ep cells in hypoxia following MyD88 knockdown, it was decided to investigate the effect of overexpression of MyD88 on NTera2 cell survival in hypoxic conditions.

MyD88 was overexpressed in NTera2 cells via transfection with an overexpression plasmid as described in Section 2.13. As in the knockdown experiments carried out

previously it was important to know the extent of the overexpression used for each functional result. As such, each biological replicate was split into thirds. One third was used to verify the overexpression and the remaining thirds were seeded overnight in plates. The following day one plate was placed in a hypoxia chamber while the other plate remained in normoxic conditions. An MTT assay was then used to compare cell survival between the two samples. NTera2 MyD88 overexpression cells did better in hypoxic conditions than in normoxic conditions; however mock transfected cells also had increased cellular proliferation. As such, increased MyD88 expression does not significantly affect cellular proliferation of NTera2 cells in hypoxia when compared to controls (Figure 4.9).

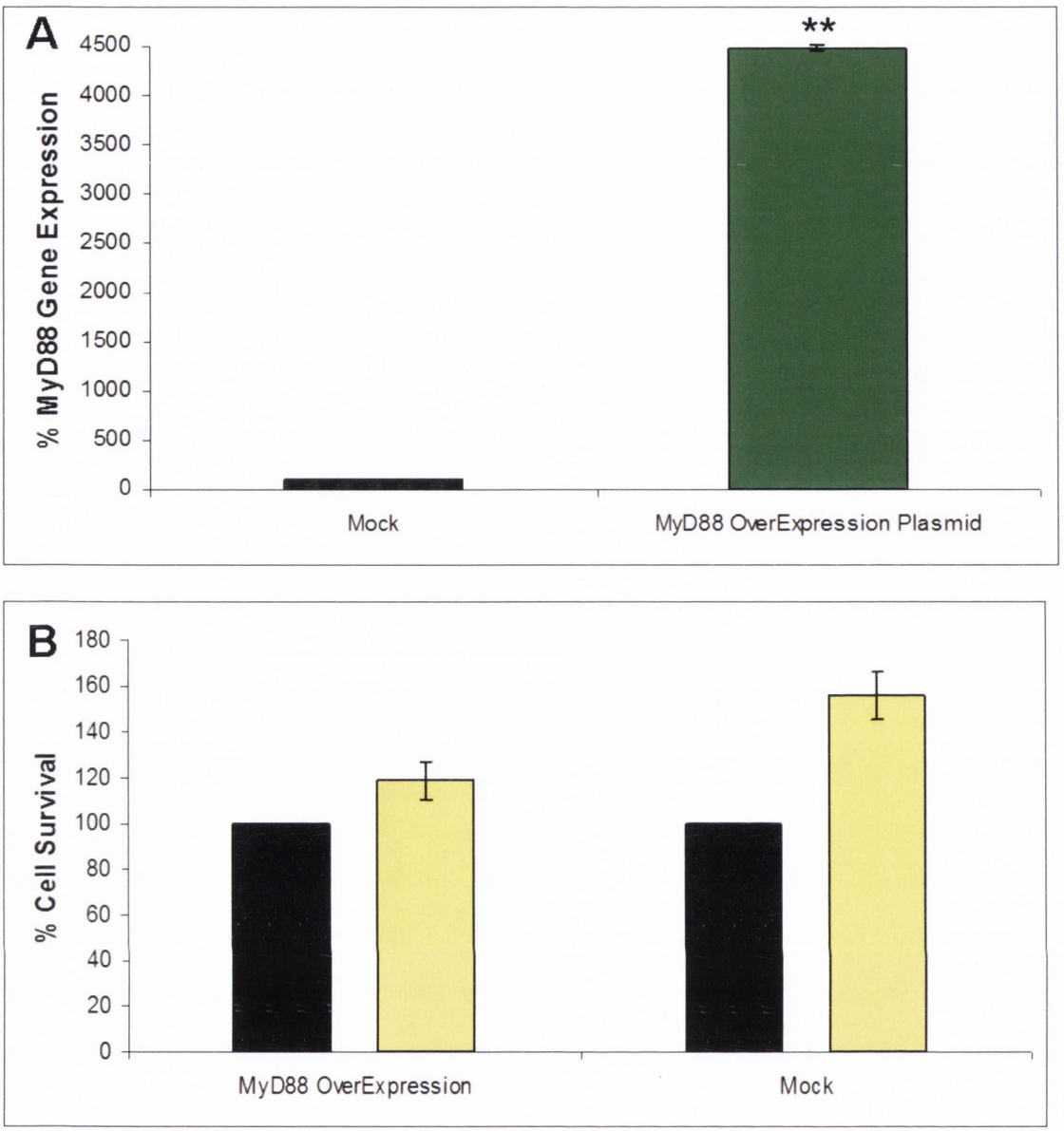

Figure 4.9: Overexpression of MyD88 in NTera2 cells and subsequent cell survival in hypoxia.

Cells were seeded overnight and transfected with MyD88 OverExpression plasmid or a mock control for three days. A) RNA was isolated from half the cells and interrogated for MyD88 (green) expression via Q-PCR relative to mock treated cells (black). B) The other half of the cells were split in half again and seeded overnight. Half of these cells were transferred to hypoxia for three days while half remained in normoxic conditions before assessing cell survival using an MTT assay. Data is displayed as cell survival in hypoxia (yellow) relative to normoxic cells (black). ** $P<0.01$

4.3.8 Survival of NTera2 overexpression cells in cisplatin.

Characterisation data from Chapter 3.3.2 had shown that NTera2 cells increase expression of MyD88 in response to treatment with cisplatin. In order to investigate if this increase in MyD88 is beneficial towards NTera2 cell survival in cisplatin, MyD88 levels were artificially increased via over expression and cell survival was assessed.

MyD88 was overexpressed in NTera2 via insertion of an overexpression plasmid (Imagene). As in the knockdown work, a correlation between overexpression and cell survival was maintained by dividing each biological replicate into thirds. RNA was isolated from one third and used to determine the extent of the overexpression. The remaining thirds were divided between two plates and treated with either a range of doses of cisplatin or a vehicle control. Cell survival was then determined using an MTT assay, comparing survival of knockdown cells in cisplatin to survival in vehicle control and doing the same for negative control transfected cells. There was no significant difference in cell survival in cisplatin between MyD88 overexpression NTera2 cells and mock treated NTera2 cells (Figure 4.10). Thus MyD88 is not sufficient to affect NTera2 cellular proliferation in hypoxic conditions.

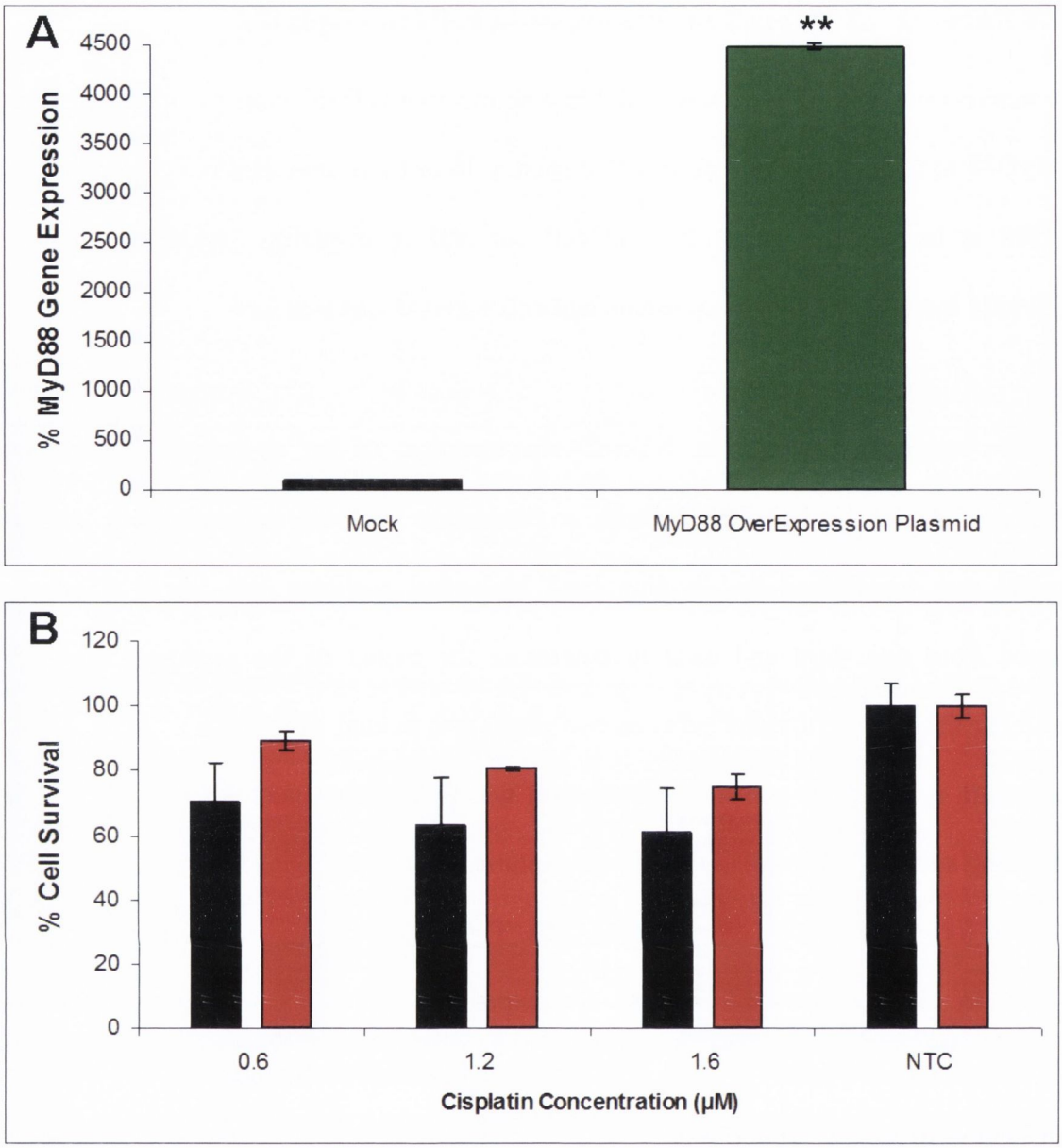

Figure 4.10: Overexpression of MyD88 in NTera2 cells and subsequent cell survival in cisplatin.

Cells were seeded overnight and transfected with MyD88 OverExpression plasmid or a mock control for three days. A) RNA was isolated from half the cells and interrogated for MyD88 (green) expression via Q-PCR relative to mock treated cells (black). B) The other half of the cells were seeded overnight, and then treated with increasing concentrations of cisplatin for three days, before assessing cell survival using an MTT assay. Data is displayed as cell survival of MyD88 Overexpression cells (red) relative to mock treated cells (black) in vehicle control. ** $P<0.01$

4.3.9 Survival of NTera2 overexpression cells in cisplatin and hypoxia.

Previous characterisation of the NTera2 response to cisplatin and hypoxia had shown different alteration in MyD88 expression in response to separate treatments (Chapter 3.3.2, Chapter 3.3.6). Functional work presented previous to this showed that MyD88 expression is not a functional determinant of NTera2 cell survival in either hypoxia (Chapter 4.3.5) or cisplatin (Chapter 4.3.6). To confirm that MyD88 was not a functional determinant of NTera2 survival in cisplatin and hypoxia, a functional experiment was performed.

This was achieved as previously by knocking down by overexpression of MyD88 in NTera2 cells via transfection with MyD88 siRNA. Transfected cells were then challenged with hypoxia and cisplatin in combination before cell survival was assessed using an MTT assay. Cell survival between MyD88 overexpression and mock control cells was then compared in order to determine any difference caused by the knockdown. In this case there was no significant difference in cell survival between cells that had been transfected with MyD88 overexpression plasmid and those that had been treated with a mock control (Figure 4.11). Thus, MyD88 is not sufficient to affect cellular proliferation in these conditions.

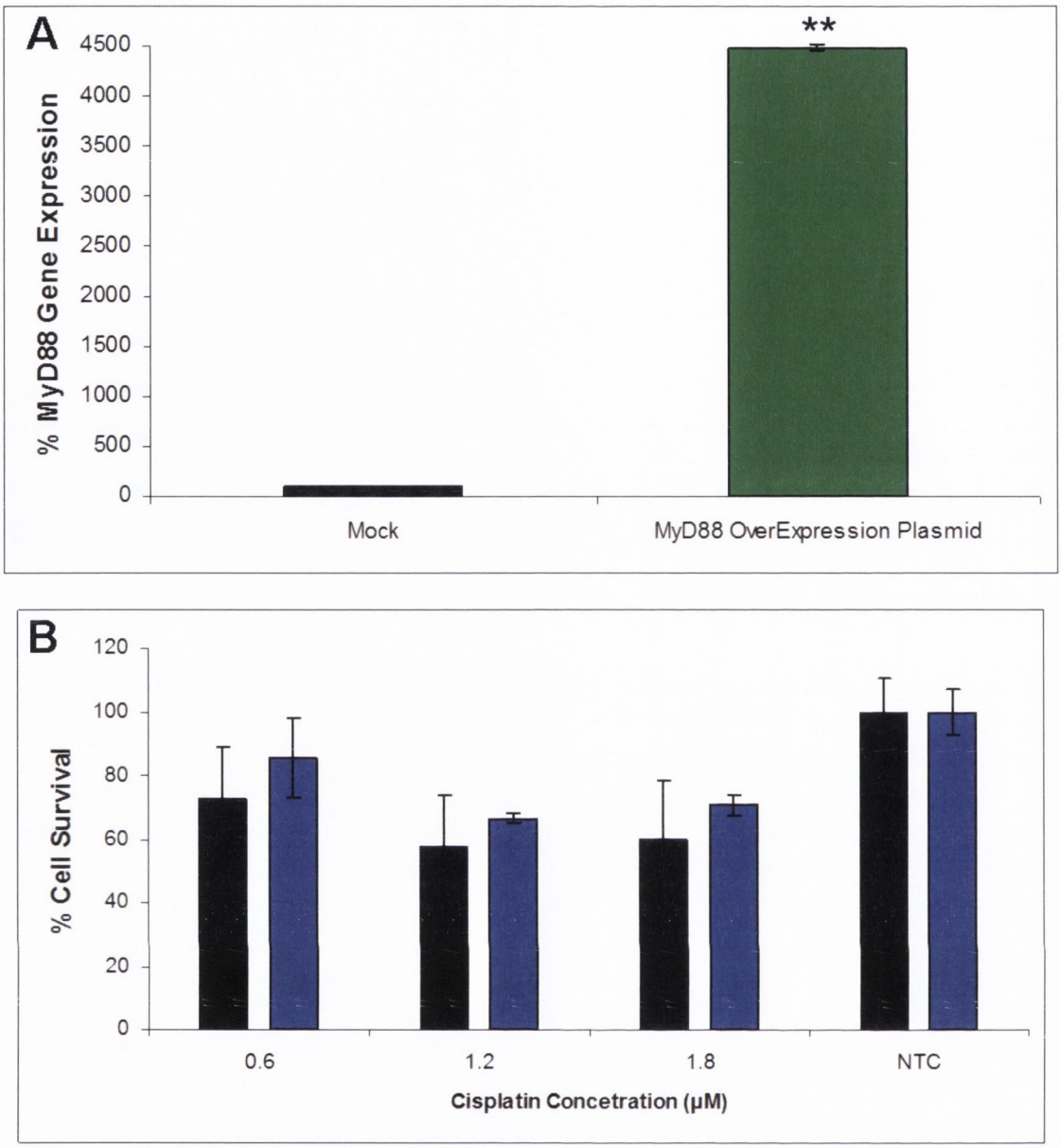

Figure 4.11: OverExpression of MyD88 in NTera2 cells and subsequent cell survival in cisplatin and hypoxia. Cells were seeded overnight and transfected with MyD88 OverExpression plasmid or mock control for three days. A) RNA was isolated from half the cells and interrogated for MyD88 (green) expression via Q-PCR relative mock treated cells (black). B)The other half of the cells were seeded overnight, and then treated with increasing concentrations of cisplatin for three days in a hypoxic chamber, before assessing cell survival using an MTT assay. Data is displayed as cell survival of MyD88 Overexpression cells (blue) relative to mock treated cells (black) in vehicle control. ** $P<0.01$

4.3.10 Differentiation Capacity of NTera2 MyD88 Overexpression cells in retinoic acid.

Previous data from the characterisation work had shown downregulation of NTera2 MyD88 signalling in response to treatment of the cells with retinoic acid. Furthermore, functional work on MyD88 knockdown had shown that loss of MyD88 in 2102Ep cells made them susceptible to differentiation via retinoic acid. To investigate whether the converse was true: i.e. that overexpression of MyD88 would make cells resistant to retinoic acid differentiation, a functional experiment was performed.

MyD88 was over expressed in NTera2 cells before placing them in media containing retinoic acid for three days. Following retinoic acid treatment the cells were transferred to a 96 well plate before being treated with an AP stain. The stain used here stains red when AP is present on the cells, therefore differentiated cells show no staining.

NTera2 cells transfected with MyD88 overexpression showed strong red staining after three days in control media (Figure 4.12C). Cells transferred to media containing retinoic acid for three days maintained their red staining (Figure 4.12F), unlike both mock (Figure 4.12E) and non- transfected control (NTC) (Figure 4. 12.D) treated cells, indicating that overexpression cells were now resistant to retinoic acid differentiation. Thus MyD88 is sufficient for maintenance of the pluripotent state in NTera2 cells.

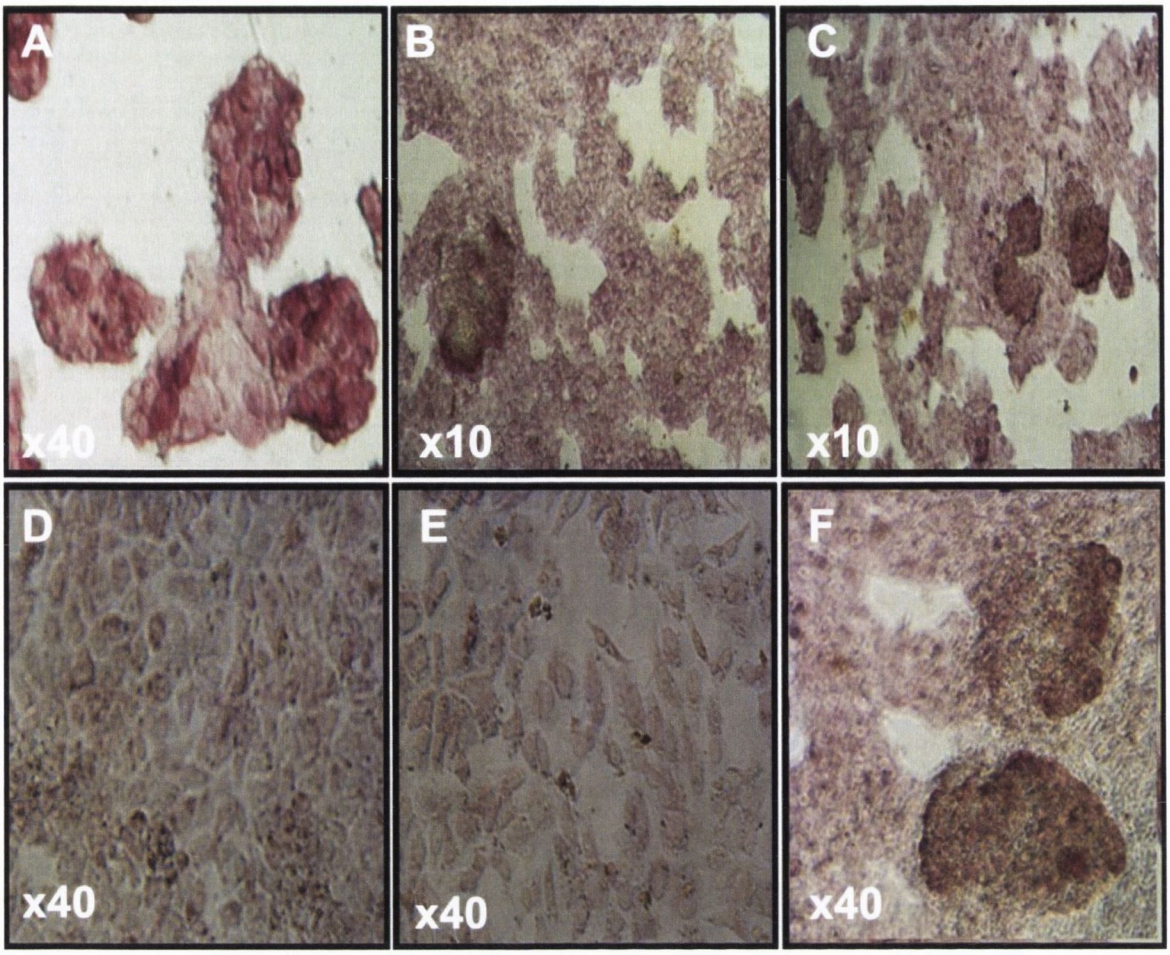

Figure 4.12: Differentiation potential of NTera2 overexpression cells. Cells were seeded overnight and either grown for three days (A,D) or mock transfected (B,E) or transfected with a MyD88 overexpression plasmid (C,F) or for three days. Media was then changed on the cells and replaced with fresh media (A-C) or media containing retinoic acid (D-F). Cells were then reseeded in a 96 well plate and stained with alkaline phosphate (AP) stain. NTera2 cells treated with MyD88 overexpression plasmid (F) continue to stain red in retinoic acid medium compared to both NTC (D) and mock (E) controls. There is no difference in AP staining between MyD88 overexpression cells in normal media (C) compared to NTC (A) and mock controls (B).

4.4 Discussion

The aim of this chapter was to investigate the functional role of MyD88 expression in the response of NTera2 and 2102Ep cells to cisplatin, hypoxia and differentiation. In order to do this, expression of MyD88 was artificially altered in both cell lines and the resulting effect on cell survival in specific conditions was established. Thus it could be determined whether MyD88 was necessary and/or sufficient for hEC CSC growth in and response to each environment.

The gene expression data obtained in Chapter 3, highlighted a number of potential functional experiments of interest. These included overexpressing MyD88 in 2102Ep cells and knocking MyD88 down in NTera2 cells. Unfortunately these were not possible within the time constraints of this project; however these experiments are currently being performed by other members of the group. The lack of assessment of transfection efficiency, may well suggest that the lack of functional effect observed in the cisplatin and hypoxia experiments is caused by inefficiencies in transfection. However this argument is counteracted by the clear functional effect observed in the retinoic acid experiments. These results suggest that transfection efficiency in both cell lines was sufficient to allow for the observation of functional effects.

The results of the MTT data for the cisplatin experiments indicate that in these cells and at these doses, MyD88 loss or gain has no specific effect on cell survival. Data from Chapter 3 had shown that MyD88 expression is altered very specifically in these cells, however the work described here has established that this alteration is neither necessary nor sufficient for cell survival. Characterisation data from chapter three also showed that TLR4 expression was altered in response to cisplatin treatment, so functional data for MyD88 presented here may suggest that it is TLR4 which is the key determining factor for these

cells. Unfortunately investigation of this was not possible within the time constraints of this project. This is a possible future experiment which has arisen from this project. In the case of MyD88 knockdown cells it is possible that either sufficient MyD88 is sequestered in the cells to cope with its knockdown or that the MyD88 independent pathway is activated via TRIF, leading to NFKB activation, albeit at a slight delay. This could be assessed by carrying out RT-PCR for TRIF or immunolocalisation studies for MyD88. Data obtained from the overexpression studies suggest that something upstream of MyD88 in the NFκB pathway must be the limiting factor. Our characterisation data would suggest that, in terms of cisplatin responses, TLRs, specifically TLR4 is key. While the relationship between TLR4-MyD88 and paclitaxel resistance is well established (Kelly et al., 2006; Silasi et al., 2006), this work is the first to suggest a link between TLR4-MyD88 and cisplatin. Future experiments from this group will focus on TLR4 in this context, and functional analysis of the effect of TLR4 knockdown and overexpression in hEC cells will be performed. This will allow for the clarification of the role of TLR4 and MyD88 in cisplatin tolerance in both 2102Ep and NTera2 cells.

The results of the MTT data for the hypoxia experiments show varying results. In the case of the 2102Ep knockdown experiments, cells that had depleted expression of MyD88 survived better in hypoxia than equivalent cells in normoxia, with a p value just approaching significance. The same was not true when comparing negative control cells in hypoxia and normoxia, these showed no difference. From this it can be inferred that low levels of MyD88 expression are advantageous to increased cell survival in hypoxic conditions. Logically speaking then, the converse result would be expected in MyD88 overexpression NTera2 cells. However this was not the case. Overexpressed cells showed increased survival in hypoxia relative normoxia; however a similar result was seen in the mock control cells, suggesting that this result is not MyD88 specific. From these results

then it can be said that 2102Ep and NTera2 cells respond to hypoxia in differently in terms of MyD88 expression. As stem cells, they would be expected to flourish in hypoxic environments (Mimeault & Batra, 2013), however an association between MyD88 expression levels and better or worse survival in hypoxia in stem cells has never been recorded previously.

The data presented from the retinoic acid functional experiments clearly show that loss of MyD88 is required for forced differentiation. Thus we can say that MyD88 plays a key role in nullipotency. Conversely in NTera2 cells, overexpression of MyD88 creates a retinoic acid differentiation resistant phenotype. Based on this we can now say that loss of MyD88 is necessary for differentiation in the pluripotent state. Identification of novel regulators of pluripotency is highly topical. However, this is paltry compared to the further significance of this data. This differentiation only takes place with the required "Push" of the presence of retinoic acid. This suggests that in the case of 2102Ep MyD88 knockdown, the resulting cells are in an intermediate state between undifferentiated and differentiated. Demonstration of such a mechanism would radically affect our understanding of the exit from the pluripotent state, which has historically been considered to be a linear process with differentiation. Our data indicate that, in hEC CSCs at least, this process is uncoupled.

Up until very recently it had been thought that all stem cells exit self-renewal and differentiated as a single fluid mechanism. Stem cells could be either undifferentiated or differentiated but there was no in between condition. However, two recent papers have argued otherwise. Silva and Smith argued for what they called the Ground State model. According to their thinking stem cells are maintained in a ground state by self-secreted Fgf/Mapk inhibitors. If these inhibitors are absent stem cells are primed for differentiation but this is in turn inhibited by Lif and Bmp4 growth factors. Absence of these growth

factors leads to differentiation along specific cell fates (Silva & Smith, 2008) This primed state is quite similar to our observations of siMyD88 treated 2012Ep cells. The opposing thought to this is the Competition Model (Loh & Lim, 2011). Under this model, cells are constantly surrounded by a range of pluripotency factors and it is the balance between these which maintains the pluripotent state. While both models are significantly different, they share one common aspect: both models hold that the exit from self-renewal and differentiation mechanisms are uncoupled.

The data presented here suggests that in these cells and in these conditions, MyD88 is the protein that governs a form of priming toward differentiation while still allowing them to proliferate. Ablation of MyD88 in these cells allows for the achievement of an intermediate state; cells do not spontaneously differentiate but are primed to differentiate in response to stimulus. This is a previously unknown function for MyD88 in stem cell biology. Furthermore, it is an area of stem cell biology for which few if any regulators have been identified. Much remains to be understood about the exact mechanisms involved but this is a novel development to arise from this work. Characterisation of this observation will continue in Chapter 5.

Chapter Five

Downstream analysis of MyD88 signalling

5.1 Introduction

Functional work performed in Chapter 4 showed that while knockdown and overexpression of MyD88 had no effect on cell survival in cisplatin, knockdown of MyD88 in 2102Ep cells did increase cell survival in hypoxia. Furthermore, alteration of MyD88 expression in both 2102Ep and NTera2 cells did have an effect on the CSC response to differentiation stimulus via retinoic acid. In order to investigate possible mechanisms that were responsible for these effects it was decided to characterise the downstream effect of MyD88 knockdown. This was achieved by three different methods: Affymetrix Array Analysis, Sequencing by Oligonucleotide Ligation and Detection (SOLiD), and Chemokine/Cytokine Arrays.

Microarray technology is a powerful tool for exploring genome wide gene expression profiles. By characterising the expression of more than 30,000 genes in each sample, it offers unique opportunities to study the interactions of genes and pathways, to characterise gene regulatory networks and to identify novel genes implicated in disease. In this study, the human Affymetrix GeneChip 1.0 ST array system was utilised. The simultaneous analysis of numerous mRNA expression patterns by microarray-based technology allows an unbiased approach to study downstream effects on gene expression alterations induced by disruption of the gene of interest which in turns allows aspects of gene function to be determined. Without this technology base, only a few genes could be studied at any one time and this means interactions between different genes or genetic pathways could be overlooked.

Gene arrays have been used in the context of ovarian cancer previous to this, most recently in studies that that obtained expression profiles in the context of cisplatin resistance (Cohen et al., 2012). Downstream analysis of MyD88 knockdown however, has never

before been studied in this manner. Whole genome analysis of this kind will allow us to determine the downstream effects of MyD88 knockdown, in particular those which lead to loss of nullipotency.

Further to the aim of elucidating the downstream effects of MyD88 knockdown at the gene level, the downstream effects of MyD88 knockdown on non coding RNA (ncRNA) species was also of interest. This was analysed by use of SOLiD sequencing. Non coding RNA species include transfer RNA (tRNAs), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA) and piwi-interacting RNA (piRNA). Expression of all of these classes of ncRNAs was of interest but most especially miRNA. The significant role that miRNAs can play in cancers is well established (Blanco-Calvo et al., 2012; Liu, 2012; McCleary-Wheeler et al., 2013). Indeed the role that miRNAs can play in ovarian cancer is also well studied (Bovicelli, D'Andrilli, & Giordano, 2011; Dahiya et al., 2008; Li, Zhang, Wang, & Wan, 2010). Previous work from this lab has shown that miRNA changes seen in the cell line studied in this work: 2102Ep cells, are mirrored in ovarian serous adenocarcinoma patients (Gallagher, Flavin, & Elbaruni, 2009). As miRNAs have been shown to be vital regulatory elements, it is widely believed that other ncRNAs will ultimately be shown to be similarly important, despite our current poor understanding of their mechanisms of action.

Because of this strong interest in the possible changes in ncRNA expression caused by MyD88 knockdown, SOLiD sequencing was performed on the same samples as those used for Affymetrix array analysis. SOLiD sequencing is a new technology which allows for comparisons of the whole transcriptome. It can be used to compare ncRNA expression between samples and controls, in this case siMyD88 and Si Negative Control treated

2102Ep cells. SOLiD sequencing has been used previously to compare Human Papilloma Virus (HPV) positive and negative head and neck squamous cell carcinomas (Nichols et al., 2012), identify genetic risk variants for deep vein thrombosis (DVT) (Lotta et al., 2012), and even deep sequence the Atlantic Cod transcriptome (Johansen et al., 2011).

miRNAs are known to alter protein expression by post-transcriptional regulation. In order to understand that interaction between both Affymetrix and SOLiD data, a protein array was carried out. Protein arrays achieve efficient and sensitive high throughput protein analysis, carrying out large numbers of determinations in parallel by automated means. Since MyD88 primarily controls chemokine and cytokine output, this method was invaluable in determining the consequence of MyD88 alteration. In Chapter 3, media from both NTera2 and 2102Ep cells treated with a range of treatments was saved and stored frozen. This biobank of samples was available for analysis via protein array meaning that the chemokine cytokine profile of both cell lines in response to a range of treatments could be analysed, in addition to the knockdown samples used on the Affymetrix arrays and SOLiD sequencing.

RayBiotech Quantibody arrays are ELISA based multiplex arrays that allow for simultaneous quantitative measurement of multiple chemokines and cytokines. Each array carries antibodies for up to 40 chemo- and cytokines. Protein arrays of this kind have been used in studies in as diverse subjects as cytokine profiling of migraines (Vause & Durham, 2012), cytokine profile of Her-e overexpressing carcinomas (Martin et al., 2008) and investigating the response to deep tissue inflammation in the rat (Watanabe et al., 2005).

By utilising these highly advanced tools and techniques it will be possible to determine the full downstream effect of MyD88 knockdown in 2102Ep cells as well as gaining a full

chemokine and cytokine profile for a wide range of cellular treatments. It is hope that the data generated in this chapter will identify the proteins specifically secreted by changes in MyD88 expression and the changes in mRNA and/or ncRNA expression that facilitate this.

5.2 Hypothesis and Aims

In chapters 3 and 4 the response and function of MyD88 was characterised in both 2102Ep and NTera2 cells. From this it was determined that although MyD88 was involved in the hEC response to cisplatin, hypoxia and retinoic acid treatment, it has a functional effect on differentiation in both nullipotent and pluripotent cell types, and a functional effect in hypoxia response in 2102Ep cells only. This led to the hypothesis that deletion of MyD88 leads to specific alterations in gene, miRNA and protein expression. Furthermore, that challenging hEC cells with a range of treatments, alone and in combination alters the secretory profile of these cells.

This led to two aims for this chapter:

- To determine and characterise the downstream effects of MyD88 knockdown in 2102Ep cells at the gene, miRNA and protein levels.
- Furthermore, to determine the chemokine and cytokine profile of hEC cells in response to cisplatin, retinoic acid and hypoxia, alone and in combination.

5.3 Results

5.3.1 Affymetrix Array Data

Microarray analysis was carried out as described in section 2.15. Briefly, 300ng of total RNA was reverse transcribed, fragmented and biotin labelled following recommended Affymetrix protocols. Single stranded fragmented, biotin labelled DNA was hybridised to GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA). Hybridised arrays were scanned on an Affymetrix GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). Six microarray experiments were performed: three biological replicates of 2102Ep cells transfected with MyD88 siRNA, and three transfected with the negative control siRNA.

The quality of the arrays was evaluated to ensure no outliers or defective arrays. Affymetrix array quality assessment procedures involve the production of a set of summary statistics for each array and graphing these for all arrays in order to provide a comparison. These quality control metrics were calculated and graphed using the Affymetrix Expression Console Software (Figure 5.1).

There was a low level of variation in all of the metrics calculated, which is as expected when working with samples from the same cell line. The pos_control and all_probset categories are used to assess the overall quality of the data from each array (Figure 5.1 A,B and E). Metrics based on these categories reflect the quality of the whole experiment including RNA quality, target preparation, chip hybridization, scanning and griding. Thus, they are useful to assess the nature of the data being used in downstream statistical analysis. The area under the curve for the signal discrimination of the positive and negative controls showed only a low amount of variation between samples (Figure 5.1A). This means there is good separation between the positive and negative controls, which are a

measure of true and false positives respectively, and indicates that the entire microarray experiment of all the samples analysed was successful. Another example of the similarity of the samples is the box plots of the relative log expression (RLE) of the probsesets (Figure 5.1B). The median RLEs should be zero, and all samples are very close to zero. Deviations from zero typically indicate a skew in the raw intensities of an array, not corrected sufficiently by normalisation. In Figure 5.1E the pm_mean appears to vary a lot between microarrays, however when the range of the signal intensity of this metric is viewed it is low (851-600) and so again reaffirms the low levels of inter-array variation.

Figures 5.1 C, D and F all show metrics for bac_spike and polya_spike controls. Figure 5.4C indicates the rank order of PolyA spikes, problems with these can indicate an issue with RNA quality or sample preparation. However, in these data, the rank orders are as expected. Figure 5.4D represents the mean absolute deviation of residuals and indicates a consistent picture between the positive controls (green), bacterial spikes (red) and polyA spikes (blue). Finally Figure 5.4E represents the rank order of the bacterial spikes. Once again these are as expected. The data represented in Figure 5.4 indicate that there are no quality control issues with the Affymetrix Array data.

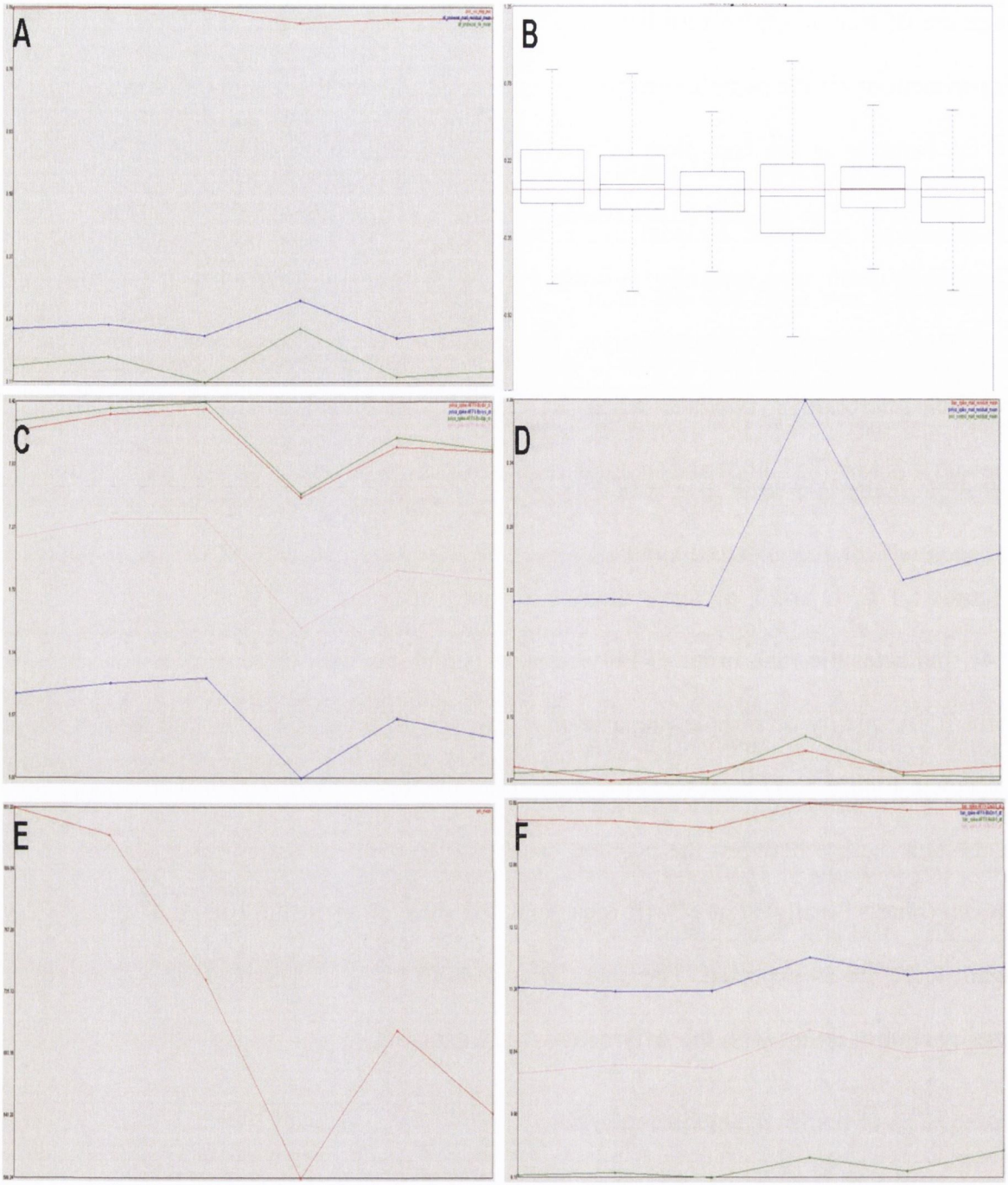

Figure 5.1: Affymetrix quality control Metrics. A) Mean absolute deviation of residuals (blue), mean absolute relative log expression (green) and the area under the receiver operator curve for signal discrimination of the positive and negative controls (red). All six samples are within acceptable parameters. B) Box plots of the relative log expression for al the probe sets analysed. Again all samples are within acceptable parameters. C) Rank order of PolyA spikes. These are all in the expected order. D) The mean absolute deviation of residuals of positive controls (green), bacterial spikes (red) and polyA spikes (blue). All samples are within acceptable parameters. E). Mean probe level intensity (Prior to background correction or normalisation.) All probes are within acceptable parameters. F) Rank order of bacterial spikes, these are all in the expected order.

In order to determine the level of differential gene expression between 2102Ep cells that had been treated with MyD88 siRNA and those treated with negative control siRNA a Pearson's correllation plot (Figure 5.2) and cluster dendrogram (Figure 5.3) were produced.

The Pearson's correlation plot is based on normalised, non-background corrected data. It is in the form of a heat map representing all possible pair-wise combinations of samples used in the microarray analysis. Close examination of Figure 5.5 indicates that samples from the same treatment group (i.e. MyD88 siRNA or Negative Control siRNA) are not as similar as would be expected. Similarly when a cluster dendrogram was produced the samples analysed did not display the expected groupings (Figure 5.3). This suggests highly similar levels of gene expression between 2102Ep MyD88 knockdown cells and 2102 Negative Control cells. The units on the scale in Figure 5.3 indicate that all samples are highly similar, rather than there being one or two outliers.

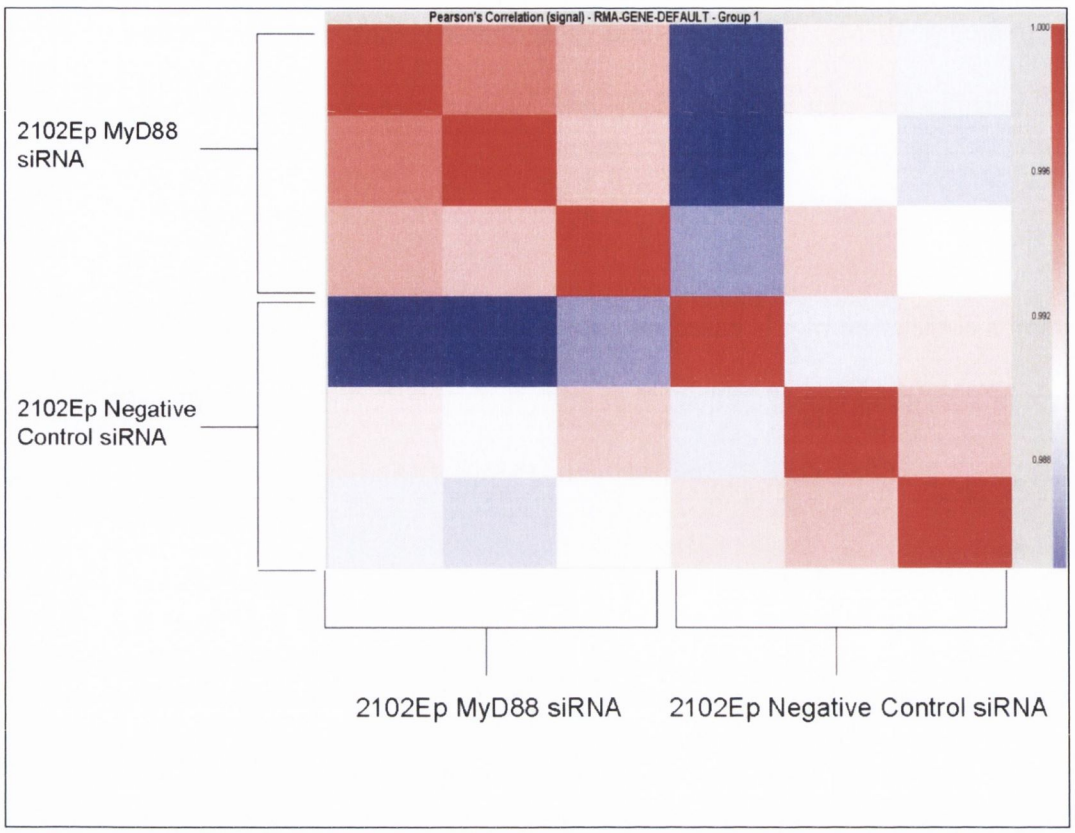

Figure 5.2: Correlation between the gene expression profile of 2102Ep cells transfected with MyD88 or Negative Control siRNA. Pearson's correlation plot of normalised non background corrected data. This plot is in the form of a heat map representing all possible pair-wise combinations of samples used in the analysis. Pearson's correlation coefficient ranges from +1 to -1, with a correlation of 0 inferring there is no relationship and a correlation of +/-1 inferring a perfect linear relationship.

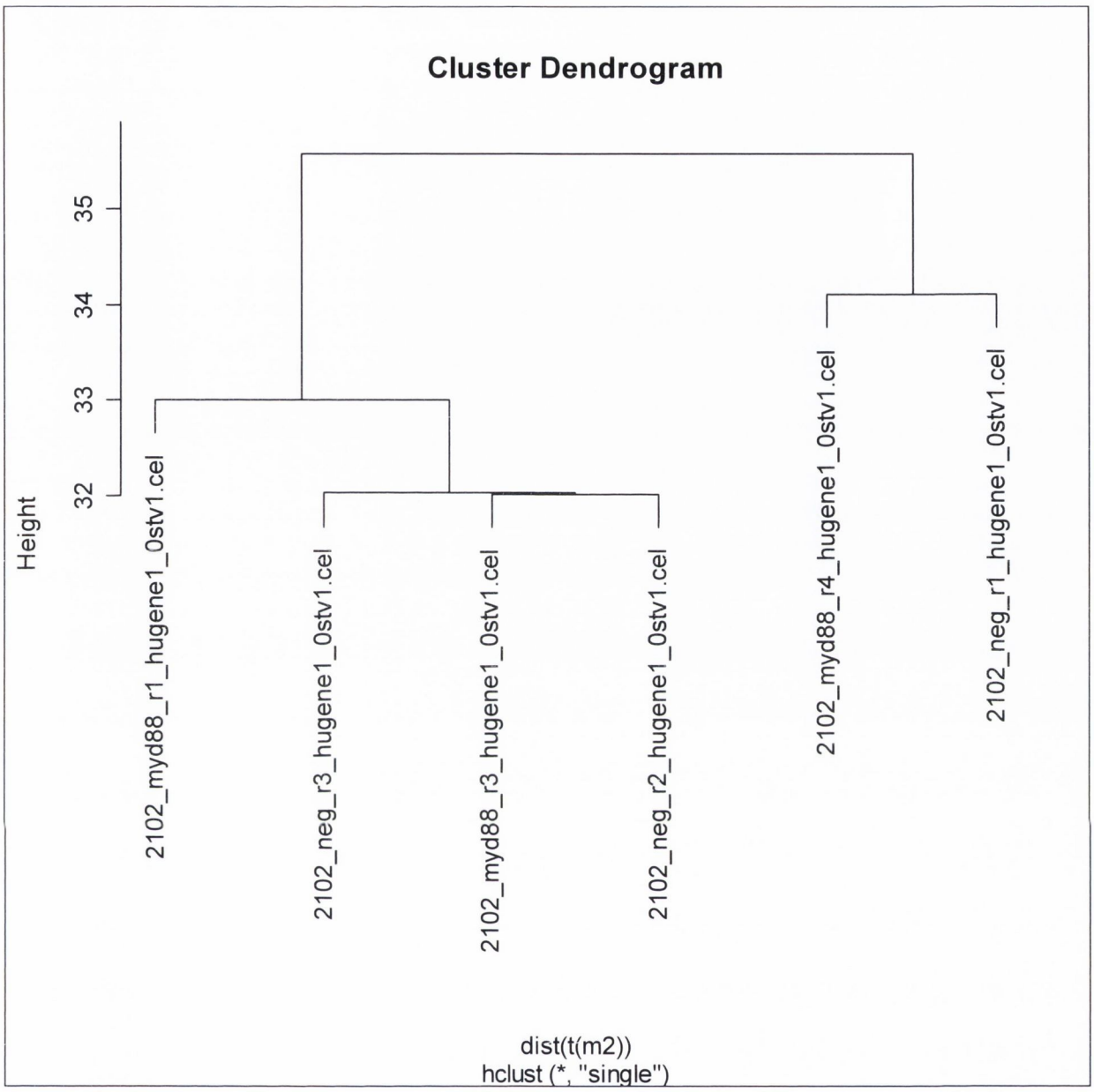

Figure 5.3: Cluster Dendrogram of MyD88 and Negative Control siRNA treated 2102Ep cells. Dendrogram was created from hierarchical clustering of the transcriptional profiles of normalised, background corrected data. In two distinct treatments such as these we would expect to see two distinct groupings, however in this case samples from both treatments are mixed together. The length of the "branches" denotes differences between groups. The branches here are short, demonstrating an unusually high level of commonality between both sets of treatments and thus all samples.

Microaray data was analysed using the XRAY version 3.99 software from Biotique Systems Inc (Reno, NV, USA). The p-value (0.05 or 0.01) has been used traditionally to control the false positive rate. Its interpretation is that from 100 tests, 5 tests would nevertheless be significant in the case of p=0.05 (i.e. false positives). Although useful in most experiments, the use of the same p-value on a microarray with 30,000 genes would yield 1,500 false positive genes, which is an unacceptable amount of false positives (Glantz, 1996). Because of this several different methods have been devised including those of Bonferroni and Sidak. However these two are generally considered to be too conservative and to have low power in microarray experiments. Instead the Benjamini and Hochberg False Discovery Rate (FDR) method outlined by Benjamini and Hochberg and originally proposed by R. J. Simes that controls the family wide error rate in a weak sense was used (Benjamini & Hochberg, 1995; Simes et al., 1986).

Fold changes for detected probes were also calculated. Fold change compares the expression level of the same gene in both groups and assesses the degree to which a gene is up- or down-regulated in one group compared to the other. Table 5.2 shows the top ten altered genes in three biological replicates of 2102Ep cells treated with MyD88 siRNA compared to three biological replicates of 2102Ep cells treated with a negative control. In order to generate this data lists were sorted by FDR in ascending order. The genes listed are thus the genes with the highest statistically significant expression change. As can be seen in Table 5.2 differentially expressed genes from this experiment had significances ranging from 0.218 to 0.412. These are above the parameters normally used as limits for statistical significance. Additionally the fold changes of these genes are all less than 2 fold, which is the minimum fold change deemed biologically significant. When comparing three biological replicates of MyD88 siRNA treated cells with negative control siRNA treated cells there is no statistical or biologically significant change in gene expression.

Table 5.1: Top ten gene expression changes in 3 biological replicates of MyD88 siRNA treated cells relative to 3 biological replicates of Negative Control siRNA treated 2102Ep cells sorted by FDR

Gene	Fold Change	FDR
cdkn2d	1.652	0.218
nek2	1.184	0.237
vars	1.116	0.293
znf584	1.084	0.299
kiaa1967	1.232	0.303
vars	1.116	0.323
nrf1	1.099	0.337
tns4	1.215	0.355
gyltl1b	1.071	0.400
rac3	1.408	0.412

This lack of significant change in gene expression when comparing three biological replicates may be a product of the high levels of similarity between samples observed in Figures 5.5 and 5.6 In order to further investigate this it was decided to compare two biological replicates of MyD88 siRNA treated cells with two biological replicates of Negative Control treated cells. The biological replicates chosen were the two sets furthest away in the cluster dendrogram (Figure 5.6). Again this list was sorted first by FDR. In this comparison, only four genes had a FDR less than 0.1. These are listed in Table 5.3. As in the three by three comparison, no genes had a fold change higher than 2.0. That said, it may be the case that proteins involved in signal transduction may undergo only minor gene expression changes that can have larger downstream effects.

MyD88 was notably absent from the list of differentially expressed genes. This was despite the fact that MyD88 knockdown of 85% had been verified by qPCR assay prior to performing the Affymetrix arrays. To clarify, a colleague repeated the qPCR on the same

samples, and verified MyD88 knockdown of 85% for each sample. The reason why MyD88 is not present on the gene array is unknown, all quality control parametrics were clear, and both Affymetrix technical support, and in-house bioinformatics were satisfied that the array protocol had been carried out successfully.

Four genes were differentially expressed in this experiment, although all four had significance less than 0.1. INO80E is the e subunit of the INO80 complex. This complex is involved in chromatin remodelling in the prevention of polyploidy (Conaway & Conaway, 2009). There is no prior evidence of a link between INO80 and MyD88 expression. GSTT2 is a protein that defends against oxidative stress. It catalyses the conjugation of reduced glutathione to a variety of electrophilic and hydrophobic compounds (Petermann et al., 2009). There is no prior link between MyD88 and GSTT2 expression. HDAC8 is known to be involved in neuroblastoma differentiation and has no prior link with MyD88 (Oehme et al., 2009). Finally, TSPAN2 belongs to a family of signal transduction proteins that regulate cell development, activation, growth and mobility (Lafleur, Xu, & Hemler, 2009). As with the previous genes, there is no prior association between TSPAN2 and MyD88.

Table 5.2: Significant gene expression in 2 biological replicates of MyD88 siRNA treated cells relative to 2 biological replicates of Negative Control siRNA treated 2102Ep cells.

Gene	Fold Change	FDR
ino80e	1.1688	0.0921
gstt2	1.1568	0.0994
Hdac8	-1.0802	0.0968
tspan2	-1.4203	0.0937

5.3.2 Regulation of non-coding RNAs by MyD88 in 2102Ep cells.

MyD88 expression was downregulated in 2102Ep cells via siRNA transfection as per the protocol described in Section 2.12. RNA was isolated from these cells using the mirVana RNA isolation kit as per manufacturer's instructions. These samples were analysed via Applied Biosystem's Sequencing by Oligonucleotide Ligation and Detection (ABI SOLiD) system. MyD88 siRNA transfected samples were compared to siNegative control transfected samples. This allowed for the detection of both up and downregulated non-coding RNAs including microRNAs (miRNAs), long-non-coding RNAs (lncRNAs) and small nucleolar RNAs (snoRNAs).

In contrast to the similarity observed at the gene expression level, substantial differences between ncRNA levels were observed. Comparison of RNA from siNegative treated cells with siMyD88 treated cells yielded a discrete list of ncRNAs that were differentially expressed (Table 5.4). The short length of this list implies that knockdown of MyD88 affects only a limited number of ncRNAs, and that changes in ncRNA expression are tightly regulated in this system. Notably absent from this list, are microRNAs known to be involved in TLR 4/MyD88 signalling: mir21 and mir146.

Table 5.3: Non-coding RNAs differentially expressed between siNegative and siMyD88 treated 2102Ep cells.

Upregulated NcRNA		Downregulated ncRNA	
ncRNA	**Fold Change**	**ncRNA**	**Fold Change**
LncRNA RP11-763E3.1	306.9	LcnRNA RP11-307E17.8	-333.3
MIR1180	111.7	AC010745.4	-43.5
MIR320A	55.5	MIR130A	-38
MIR744	6.2	AL157827.2	-25
MIR7-3	4.5	RP4-798A10.5	-16.7
MIR769	4.2	NEAT1	-9.9
MIR498	3.6	MIR519E	-7.4
MIR125B2	3.6	AL132988.3	-7
MIR573	3.5	CTD-2029E14.1	-4.7
MIR519B	3.5	SNORA26	-3.9
MIR494	3.2	RP4-550H1.6	-3.7
RNU12	3	SNORA42	-3.5
MIR27B	2.7	RP11-766N7.3	-3.4
MIR1296	2.6	MIR9-1	-3.2
MIR1272	2.3	MIR184	-3.1
SNORD94	2.3	SNORD101	-2.9
MIR526A2	2.2	MIR524	-2.8
MIR491	2.2	SNORA66	-2.8
MIR181B1	2.1	MALAT1	-2.6
MIR124-3	2.1	SNORA71D	-2.6
RMRP	2.1	SNORD116-24	-2.4
SCARNA12	2.1	SNORD42A	-2.2
MIR589	2	SNORD116-23	-2.1
MIR877	2	MIR29A	-2
MIR1301	2	SNORD59A	-2
MIR33B	2		

The top ten upregulated non-coding upregulated RNAs are shown in Table 5.5 along with their fold changes. miRNAs were primarily upregulated in MyD88 siRNA treated cells.

Specific examples of biological interest include miR-1180, which is linked to chemo-resistance in breast cancer (Cittelly et al., 2010) and miR-320, which is a regulator of PTEN controlled protein secretion in the breast cancer microenvironment (Bronisz, Godlewski, & Wallace, 2011). This intriguing result is strong evidence for an involvement of TLR-MyD88 as modulators of (PTEN-controlled) protein secretion in the tumour microenvironment. Also upregulated are miR-774, which is a regulator of TGF-β signaling, miR-125b2, the deletion of which is linked to lung cancer (Nagayama et al., 2007), and miR-7-3, a member of the miR-7 family that has links to numerous cancers.

Table 5.4: Top 10 non coding upregulated RNAs

Gene Name	Fold Change
LncRNA RP11-763E3.1	306.9
MIR1180	111.7
MIR320A	55.5
MIR744	6.2
MIR7-3	4.5
MIR769	4.2
MIR498	3.6
MIR125B2	3.6
MIR573	3.5
MIR519B	3.5

Non-coding RNAs that were downregulated in siMyD88 treated 2102Ep cells are listed in Table 5.6 alongside their fold change. Downregulated small RNA species have not been previously described due to their recent discovery. One exception is miRNA-130a, which has shown to be involved in cervical cancer (Lui et al 07) and also in embryonic stem cells (Suh et al 04). Thus, second generation sequencing has highlighted a collection of novel MyD88-regulated small RNA species.

Table 5.5: Top 10 non coding downregulated RNAs

Gene Name	Fold Change
LcnRNA RP11-307E17.8	-333.3
AC010745.4	-43.5
MIR130A	-38
AL157827.2	-25
RP4-798A10.5	-16.7
NEAT1	-9.9
MIR519E	-7.4
AL132988.3	-7
CTD-2029E14.1	-4.7
SNORA26	-3.9

5.3.3 RayBiotech Array Results

5.3.3.1 TNF-α ELISA on hEC media samples.

In chapter 3 cells were treated with a range of treatments and changes of TLR4-MyD88 expression were monitored. Media from those treatments was collected and stored frozen for chemokine cytokine analysis. In order to test that the protein concentration in the hEC media samples was appropriate for the protein arrays, a TNF-α ELISA was performed on a representative set of samples. Results from this experiment showed that protein concentrations were at the minimum levels for the TNF-α ELISA to detect (Figure 5.4). This was resolved by passing all samples through a Amicon Ultracel spin filter, to increase protein concentration.

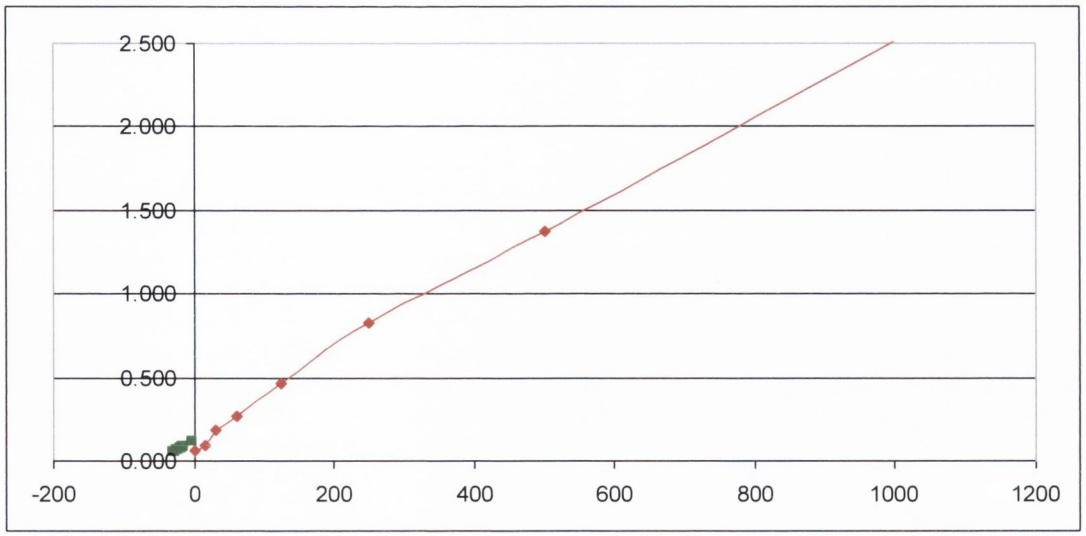

Figure 5.4: TNF-α concentration in chemokine and cytokine media samples. The standard curve generated by manufacturer supplied standards is shown in red, while representative data from chemokine/cytokine array samples is shown in green.

In order to increase the concentration of chemokines and cytokines in the media to a level detectable by the protein arrays, all samples were centrifuged through Amicon Ultracel 3kDa spin filters. 500µl of each sample was added to a filter and centrifuged at 14,000g for 30 seconds. The filter was removed and placed in reverse in a fresh sample tube. This was then centrifuged at 1,000g for 10 seconds. In this way the concentration of proteins within each sample was increased by a factor of 20. Samples were concentrated to the minimum volume required for chemokine/cytokine analysis, which maximised detection potential.

5.3.3.2 Interpretation of RayBiotech Arrays

Banked media samples were loaded on the RayBiotech protein array slides as per manufacturer's protocol and scanned using a GenePix Scanner. The results file produced was then analysed using RayBiotech provided software. Unfortunately, this revealed high levels of variability between biological replicates (Figure 5.5). Accordingly it was decided to present the data as qualitative rather than quantitative. In keeping with accepted standards for RNA data, a chemokine was defined as increased if there was a greater than

two fold difference between the mean of the treated biological samples, and the mean of untreated samples. In Figure 5.5 therefore, Activin A, ANG and Angiostatin were defined as increased. Full raw data for the protein arrays can be found in Appendix I.

(pg/ml)	2102 1	2102 2	2102 3	2102 Cis 1	2102 Cis 2	2102 Cis 3
Activin A	46.9	0.0	0.0	445.8	337.5	119.0
AgRP	0.0	0.0	7.2	15.0	10.9	8.5
ANG	458.5	606.7	655.7	1261.0	1263.6	1158.7
ANG-1	201.9	521.9	117.4	456.0	493.0	0.0
Angiostatin	559.4	774.9	641.6	983.1	1657.3	1453.2
Catheprin S	11.3	20.4	4.9	27.0	70.3	30.4

Figure 5.5: Output from RayBioTech Software

5.3.3.3 Chemokine and Cytokine profile of cisplatin treated CSCs.

In Section 3.3.2, cells were treated with cisplatin for three days and changes of expression of TLR4-MyD88 were monitored. At that time the media from these treatments was collected and stored frozen for chemokine cytokine analysis. These samples were assessed for changes in concentration of a panel of chemokines and cytokines using protein array technology.

Chemokines and cytokines whose concentration was affected by cisplatin treatment are shown in table 5.6 (2102Ep) and 5.7 (NTera2). Twenty-eight proteins were altered in 2102Ep cells, twenty-six of which were at higher concentrations in cisplatin treated cells while the remaining two were at lower concentrations in treated cells. This indicates that 2102Ep cells alters secretion of these proteins in response to cisplatin treatment. This list largely consisted of growth factor pathway and receptor proteins (Table 5.6). Specifically Bone Morphogenic Protein (BMP), Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor Beta (TGFβ) growth factor pathways were upregulated. Fourteen proteins were altered in NTera2 cells, twelve of which were increased and two of

which were decreased in cisplatin treated cell samples. This indicates that NTera2 cells respond to cisplatin treatment by increasing secretion of NT-4 and TGF-α, while decreasing secretion of NT-3. Alteration of neurotrophin growth factors NT-3 and NT-4 is consistent with NTera2 differentiation mechanisms, which bias neuronal development. Increased secretion of TGF-α is similarly consistent with our groups NTera2 differentiation data which has demonstrated differential alteration of TGF signalling in NTera2 versus 2102Ep cells. Comparing 2102Ep to NTera2 cells, the data demonstrate common (NT-4), opposite (NT-3) and 2102Ep-specific and NTera2-specific protein secretion. These specific profiles concur with alternate TLR4-MyD88 gene expression profiles described in Chapter 3 which will be discussed in section 5.5. The presence of receptor molecules in samples secreted by the cell appears contradictory. However secretion of receptors has been described in several cell types as will be discussed in section 5.4.

Table 5.6: Chemokines and cytokines secreted by cisplatin treated 2102Ep cells compared to untreated 2102Ep cells.

Increased	Decreased
Activin A	GROa
ANG	TIMP-4
Angiostatin	
BMP-7	
DKK-1	
E-Cadherin	
EG-VEGF	
Follistatin	
Galectin-7	
ICAM-2	
LAP	
MCF R	
MMP-10	
NSE	
NT-3	
NT-4	
OPG	
PDGF-AB	
sgp130	
Siglec-5	
TGF-b2	
TGFb3	
TPO	
VEGF	
VEGF R1	
VEGF R3	

Table 5.7: Chemokines and cytokines secreted by cisplatin treated NTera2 cells compared to untreated NTera2 cells.

Increased	Decreased
ANG-1	MMP-2
Galectin-7	NT-3
IL-13 R1	
IL-13 R2	
IL-2 Rb	
NT-4	
PDGF-AB	
Procalcitonin	
sgp130	
Siglec-5	
TGFa	
TIMP-4	

5.3.3.4 Chemokine Cytokine profile of retinoic acid treated CSCs

In section 3.3.4, both 2102Ep and NTera2 cells were treated with retinoic acid for three days and characterisation of the TLR4-MyD88 response was performed. Media was retained from these experiments and stored frozen for chemokine cytokine analysis. These samples were tested for changes in chemokine cytokine profile relative to media from untreated cells using protein array technology.

Chemokines and cytokines whose concentration was affected by retinoic acid treatment are shown in Table 5.8 (2102Ep) and Table 5.9 (NTera2). Fourteen different chemokines and cytokines were increased in retinoic acid treated 2102Ep cells while none were decreased (Table 5.8). This indicates that 2102Ep cells respond to differentiation stimulus via retinoic acid by increasing secretion of these proteins. Proteins upregulated included several different growth factors involved in regulation of neovascularisation (ANG, ANG-1 and

Angiostatin). NTera2 cells responded to retinoic acid treatment by increasing secretion of fourteen chemokine and cytokines and decreasing secretion of ten. Despite the fact that 2102Ep cells resist differentiation via retinoic acid while NTera2 cells do not, there is some overlap in their secretory profile. Seven chemokine and cytokines are common between the two, which would suggest that it is the remaining seventeen unique changes that account for the differing response. As previously, some receptors were found in the secreted protein profile in both 2102Ep and NTera2 samples. This will be discussed further in section 5.5.

Table 5.8: Chemokines and Cytokines secreted by retinoic acid treated 2102Ep cells compared to untreated 2102Ep cells.

Increased
ANG
ANG-1
Angiostatin
DAN
E-Cadherin
ICAM-2
IL-13 R1
LAP
NSE
PDGF-AB
Thyroglobulin
TPO
TREM-1
VEGF R1

Table 5.9: Chemokines and cytokines secreted by retinoic acid treated NTera2 cells compared to untreated NTera2 cells.

Increased	Decreased
ANG	B2M
ANG-1	Cripto-1
ANGPTL4	Follistatin
E-Cadherin	MMP-2
ICAM-2	NCAM-1
IL-13 R1	PAI-I
IL-13 R2	Procalcitonin
IL-2 Rb	Resistin
LAP	TIMP-4
NSE	VEGF R1
PDGF-AB	
sgp130	
TGFa	
TPO	

5.3.3.5 Chemokine Cytokine profile of hypoxia treated CSCs

In section 3.3.6, both 2102Ep and NTera2 cells were grown in hypoxic conditions ($0.5\%O_2$) and characterisation of the TLR4-MyD88 response was performed. Media was retained from these experiments and stored frozen for chemokine cytokine analysis. These samples were tested for changes in chemokine cytokine profile relative to media from cells grown in normoxic conditions using protein array technology.

Chemokines and cytokines altered by cells grown in hypoxia are shown in Table 5.10 (2102Ep) and Table 5.11 (NTera2). Secretion of nineteen chemokines and cytokines was increased by 2102Ep cells in response to hypoxia while secretion of three chemokines and cytokines were decreased (Table 5.10). Amongst those altered were growth factor pathways involved in neovascularisation as well as differentiation regulation. There were

five chemokines and cytokines whose concentration was increased in both hypoxia and cisplatin treated 2102Ep cells (Table 5. 10 and Table 5.6). These were ANG, Angiostatin, LAP, NT-3 and NT-4. This could suggest these cytokines as a specific stress response in 2102Ep cells.

Secretion of fifteen chemokines and cytokines was increased by NTera2 cells in response to hypoxia while secretion of ten chemokines and cytokines were decreased (Table 5.11). Alterations included those to growth factors and chemokines involved in the immune response such as Ferritin. Increase in Ferritin production has been shown to be associated with infection. As with 2102Ep cells there was limited overlap between chemokines and cytokines implicated in the NTera2 response to both hypoxia and cisplatin (Table 5.11 and 5.7). Concentrations of three proteins were shown to be increased in both treatments, these were IL-13R2, NT-4 and TIMP4.

Table 5.10: Chemokines and cytokines secreted by hypoxia treated 2102Ep cells compared to untreated 2102Ep cells.

Increased	Decreased
ANG	b-NGF
Angiostatin	E-Cadherin
BCAM	MMP-3
BMP-5	
FGF-4	
hCGb	
HGF	
IGFBP-3	
IGF-I	
IL-17B	
LAP	
NGF R	
Nidogen-1	
NT-3	
NT-4	
Resistin	
SCF	
TPO	
VEGF R2	

Table 5.11: Chemokines and Cytokines Secreted by hypoxia treated NTera2 cells compared to untreated NTera2 cells.

Increased	Decreased
Adipsin	BMP-7
ANGPTL4	Cripto-1
BCAM	DKK-1
CA125	Follistatin
EG-VEGF	Galectin-7
Ferritin	LAP
GROa	NCAM-1
hCGb	PDGF-AB
IL-13 R2	TGFa
IL-21	VEGF R1
MMP-9	
NT-3	
NT-4	
Procalcitonin	
TIMP-4	

5.3.3.6 Chemokine Cytokine profile of retinoic acid pre-treated cisplatin treated CSCs

In section 3.3.7, both 2102Ep and NTera2 cells were pre-treated treated with retinoic acid for three days prior to treatment with cisplatin, and characterisation of the TLR4-MyD88 response was performed. Media was retained from these experiments and stored frozen for chemokine cytokine analysis. These samples were tested for changes in chemokine cytokine profile relative to media from cells grown in normal media.

Chemokines and cytokines altered by cells pre-treated with retinoic acid before being treated with cisplatin are shown in Table 5.12 (2102Ep) and Table 5.13 (NTera2).

Secretion of nine chemokines and cytokines was increased by 2102Ep cells in response to cisplatin following retinoic acid pre-treatment while secretion of seven chemokines and cytokines were decreased (Table 5.12). Amongst those altered were growth factor pathways involved in neovascularisation as well as differentiation regulation. Of those nine which were increased, six were chemokines and cytokines that showed no alteration in either retinoic acid or cisplatin treatment alone. Similarly all seven which were decreased were unique to the combination treatment.

Secretion of eight chemokines and cytokines was increased by NTera2 cells in response to cisplatin following retinoic acid pre-treatment while secretion of seven chemokines and cytokines were decreased (Table 5.13). There was no overlap in secretory profile between 2102Ep and NTera2 cells, which may reflect there different pluripotencies. However, similar to 2102Ep cells, the secretory profile of combination treated NTera2 cells is substantially different to either treatment in isolation. Only NT-3, concentration of which is decreased in combination treated NTera2 cells, is common between combination treatments and NTera2 cells treated with cisplatin alone. All other of the secretory alterations are unique to NTera2 cells pre-treated with retinoic acid followed by cisplatin.

Table 5.12: Chemokines and cytokines secreted by retinoic acid pre-treated cisplatin treated 2102Ep cells compared to untreated 2102Ep cells.

Increased	Decreased
Adipsin	DKK-1
BCAM	CRP
BMP-7	EG-VEGF
Catheprin S	Prolactin
IL-2 Rb	TACE
MMP-13	TGFa
NT-4	TGF-b2
Resistin	
sgp130	

Table 5.13 Chemokines and cytokines secreted by retinoic acid pre-treated cisplatin treated NTera2 cells compared to untreated NTera2 cells.

Increased	Decreased
CRP	Activin A
DAN	AR
DKK-1	MMP-10
EG-VEGF	NT-3
MMP-1	PDGF-AB
NCAM-1	TACE
Prolactin	
Thyroglobulin	

5.3.3.7 Chemokine Cytokine profile of 3 day MyD88 siRNA transfected 2102Ep cells

In section 4.3.1, MyD88 was knocked down in 2102Ep cells via siRNA. Media was retained from these experiments and stored frozen for chemokine cytokine analysis. These samples were tested for changes in chemokine cytokine profile relative to media from cells treated with Negative Control siRNA using protein array technology.

Chemokines and cytokines altered by 2102Ep MyD88 knockdown cells are shown in Table 5.14. Secretion of eleven chemokines and cytokines was increased by these cells, there were no decreases in secretion. As previously, many of those increased have functions in cell growth factor pathways. Six of the altered proteins are unique to the MyD88 knockdown treatment (i.e. they were not increased in response to any other treatment) suggesting possible specificity. The six which are unique to this treatment are: ANGPTL4, b-NGF, EG-VEGF, Procalcitonin, Siglec-9 and TREM-1.

Table 5.14: Chemokines and cytokines secreted by MyD88 siRNA transfected 2102Ep cells compared to Negative siRNA transfected 2102Ep cells.

Increased
ANGPTL4
b-NGF
DKK-1
EG-VEGF
Galectin-7
IL-17B
Procalcitonin
Siglec-9
TGFb3
Thyroglobulin
TREM-1

5.3.3.8 Chemokine Cytokine profile of 6 day MyD88 siRNA transfected 2102Ep cells

In section 4.3.4, MyD88 was knocked down in 2102Ep cells via siRNA for six days. Media was retained from these experiments and stored frozen for chemokine cytokine analysis. These samples were tested for changes in chemokine cytokine profile relative to media from cells treated with Negative Control siRNA using protein array technology.

Chemokines and cytokines altered by 2102Ep MyD88 knockdown 6 day cells are shown in Table 5.15 Secretion of six chemokines and cytokines was increased by these cells, while secretion of seven chemokines and cytokines was decreased. This is strikingly different to cells treated with MyD88 siRNA for just three days in which no decrease in concentration was detected (Table 5.14). The only chemokine in common between the two different time points is Procalcitonin, concentration of which is increased in both 3day and 6day MyD88 siRNA treatment (Table 5.14 and 5.15). The secretory profile of MyD88 siRNA can thus be said to be substantially altered, depending on the time point at which it is assessed.

Table 5.15: Chemokines and cytokines secreted by 2102Ep cells transfected with siMYD88 for six days compared to 2102Ep cells transfected with siNegative for six days.

Increased	Decreased
AR	Adipsin
CA15-3	BCAM
Ferritin	BMP-7
IL-21	CA125
Procalcitonin	NSE
VEGF	PDGF-AB
	Siglec-5

5.3.3.9 Chemokine Cytokine profile of 6 day MyD88 siRNA transfected 2102Ep cells treated with retinoic acid

In section 4.3.4 MyD88 was knocked down in 2102Ep cells via siRNA for three days before treating them with retinoic acid. Media was retained from these experiments and stored frozen for chemokine cytokine analysis. These samples were tested for changes in

chemokine cytokine profile relative to media from cells treated with Negative Control siRNA followed by retinoic acid using protein array technology.

Chemokines and cytokines altered by 2102Ep MyD88 knockdown cells are shown in Table 5.16 Secretion of six chemokines and cytokines was increased by these cells, while secretion of five chemokines and cytokines was decreased. Three of the proteins whose concentration increased are not increased in 6day knockdown cells not treated with retinoic acid, suggesting that an increased in concentration of these proteins may be linked with the loss of pluripotency seen in previous results (Section 4.4.4).

Table 5.16: Chemokines and cytokines secreted by 2102Ep cells transfected with siMYD88 for six days and treated with retinoic acid compared to 2102Ep cells transfected with siNegative for six days and treated with retinoic acid.

Increased	Decreased
AR	ICAM-2
Ferritin	IL-13 R1
GROa	IL-13 R2
Procalcitonin	IL-17B
Prolactin	
TIMP-4	

5.4 Discussion

In Chapters 3 and 4, MyD88 was characterised and functionally assessed in two cancer stem cell lines. Following on from this work, the aim of this chapter was to utilise all three technologies: Affymetrix Array, SOLiD sequencing and Ray Biotech Protein Arrays to provide a full downstream analysis of the effects of MyD88 knockdown at the gene, miRNA and protein level. A further aim in the case of the Ray Biotech arrays was to provide a full secretory profile of 2102Ep and NTera2 cells in response to a range of stimulus.

Affymetrix array data described in Section 5.3.1 shows no significant difference in gene expression between 2102Ep cells treated with MyD88 siRNA and those treated with Negative Control siRNA when comparing three biological replicates. If the comparison is reduced to two biological replicates we obtain only four genes whose expression is altered with a less stringent FDR of <0.1: these genes are altered less than two fold. These results are highly unexpected, leading to external examination by bioinformatics collaborators from the Higgins group in University College Dublin. Quality control data from the arrays was also examined independently by technicians from Affymetrix, who concluded that there was no issue with the quality of the arrays. Extensive analysis by the Higgins group could find no alternative method of analysis that would generate an improved genelist without manipulating the data beyond recognition.

From this then, we can conclude that 2102Ep cells treated with MyD88 siRNA and those treated with negative control siRNA show no significant substantial differential gene expression under these experimental conditions. There are several possibilities as to why this could occur. One possibility is that cells treated with MyD88 siRNA experience alterations of gene expression that are too subtle for the Affymetrix array technology and

software to detect. While convention has set the limit for gene expression changes to be considered biologically significant at +/- 2fold, current thinking is that in the case of signalling pathway modulators, subtle changes (i.e. less than 2 fold in either direction) can cause much greater downstream effects. Alteration in MyD88 levels may also have post translational effects that would be undetectable using gene arrays such as phosphorylation of downstream proteins or miRNA interference. Although it is an unusual finding, all data appear to have been correctly analysed and indicate that 85% loss of MyD88 does not cause significant changes in downstream gene expression, even though it is sufficient for a functional effect on the RA-induced differentiation response of 2102Ep cells.

The data generated by SOLiD sequencing shows that in the 2102Ep cells which have MyD88 knocked down by a minimum of 85%, there are specific, significant alterations in expression of ncRNAs. These include both ncRNAs that are upregulated as well as downregulated, and range from well-studied miRNAs to those that are virtually unknown. In the case of both upregulation and downregulation the two most altered ncRNAs are both unstudied (LncRNA RP11-763E3.1 and LcnRNA RP11-307E17.8 respectively). Both of these were highly altered between MyD88 knockdown cells and negative controls suggesting a novel role downstream of MyD88 signalling. The role of LncRNAs is poorly understood. However, it is already understood that LncRNAs have important regulatory roles in cells. For example, LncRNAs have been shown to regulate pluripotency master gene Oct4 (Baker 2011). The ncRNAs identified in this study are now available for future analysis into their role in cancer, stem cells and cancer stem cells. Another interesting feature of these data is the lack of well-established ncRNAs which were present in the data sets. For example mir21 and mir146 are well established regulators of TLR4MyD88 signalling, however they were conspicuously absent from the list of altered ncRNAs. This observation further emphasises the novel nature of this data, and emphasises the potential

impact that these findings will have on future research directions. The distinct alterations observed at the ncRNA level further support the hypothesis that alteration of MyD88 expression in these cells has downstream post translational effects, rather than downstream transcriptional effects.

The chemokine and cytokine array data presented here, describes for the first time the secretory profile of a nullipotent and pluripotent cell line in response to a range of treatments, as well as the secretory profile of 2102Ep cells with MyD88 knockdown cells for both three and six days, and in retinoic acid. These data are invaluable, and comparisons between them will allow for the identification of novel relationships between treatments and chemo-/cytokines. These will be discussed briefly here, and in greater detail in Chapter 6. When comparing the NTera2 response to both hypoxia and cisplatin a number of cytokines are common to both. These include IL-13Receptor 1, NT-4, Procalcitonin, and TIMP-4. Common increase in secretion in response to such different treatments as cisplatin and hypoxia, may suggest a common, general stress response for the cell line. Interleukin 13 (IL-13) is a protein known to have anti-inflammatory effects *in vivo* (Townley et al., 2011). Increase in secretion of its receptor may act to block its action, thus having a net pro-inflammatory effect. This would be consistent with current thinking on inflammation's role in oncogenesis (i.e. that inflammatory conditions are beneficial to the proliferation of cancer cells).

In addition to comparisons between treatments in the same cell line, comparisons between cell lines may also lead to interesting conclusions. For example in NTera2 cells treated with retinoic acid secretion of a number of proteins is reduced, unlike 2102Ep cells which respond to retinoic acid only by increasing secretions. One of the proteins whose secretion is decreased by NTera2 cells is Cripto-1, a protein thought to play a role in the

maintenance of the stem like state (Bianco et al., 2010). Downregulation such as this would be consistent with its known function. Cripto-1 has been previously shown by the O'Leary group to be one of the one of the highest altered genes when comparing three day differentiated NTera2 cells with undifferentiated (M Gallagher, unpublished data).

As mentioned in Section 5.3, secretion of receptors was common to all treatments. Historically, it was assumed that receptor proteins would only be encountered at the cell surface and not secreted. Recent work however has reported secretion of soluble receptors (Jung et al., 2012). Secreted VEGF receptors 1 and 2 has previously been described in endothelial cells, however this is the first time such secretions have been documented in a cancer stem cell model. The functions of these secreted receptors are as yet poorly understood however it is thought that they act to bind and sequester VEGF (Barleon et al., 2001; Jung et al., 2012; Stachon et al., 2009). In the CSC context, this may present an interesting putative mechanism whereby receptor proteins are secreted by these hEC cells to act as biochemical regulators. Such secreted receptors have been proposed to act as chaperones, to stabilise ligand molecules, or to bind to ligands or receptors to compete for or inhibit ligand-receptor binding. In this context, the high level of receptor secretion observed as a result of these treatments, is of substantial interest.

The data presented here demonstrate that it was possible to identify MyD88-specific downstream events. SOLiD sequencing data demonstrates that MyD88 knockdown led to specific alterations in ncRNAs. Furthermore alterations in MyD88 levels led to changes in the concentrations of chemokines and cytokines in the media, which demonstrates that the MyD88 pathway is functional and regulating the cells at high levels. The characterisation of these specific molecular events is now available for further study by the O'Leary lab, which is an important legacy of this work. This chapter integrates with chapters 3 and 4 to

provide a clearer understanding of the MyD88 downstream regulatory network. This will be presented in the general discussion

Chapter Six

General Discussion

6.1 The hEC response to cisplatin

The data presented here define the response of two different CSC cell lines to a range of challenges both in terms of their TLR4/MyD88 response, and alteration in chemokine and cytokine secretion. In this chapter these results will be analysed, both in terms of similarities and contrasts between treatments, and between cell lines.

2102Ep cells decreased expression of MyD88 only in response to treatment with cisplatin (Figure 3.3). MyD88 downregulation was shown by functional analysis in 2102Ep cells to be non-functional for cell survival in cisplatin (Figure 4.5). 2102Ep cells treated with cisplatin resulted in substantial increase in chemokine and cytokine secretion, as well as decrease in secretion of two proteins GROa and TIMP4 (Table 5.6). In contrast to this response, NTera2 cells upregulated both TLR4 and MyD88 in response to treatment with cisplatin (Figure 3.3). Overexpression of MyD88 in these cells had no effect on cell survival in cisplatin (Figure 4.10). At the chemokine and cytokine level, there was both increase and decrease in secretion of specific proteins (Table 5.7). When comparing both cell lines, there were five common secretory changes in common between them. These were: Galectin-7, NT-4, PDGF-AB, sgp130 and siglec-5. These may suggest a common hEC response to challenge with cisplatin. In overview, the collective data indicate that TLR-MyD88 signaling is involved in the cisplatin-response of hEC cells and suggest that the downstream effect is the expression of an altered profile of chemokines and cytokines. It is likely that this protein profile contributes to hEC chemo-resistance. As such, targeting this mechanism may be essential for improved cancer treatments.

6.2 The hEC response to retinoic acid

2102Ep cells decreased expression of both MyD88 and TLR4 in response to treatment with retinoic acid (Figure 3.6). Knockdown of MyD88 in these cells made them susceptible to differentiation via retinoic acid (Figure 4.7). 2102Ep cells treated with retinoic acid increased expression of fourteen different chemokines and cytokines in response to treatment with retinoic acid (Table 5.8). Similarly NTera2 cells downregulated both TLR4 and MyD88 in response to retinoic acid treatment (Figure 3.6). Functional experiments showed that in NTera2 cells if MyD88 was overexpressed the NTera2 cells acquired nullipotency (Figure 4.12). NTera2 cells increased secretion of fourteen chemokines and cytokines and decreased secretion of ten in response to retinoic acid treatment (Table 5.9). There is some overlap between the secretory profiles in both cell lines. Uniquely, 2102Ep cells increased secretion of Angiostatin, DAN, Thyroglobulin, TPO, TREM-1, VEGF R1 which may suggest an association between their increase and maintenance of pluripotency. In contrast, NTera2 increased expression of ANGPT-4, IL-13R2, IL-2Rb, sgp130, TGFa, and TPO uniquely. This may suggest a role for them in regulating retinoic acid mediated differentiation. Decreased secretion of ten chemokines and cytokines may suggest a role for them in maintenance of pluripotency. In overview, the identification of MyD88 as a gatekeeper of hEC retinoic acid-induced differentiation is a significant stem cell discovery. Pluripotency is highly topical presently, with increased interest in induced pluripotent stem (iPS) cell technology in particular. It has been known for some time that pluripotency if governed by a concert of Oct4, Sox2 and Nanog. However, the upstream regulators of these regulatory proteins have eluded identification. This study has identified MyD88 as a likely upstream regulator of Oct4, Sox2 and/or Nanog. Publication of this mechanism is likely to have dramatic effects on iPS and embryonic stem cell research across the world.

In Chapter 4, the role that MyD88 plays in hEC pluripotency was demonstrated via knockdown and overexpression in 2102Ep and NTera2 cell lines respectively (Figure 4.7, Figure 4.12). These data demonstrated that MyD88 regulates the differentiation potential of these two cell lines. In its absence, the normally pluripotent 2102Ep cells become nullipotent, and when it is overexpressed, normally nullipotent NTera2 cells become pluripotent. This is of special interest given that unusually, 2102Ep MyD88 knockdown cells are not differentiating spontaneously, which would be the usual response when a factor necessary for maintenance of the self-renewal state was lost. Instead they appear primed for differentiation via retinoic acid. This appears to be in line with the 'Ground State' hypothesis for pluripotency proposed by Silva and Smith (2008). However, a primed pluripotent state has never been achieved *in vitro* without the addition of growth factors to the cell media. To further characterize this novel function for MyD88 in this system, chemokine and cytokine arrays were carried out. To this end, three sets of samples were tested for this characterisation: these were taken from 2102Ep cells three days after MyD88 siRNA transfection (Table 5.14), six days after MyD88 siRNA transfection (Table 5.15), and six days after MyD88 siRNA transfection with the addition of retinoic acid (Table 5.16). Comparison of these samples allows for the drawing of interesting conclusions regarding the functionality of these secreted proteins. Comparing the 3 day post knockdown profile to the six day post knockdown profile it is clear that there only an increase in Procalcitonin is common to both. This suggests that changes in secretions are fluid and not fixed. When profiles for MyD88 knockdown 2102Ep cells with and without retinoic acid are compared, there is some overlap. Increase in secretion of AR, Ferritin and Procalcitonin was maintained in both treatments. If we assume that six day MyD88 knockdown treated cells are primed to differentiate, then any differences between the profile of these cells and those treated with retinoic acid must be involved in this differentiation response. Thus we can say that increased secretion of CA15-3, IL-21 and

VEGF alongside decreased secretion of Adipsin, BCAM, BMP-7, CA125, NSE, PDGF-AB and Siglec 5 are associated with maintenance of this primed state. Furthermore, increase of GROa, Prolactin and TIMP-4 alongside decrease of ICAM-2, IL-13 R1, IL-13R2 and IL-17B are associated with departure from this primed state into differentiation.

6.3 The hEC response to hypoxia

When 2102Ep cells were grown in hypoxic conditions they downregulated expression of both TLR4 and MyD88 (Figure 3.9). When MyD88 expression was knocked down in these same cells, they had improved survival in hypoxia (Figure 4.4). Secretion of nineteen chemokines and cytokines was increased in response to hypoxia treatment while secretion of three was decreased (Table 5.10). In contrast, NTera2 cells displayed no change in gene expression of TLR4 or MyD88 in response to growth in hypoxic conditions (Figure 3.9). Overexpression of MyD88 had no impact on cell survival in hypoxia. NTera2 cells grown in hypoxia increased secretion of fifteen chemokines and cytokines while decreasing secretion of ten (Table 5.11). Increase of secretion of three chemokines and cytokines was common to both cell lines: BCAM, NT-3 and NT-4. LAP secretion was increased in 2102Ep cells but decreased in NTera2 cells which may suggest a role for it in MyD88 mediated cell survival in hypoxia. From the functional analysis in 2102Ep cells, there is an association between downregulation of MyD88 and increase in sell survival in hypoxic conditions.

6.4 Pre-treatment alters hEC responses

2102Ep cells pre-treated with retinoic acid prior to treatment with cisplatin demonstrated no change in TLR4 or MyD88 expression (Figure 3.10). This represents an alteration in the "normal" 2102Ep response to challenge with either retinoic acid or cisplatin alone. This alteration was also seen at the protein level. Secretion of nine chemokines and cytokines

was increased, while secretion of seven chemokines and cytokines were decreased (Table 5.12). Unexpectedly there was little overlap between the combination treatment and the individual treatments. Only three chemokines and cytokines were common to both the combination treatment and retinoic acid alone. These were BMP-7, NT-4 and sgp140, secretion of which was increased. Both EG-VEGF and TGFb2 were differentially regulated between samples, i.e. they were increased in retinoic acid treatment alone and decreased in the case of combination treatments. This suggests that in these cells, pre-treatment affects their ability to respond to chemotherapy. Under normal physiological conditions in an *in vivo* situation, cells will be constantly surrounded by a range of signals. This data suggest that this variation in signal exposure affects the response to chemotherapy, a suggestion which has clinical significance. The alternative secreted protein profiles may reflect the ability of nullipotent hEC cells to resist differentiation and cisplatin treatment more that pluripotent cells.

NTera2 cells pre-treated with retinoic acid experienced no change in TLR4 or MyD88 expression (Figure 3.10). This represented an alteration to their "normal" response to either treatment in isolation. When chemokine and cytokine secretion profiles were analysed they displayed a similar change (Table 5.13). Only PDGF-AB was common to both the combination treatment and cisplatin treatment. However, while its secretion was increased in cisplatin treatment only, in the combination treatments its secretion was decreased. When comparing the secretory profiles of both cell lines in these combination treatments, there are some overlaps. TACE secretion is decreased in both 2102Ep and NTera2 cells in response to combination treatment. Secretion of CRP, DKK-1, EG-VEGF and Prolactin was altered in both cell lines, however in opposite directions.

The opposite combination treatment had a similar effect on 2102Ep cells i.e.: when they were pre-treated with cisplatin before differentiation stimulus they showed no significant change in TLR4 or MyD88 gene expression (Figure 3.11). Similarly when NTera2 cells were treated with cisplatin prior to retinoic acid treatment they altered their "normal" response (Figure 3.11). Unfortunately due to financial and time constraints it was not possible to characterise the downstream effect of this alteration. However, given the alteration seen at the gene level, a substantially different protein secretory profile would be expected. In overview, it is clear that hEC cells can respond to multiple cancer-related stimuli individually, in combination or sequentially. hEC cells do not absolutely commit to one response: they maintain the ability to alter their responses to subsequent stimuli. It is obvious that such a property would be highly advantageous for tumourigenic cells. The targeting of such mechanisms may be important for improved cancer treatments.

6.5 The role of MyD88 in hEC resistance to chemotherapy, differentiation and hypoxia

The question of the nature of the role of MyD88 in hEC responses to chemotherapy, differentiation and hypoxia, is evidently a complex one. This is made clear by comparison between the protein arrays in Chapter 5 and the gene expression data from Chapter 3. If protein array data is compared between treatments which resulted in similar gene expression changes (Retinoic Acid treatment of 2102Ep and NTera2 cells, Figure 3.6B,D decrease in expression of MyD88), it is clear that the similarity between gene expression data for both cell lines, is not mirrored in the protein array data (Tables 5.8 and 5.9). These in turn, are different to the protein secretion profile of 2102Ep cells that have been treated with siMyD88 (Table 5. 14). This demonstrates the complexity of this mechanism. It is not as straightforward as treatment with X causes an increase/decrease in MyD88 expression which in turn causes an increase/decrease in A, B or C secretion. However by careful

comparison between protein array lists, some chemokines can be picked out which seem to be specific to one treatment.

If a comparison is made between the list of chemokines secreted by 2102Ep siMyD88 treated cells, and retinoic acid treated 2102Ep cells a list of chemokines unique to retinoic acid treated cells emerge. These are shown in Table 6.1.

Table 6.1 Chemokine and cytokines unique to retinoic acid treated 2102Ep cells

Increased
ANG
ANG-1
Angiostatin
DAN
E-Cadherin
ICAM-2
IL-13 R!
LAP
NSE
PDGF-AB
TPO
VEGF –R1

Chemokines and cytokines associated with neovascularisation including ANG, ANG-1, Angiostatin and VEGF are amongst those highlighted in this list. This suggests that the differentiation avoidance mechanism utilised by 2102Ep cells when treated with retinoic acid, involves the induction of neo-vascularisation pathways. This is previously unheard of.

Comparisons between lists generated from these experiments, should yield a rich source of future targets for investigation. This is a key legacy of this work. In summary, the role of MyD88 in the differentiation process in hEC cells is a completely novel finding of this work. Alongside this key finding, this work has also produced a rich legacy of future targets for investigation.

6.6 Limitations of this work

The initial approach of this work, to characterise the changes in expression of TLR4 and MyD88 in response to a range of chemotherapeutic, differentiation and hypoxia treatments allowed for large scale assessment of TLR4-MyD88 responses in a time and cost effective manner. This allowed for the selection of target for future functional analysis. However, it is important to be aware that gene expression data alone is not sufficient to determine functionality. To this end, functional experiments were performed, again within the bounds of feasibility within the time and cost restrains of this project. There remains much to be investigated in terms of alternative functional analysis (specifically overexpression of MyD88 in 2102Ep cells, and knockdown of MyD88 in NTera2 cells).

The protein array results obtained were unfortunately, non-quantitative. High levels of variation seen between replicates, indicates that changes in protein secretion are not as readily quantifiable in this system as changes in gene expression. To this end, it was decided to categorise changes in protein secretion as either an increase or decrease relative to control. Similar cut-offs for biological significance were used as those used in gene expression studies i.e.: two-fold increase or decrease was considered significant in terms of biological impact. For the purposes of this project, i.e.: to obtain a novel secretory profile of both cell lines in a range of conditions, this method was sufficient. However, in order to understand the biological mechanisms involved, a quantifiable assessment of secretion levels would be preferable e.g. via ELISA. However for this project, ELISAs were not a viable option, given the novel nature of the secretions.

6.7 Future Work

The Affymetrix Gene Array carried out in chapter 4 indicated a high level of similarity between three day MyD88 siRNA treated cells and Negative Controls. The protein array on these samples demonstrated that there was significant alteration in downstream expression patterns following three day knockdown. Furthermore, these alterations were substantially different again in cells analysed six days after knockdown. These data suggest that further investigation of this mechanism is required. This could be achieved either by using the array system to analyse RNA isolated from cells six days after knockdown, or alternatively by using shRNA to knockdown MyD88 expression in 2102Ep cells, thus creating a MyD88 negative hEC cell line. In this study, six day knockdown samples were not used for two reasons. Firstly, three days knockdown was sufficient to allow differentiation of the cells upon addition of retinoic acid. Secondly, assaying the earlier time point is always more effective in terms of identifying the immediate targets of the gene of interest while later time points will include indirect downstream effects. It appears that the three day knockdown effect may be too subtle for this technology. As such, a six day knockdown that exaggerates the differences between treatments and controls may permit identification of the targets of MyD88 in hEC cells. Affymetrix array analysis comparing this to its parent cell line would allow for the identification of the specific changes mediated by MyD88 in this system.

The results from the functional analysis demonstrate that knockdown of MyD88 improves 2102Ep resistance to hypoxia. Ovarian tumours have a strong association with hypoxic conditions (Seeber et al 2010). Previous work from this laboratory has indicated a strong correlation between MyD88 expression in the tumour as a whole and poor long term survival in ovarian cancer patients (d'Adhemar et al, in preparation). These two sets of data may suggest a disconnect between the importance of MyD88 expression in the cancer stem

cell component of a tumour and in the normal cancer cell population. If MyD88 downregulation increases hypoxia survival in 2102Ep cells, then specific targeting of this via overexpression of MyD88 in the stem cell component represents a therapeutic avenue of considerable interest.

Specific targeting of CSCs has previously been identified as a potential way of improving ovarian cancer survival rates. The data presented here have greatly improved the understanding of CSCs in relation to ovarian cancer and its treatments. TLR4-MyD88 has been shown to be consistently involved in the CSC response to a range of treatments, underlining its importance. Since MyD88 signalling is central to so many pathways in normal cellular function, it may prove that specific targeting of MyD88 directly could be prohibitively impractical *in vivo*. In this case, the legacy of this data is to provide a thorough outline of potential downstream targets, regulated by MyD88 alteration that could be explored as potential treatments. These data from the chemokine and cytokine arrays represent a completely novel set of potential targets. Secretion of receptors in particular were observed across all treatments. These receptors are now identified and the effect of their artificial decrease or increase, can now be investigated *in vitro*.

Other projects currently undertaken in the O'Leary lab include the isolation of stem cells from ovarian cancer cells. Since expression of MyD88 has previously been used to classify epithelial ovarian cells as either a stem-like or non-stem like phenotype (Chen et al 2008), it is possible that MyD88 presence or absence may represent a possible screening method for these CSCs.

6.8 Conclusion

The data presented here represent a thorough characterisation, functional analysis and downstream analysis of MyD88 signalling in CSCS in the context of ovarian cancer. For the first time MyD88 has been demonstrated as mediating 2102Ep cell survival in hypoxia. Furthermore MyD88 has been identified as a regulator of pluripotency. Loss of MyD88 in pluripotent 2102Ep cells has been proven to prime them for retinoic acid mediated differentiation, while overexpression of MyD88 NTera2 has been shown to achieve retinoic acid resistance. This work represents the first time this association has been made. As CSCs lose their tumourigenic potential upon differentiation, targeting of MyD88 in CSCs may represent a potential avenue of investigation in terms of novel therapeutic approaches. Novel ncRNAs regulated by MyD88 in pluripotent hEC cells have been identified. Furthermore a comprehensive secretory profile for both pluripotent and nullipotent cells has been produced which will provide a legacy of potential targets.

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Appendix

RayBiotech Protein Array Data

Table 7.1 RayBioTech Growth Factor Array
2102Ep Vs 2102Ep 3Day RA Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 RA 1	2102 RA 2	2102 RA 3
AR	348.9	437.8	586.9	457.7	466.8	591.3
BDNF	0.0	0.0	0.0	0.0	0.0	0.0
bFGF	5.7	324.3	0.0	0.0	0.0	463.9
BMP-4	0.0	0.0	0.0	0.0	0.0	284.6
BMP-5	0.0	0.0	0.0	0.0	0.0	0.0
BMP-7	14148.8	11138.0	10313.5	12860.5	10352.4	13168.9
b-NGF	1263.1	935.7	701.1	977.0	800.9	993.3
EGF	13.4	6.3	5.7	23.9	18.9	21.7
EGF R	0.0	0.0	0.0	0.0	0.0	43.2
EG-VEGF	22092.9	18248.3	14501.9	18243.0	17794.0	28935.3
FGF-4	0.0	0.0	0.0	0.0	5.9	21.5
FGF-7	0.0	0.0	0.0	0.0	70.5	20.6
GDF-15	0.0	0.0	0.0	8.7	16.5	62.8
GDNF	0.0	0.0	0.0	0.0	0.0	0.7
GH	0.0	0.0	0.0	0.0	0.0	14.1
HB-EGF	0.0	0.0	0.0	0.0	0.0	0.0
HGF	0.0	0.0	0.0	0.0	5.0	4.2
IGFBP-1	0.0	0.0	0.0	0.7	5.0	1.6
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-3	0.0	0.0	0.0	0.0	0.0	465.7
IGFBP-4	0.0	0.0	0.0	0.0	0.0	63.9
IGFBP-6	0.0	0.0	0.0	0.0	0.0	529.2
IGF-I	0.0	0.0	0.0	0.0	0.0	0.0
Insulin	0.0	0.0	0.0	0.0	0.0	9.8
MCF R	40.9	40.6	21.0	66.5	0.0	280.6
NGF R	0.0	0.0	0.0	0.0	2.4	0.0
NT-3	2612.8	1867.1	2324.1	3471.8	2644.6	2705.3
NT-4	1953.5	670.9	1524.2	1372.3	395.5	2404.0
OPG	1.8	0.0	0.0	0.7	1.2	10.2
PDGF-AA	0.0	0.0	0.0	0.0	0.0	16.8
PIGF	0.0	0.0	0.0	0.0	0.0	0.0
SCF	0.0	0.0	0.0	0.0	1.3	4.3
SCF R	25.4	0.0	1.2	9.2	6.1	27.6
TGFa	6474.8	5758.7	6308.2	5979.3	6669.6	7543.8

TGFb1	*0.0*	*0.0*	*0.0*	*84.3*	*35.4*	*5.7*
TGFb3	*0.0*	*0.0*	*0.0*	*16.8*	*72.8*	*0.0*
VEGF	*14.6*	*0.0*	*0.0*	262.7	151.9	*0.0*
VEGF R2	*0.0*	*0.0*	*111.4*	*22.0*	*11.0*	*32.2*
VEGF R3	*0.0*	*0.0*	*0.0*	*0.0*	29.3	*0.0*
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.2 RayBiotech Cytokine Array 1
2102Ep Vs 2102Ep 3day RA Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 RA 1	2102 RA 2	2102 RA 3
Activin A	46.9	0.0	0.0	115.4	13.5	0.0
AgRP	0.0	0.0	7.2	0.0	0.0	37.6
ANG	458.5	606.7	655.7	1307.5	1021.8	1051.0
ANG-1	201.9	521.9	117.4	651.7	628.5	1698.5
Angiostatin	559.4	774.9	641.6	934.6	1192.3	2343.2
Catheprin S	11.3	20.4	4.9	19.8	30.8	62.5
CD 40	0.0	11.9	0.0	20.8	24.0	115.3
Cripto-1	7600.7	9804.7	10216.9	9689.2	8392.8	13205.9
DAN	26.6	24.3	0.0	157.3	211.4	458.7
DKK-1	0.0	527.2	183.3	979.8	181.3	1702.5
E-Cadherin	187.8	595.1	317.3	0.0	942.1	1740.0
EpCAM	321.8	283.2	375.1	288.2	256.5	954.9
FAS L	4.1	4.5	2.1	8.8	9.4	26.8
Fcr RIIB/C	4.0	5.9	6.5	13.4	7.1	19.1
Follistatin	4397.6	4704.4	5252.7	9075.3	7175.8	12442.3
Galectin-7	1184.5	3101.7	2211.7	1475.2	1494.1	3059.4
ICAM-2	273.7	282.6	324.0	657.0	692.9	1637.0
IL-13 R1	219.3	391.3	173.4	350.4	343.6	1545.2
IL-13 R2	37.5	235.1	85.1	47.8	66.6	1054.6
IL-17B	112.4	128.3	0.0	177.1	317.1	507.3
IL-2 Ra	17.4	74.7	44.5	45.4	33.3	119.4
IL-2 Rb	121.6	697.0	217.5	566.4	667.6	1772.8
IL-23	0.0	0.0	0.0	0.0	0.0	118.7
LAP	165.9	218.3	210.1	300.7	210.1	516.1
NrCAM	17.8	18.5	3.0	18.4	21.9	75.2
PAI-I	19631.2	25745.0	31629.7	24643.3	19954.1	31890.1
PDGF-AB	349.3	505.4	509.3	838.8	594.4	822.0
Resistin	21.3	154.3	114.5	188.8	166.0	214.8
SDF-1b	0.0	17.8	0.0	0.0	4.8	97.5
sgp130	0.0	136.0	0.0	292.3	97.5	761.7
Shh N	0.0	0.0	0.0	0.0	0.0	39.1
Siglec-5	456.6	535.2	756.8	805.9	496.3	870.6
ST2	0.0	0.0	0.0	8.8	0.0	0.0
TGF-b2	0.0	31.0	0.0	49.1	94.5	234.7

Tie-2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	255.8
TPO	*31.3*	*50.4*	*30.4*	*181.9*	*133.1*	343.0
TRAIL-R4	*0.0*	*10.6*	*0.0*	*12.5*	*22.0*	145.2
TREM-1	*0.0*	*0.0*	*0.0*	*0.0*	*220.2*	423.9
VEGF-C	*0.0*	*0.0*	*0.0*	*31.5*	*32.6*	*55.6*
VEGF R1	865.1	1597.9	1195.9	2198.9	2895.6	3413.6

Table 7.3 RayBiotech Cytokine Array 2
2102Ep Vs 2102Ep 3day RA Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 RA 1	2102 RA 2	2102 RA 3
Adiponectin	13.1	0.0	0.0	0.0	0.0	0.0
Adipsin	46.1	0.0	13.0	60.9	17.9	129.4
AFP	0.0	0.0	0.0	0.0	0.0	0.0
ANGPTL4	76082.8	50848.3	52577.4	113158.2	42793.6	42929.7
B2M	5474.7	6463.1	6615.4	6342.8	5966.2	3091.0
BCAM	597.8	760.3	1641.3	1761.6	1091.3	963.5
CA125	0.0	0.0	10.6	0.0	0.0	71.1
CA15-3	0.0	659.8	0.0	464.3	112.9	0.0
CEA	0.0	26.3	6.5	0.0	0.0	0.0
CRP	0.0	0.0	0.0	0.0	0.0	0.0
ErbB2	0.0	0.0	0.0	0.0	0.0	0.0
Ferritin	0.0	0.0	0.0	0.0	0.0	9027.9
FSH	0.0	0.4	0.0	0.0	0.0	0.0
GROa	1999.8	6878.8	9381.0	3954.0	5276.8	5828.0
hCGb	16.0	42.2	88.9	89.9	62.3	0.0
IGF-I SR	0.0	0.0	0.0	0.0	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	0.0	6.7	16.7
IL-18 Rb	0.0	31.7	28.7	47.7	0.0	0.0
IL-21	271.5	0.0	0.0	162.3	197.6	174.7
Leptin	0.0	0.0	0.0	0.0	0.0	0.0
MMP-1	0.0	16.5	12.0	0.0	7.5	0.0
MMP-2	0.0	0.0	0.0	198.1	157.5	0.0
MMP-3	174.3	317.5	460.7	710.2	549.4	293.7
MMP-8	0.0	6.8	2.9	0.0	13.0	0.0
MMP-9	90.6	79.4	131.8	105.4	44.8	64.8
MMP-10	78.7	79.6	114.6	107.6	90.0	92.7
MMP-13	0.0	2.2	1.4	1.1	0.4	0.0
NCAM-1	16560.0	20872.5	22554.2	21997.2	19086.0	18606.8
Nidogen-1	17374.8	19889.6	20901.8	24374.7	21985.7	21615.8
NSE	32187.8	36130.1	60173.2	81178.3	73923.0	77628.8
OSM	85.0	0.0	0.0	0.0	0.0	52.6
Procalcitonin	0.0	372.8	436.5	621.2	883.1	323.0

Prolactin	*0.0*	*166.7*	*227.3*	*186.2*	*214.0*	1732.6
PSA	*0.0*	*0.0*	*0.0*	*0.0*	*53.8*	*0.0*
Siglec-9	*18.3*	*72.4*	*52.0*	*17.7*	*59.5*	*29.4*
TACE	*0.0*	*0.0*	*0.0*	*0.0*	*25.7*	*0.0*
Thyroglobulin	*70.8*	*0.0*	*242.1*	1080.2	2654.9	*0.0*
TIMP-4	18490.8	21808.5	22602.7	23524.3	21844.9	20314.0
TSH	*0.0*	*3.0*	*0.0*	*15.4*	*26.0*	*0.0*

Table 7.4 RayBiotech Growth Factor Array
NTera2 Vs NTera2 3day RA Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera RA 1	NTera RA 2	NTera RA 3
AR	416.7	519.8	1118.1	595.2	493.2	168.9
BDNF	0.0	0.0	0.0	0.0	0.0	0.0
bFGF	862.4	10.6	0.0	34.8	152.5	38.4
BMP-4	0.0	0.0	2973.0	0.0	0.0	0.0
BMP-5	0.0	0.0	0.0	0.0	0.0	0.0
BMP-7	1419.3	669.5	643.7	827.7	659.1	421.9
b-NGF	0.0	1.3	553.1	0.0	21.5	6.8
EGF	12.4	3.6	3.2	24.8	8.4	1.5
EGF R	0.0	0.0	0.0	0.0	0.0	0.0
EG-VEGF	2369.1	2373.4	2078.5	2852.7	3003.7	2343.9
FGF-4	0.0	0.0	0.0	0.0	0.0	0.0
FGF-7	0.0	0.0	0.0	0.0	0.0	0.0
GDF-15	0.0	22.1	59.9	0.0	0.0	0.0
GDNF	0.0	0.0	0.0	0.0	0.0	0.0
GH	0.0	17.4	0.0	0.0	0.0	0.0
HB-EGF	0.0	1.1	0.0	0.0	0.0	0.0
HGF	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-1	7.1	8.3	0.0	0.0	0.3	0.0
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-3	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-4	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-6	0.0	0.0	0.0	0.0	0.0	0.0
IGF-I	0.0	0.0	0.0	0.0	0.0	0.0
Insulin	0.0	0.0	0.0	0.0	0.0	0.0
MCF R	0.0	0.0	27.5	25.2	47.7	125.9
NGF R	0.0	0.0	0.0	0.0	0.0	0.0
NT-3	6577.8	4869.8	6181.8	6457.6	5289.4	4228.3
NT-4	1150.6	427.5	1166.5	516.1	778.0	955.5
OPG	0.0	0.0	0.0	0.0	0.0	0.0
PDGF-AA	0.0	0.0	0.0	0.0	0.0	0.0
PIGF	0.0	0.0	0.0	0.0	0.3	0.0
SCF	0.0	11.5	0.0	0.0	0.0	0.0
SCF R	0.0	12.4	0.0	4.9	0.0	0.0
TGFa	3673.2	2533.7	3051.2	3757.5	4596.6	4638.3

TGFb1	*0.0*	*71.6*	*0.0*	*0.0*	*0.0*	*0.0*
TGFb3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF	*0.0*	1695.0	*41.6*	*0.0*	*0.0*	*0.0*
VEGF R2	*0.0*	*0.0*	*0.0*	*0.5*	*0.0*	*0.0*
VEGF R3	*0.0*	*15.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.5 RayBiotech Cytokine Array 1
NTera2 Vs NTera2 3day RA Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera RA 1	NTera RA 2	NTera RA 3
Activin A	0.0	0.0	0.0	0.0	75.1	0.0
AgRP	0.4	0.0	0.0	12.0	6.4	3.6
ANG	217.6	151.4	201.1	372.0	421.7	361.6
ANG-1	49.2	33.4	0.0	191.8	271.3	160.0
Angiostatin	889.4	500.6	995.1	1128.9	933.7	772.4
Catheprin S	0.0	0.0	3.6	5.6	10.4	7.3
CD 40	0.0	0.0	0.0	18.1	22.4	4.9
Cripto-1	9960.4	8783.1	9855.1	5359.3	5717.5	4185.7
DAN	0.0	0.0	0.0	48.3	0.0	0.0
DKK-1	0.0	250.3	255.5	30.4	636.8	758.4
E-Cadherin	360.6	277.1	97.8	665.2	692.1	646.8
EpCAM	5.1	0.0	0.0	7.7	5.1	2.2
FAS L	0.0	0.0	0.0	6.3	3.6	0.0
Fcr RIIB/C	3.7	0.0	0.0	7.0	8.2	6.2
Follistatin	47034.9	39047.3	46796.4	30457.2	35625.9	31638.8
Galectin-7	4623.3	3984.8	4919.0	4077.7	4002.6	3567.1
ICAM-2	172.1	0.0	0.0	186.1	448.1	206.9
IL-13 R1	0.0	0.0	0.0	278.4	304.3	295.6
IL-13 R2	0.0	0.0	0.0	204.9	324.6	260.2
IL-17B	43.0	0.0	0.0	44.8	36.2	0.0
IL-2 Ra	31.0	24.6	46.6	30.8	37.9	39.7
IL-2 Rb	0.0	0.0	87.4	316.3	441.2	240.7
IL-23	0.0	0.0	0.0	0.0	0.0	0.0
LAP	591.1	430.8	497.1	704.4	626.5	596.5
NrCAM	0.0	0.0	0.0	25.2	16.5	7.0
PAI-I	25733.8	25049.7	28631.9	18489.4	21342.1	20520.6
PDGF-AB	1324.0	1178.1	1043.8	1657.6	1987.4	1807.7
Resistin	131.8	115.7	267.7	26.7	51.0	10.4
SDF-1b	79.6	36.2	40.1	7.6	23.3	0.0
sgp130	0.0	0.0	0.0	111.0	287.1	192.8
Shh N	0.0	0.0	0.0	0.0	0.0	0.0
Siglec-5	71.3	47.3	62.8	36.8	32.8	35.0
ST2	0.0	0.0	0.0	0.0	0.0	0.0
TGF-b2	0.0	0.0	0.0	33.0	35.8	0.0

Tie-2	*0.0*	*0.0*	*0.0*	117.0	*24.4*	90.8
TPO	*0.0*	*0.0*	*0.0*	*122.4*	*106.7*	*106.2*
TRAIL-R4	*0.0*	*0.0*	*0.0*	*0.0*	*2.2*	*13.4*
TREM-1	*0.0*	*0.0*	*0.0*	*0.0*	*161.5*	*0.0*
VEGF-C	*0.0*	*0.0*	*0.0*	*11.0*	*12.0*	*2.3*
VEGF R1	24892.9	21547.6	27441.9	5825.3	7401.1	8909.2

Table 7.6 RayBiotech Cytokine Array 2
NTera2 Vs NTera2 3day RA Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera RA 1	NTera RA 2	NTera RA 3
Adiponectin	0.0	0.0	0.0	48.1	0.0	0.0
Adipsin	0.0	0.0	0.0	0.0	0.0	0.0
AFP	0.0	0.0	0.0	0.0	0.0	0.0
ANGPTL4	0.0	1273.5	0.0	1031.2	8946.5	46608.6
B2M	6552.7	6164.9	5707.6	4864.3	4914.9	4779.4
BCAM	725.2	341.5	307.6	578.6	371.2	242.0
CA125	0.0	0.0	0.0	0.0	0.0	0.0
CA15-3	0.0	0.0	0.0	0.0	0.0	0.0
CEA	0.0	0.0	0.0	0.0	0.0	0.0
CRP	0.0	0.0	0.0	0.0	0.0	0.0
ErbB2	0.0	0.0	0.0	0.0	0.0	0.0
Ferritin	0.0	0.0	0.0	0.0	0.0	0.0
FSH	0.0	0.0	0.0	0.0	0.0	0.0
GROa	1414.6	0.0	671.9	749.2	0.0	3910.8
hCGb	38.4	38.8	25.4	46.0	31.2	14.2
IGF-I SR	0.0	0.0	0.0	48.1	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	1.2	0.0	0.0
IL-18 Rb	0.0	121.2	8.1	0.0	0.0	0.0
IL-21	0.0	0.0	0.0	0.0	254.9	32.7
Leptin	0.0	0.0	0.0	0.0	0.0	0.0
MMP-1	0.0	11.1	0.0	9.8	0.0	21.6
MMP-2	0.0	1073.6	2310.0	0.0	0.0	0.0
MMP-3	0.0	0.0	0.0	0.0	0.0	1739.8
MMP-8	0.0	0.0	0.0	0.0	0.0	0.0
MMP-9	26.9	33.1	25.5	41.8	37.2	90.3
MMP-10	327.5	297.4	273.5	162.1	143.6	581.4
MMP-13	0.0	0.0	0.0	0.0	0.0	11.0
NCAM-1	22970.7	23578.0	21297.5	19825.2	20838.8	19555.1
Nidogen-1	27264.2	25867.1	23986.1	21976.9	26475.5	20810.4
NSE	17533.4	11250.5	13830.9	29300.9	24599.8	72858.3
OSM	0.0	0.0	0.0	0.0	0.0	0.0
Procalcitonin	617.1	651.4	322.7	273.6	190.5	216.8
Prolactin	52.1	164.7	0.0	0.0	0.0	0.0
PSA	0.0	11.2	0.0	0.0	0.0	0.0

Siglec-9	*35.0*	*51.2*	*16.0*	*30.4*	*12.3*	*0.0*
TACE	*0.0*	*0.0*	*0.0*	*96.8*	*0.0*	*0.0*
Thyroglobulin	836.9	*279.3*	*0.0*	*0.0*	*0.0*	*0.0*
TIMP-4	19671.8	16470.2	16659.1	9649.0	9921.8	14414.1
TSH	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*9.6*

Table 7.7 RayBiotech Growth Factor Array
2102Ep Vs 2102Ep 3day Cisplatin Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 Cis 1	2102 Cis 2
AR	348.9	437.8	586.9	452.0	339.7
BDNF	0.0	0.0	0.0	2.3	0.0
bFGF	5.7	324.3	0.0	214.8	0.0
BMP-4	0.0	0.0	0.0	498.1	236.3
BMP-5	0.0	0.0	0.0	0.0	0.0
BMP-7	14148.8	11138.0	10313.5	24093.9	19879.7
b-NGF	1263.1	935.7	701.1	1914.6	1824.4
EGF	13.4	6.3	5.7	40.0	26.6
EGF R	0.0	0.0	0.0	0.0	0.0
EG-VEGF	22092.9	18248.3	14501.9	36375.0	26487.7
FGF-4	0.0	0.0	0.0	0.0	0.0
FGF-7	0.0	0.0	0.0	0.0	0.0
GDF-15	0.0	0.0	0.0	11.8	0.0
GDNF	0.0	0.0	0.0	0.0	0.0
GH	0.0	0.0	0.0	0.0	0.0
HB-EGF	0.0	0.0	0.0	3.1	0.0
HGF	0.0	0.0	0.0	0.0	0.0
IGFBP-1	0.0	0.0	0.0	0.0	0.0
IGFBP-2	0.0	0.0	0.0	1027.0	0.0
IGFBP-3	0.0	0.0	0.0	0.0	0.0
IGFBP-4	0.0	0.0	0.0	0.0	0.0
IGFBP-6	0.0	0.0	0.0	56.2	0.0
IGF-I	0.0	0.0	0.0	0.0	0.0
Insulin	0.0	0.0	0.0	0.0	0.0
MCF R	40.9	40.6	21.0	542.0	427.8
NGF R	0.0	0.0	0.0	0.0	0.0
NT-3	2612.8	1867.1	2324.1	4612.0	4142.5
NT-4	1953.5	670.9	1524.2	3167.5	2360.1
OPG	1.8	0.0	0.0	117.0	107.0
PDGF-AA	0.0	0.0	0.0	0.0	0.0
PIGF	0.0	0.0	0.0	15.7	9.8
SCF	0.0	0.0	0.0	0.0	0.0
SCF R	25.4	0.0	1.2	57.9	37.3
TGFa	6474.8	5758.7	6308.2	6025.0	5301.7

TGFb1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
TGFb3	*0.0*	*0.0*	*0.0*	*164.1*	*148.4*
VEGF	*14.6*	*0.0*	*0.0*	*31.2*	171.0
VEGF R2	*0.0*	*0.0*	*111.4*	*7.0*	*69.9*
VEGF R3	*0.0*	*0.0*	*0.0*	301.1	371.4
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.8 RayBiotech Cytokine Array 1
2102Ep Vs 2102Ep 3day Cisplatin treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 Cis 1	2102 Cis 2	2102 Cis 3
Activin A	46.9	0.0	0.0	445.8	337.5	119.0
AgRP	0.0	0.0	7.2	15.0	10.9	8.5
ANG	458.5	606.7	655.7	1261.0	1263.6	1158.7
ANG-1	201.9	521.9	117.4	456.0	493.0	0.0
Angiostatin	559.4	774.9	641.6	983.1	1657.3	1453.2
Catheprin S	11.3	20.4	4.9	27.0	70.3	30.4
CD 40	0.0	11.9	0.0	64.4	40.2	55.9
Cripto-1	7600.7	9804.7	10216.9	8886.4	10968.8	8199.1
DAN	26.6	24.3	0.0	50.8	63.4	75.5
DKK-1	0.0	527.2	183.3	1533.9	1017.5	833.8
E-Cadherin	187.8	595.1	317.3	978.7	1711.9	1060.8
EpCAM	321.8	283.2	375.1	361.7	494.0	321.3
FAS L	4.1	4.5	2.1	3.8	4.1	10.2
Fcr RIIB/C	4.0	5.9	6.5	5.5	11.5	7.9
Follistatin	4397.6	4704.4	5252.7	16019.2	21966.8	34046.5
Galectin-7	1184.5	3101.7	2211.7	13597.0	18390.6	10842.0
ICAM-2	273.7	282.6	324.0	769.1	2118.4	799.4
IL-13 R1	219.3	391.3	173.4	477.3	1005.3	558.2
IL-13 R2	37.5	235.1	85.1	289.1	720.6	371.9
IL-17B	112.4	128.3	0.0	131.2	230.5	348.1
IL-2 Ra	17.4	74.7	44.5	31.2	65.3	31.3
IL-2 Rb	121.6	697.0	217.5	462.5	1257.7	701.9
IL-23	0.0	0.0	0.0	0.0	116.6	0.0
LAP	165.9	218.3	210.1	624.0	922.1	788.5
NrCAM	17.8	18.5	3.0	19.3	39.0	40.0
PAI-I	19631.2	25745.0	31629.7	35465.8	46526.0	31396.8
PDGF-AB	349.3	505.4	509.3	2901.8	4399.5	3543.7
Resistin	21.3	154.3	114.5	17.4	193.6	54.4
SDF-1b	0.0	17.8	0.0	16.0	37.4	18.8
sgp130	0.0	136.0	0.0	310.7	979.2	604.9
Shh N	0.0	0.0	0.0	0.0	12.2	0.0
Siglec-5	456.6	535.2	756.8	5749.3	6038.8	3452.8
ST2	0.0	0.0	0.0	28.9	17.8	0.0
TGF-b2	0.0	31.0	0.0	97.0	211.2	462.3

Tie-2	*0.0*	*0.0*	*0.0*	96.3	189.1	*17.7*
TPO	*31.3*	*50.4*	*30.4*	*187.6*	*242.7*	300.2
TRAIL-R4	*0.0*	*10.6*	*0.0*	55.7	*34.1*	*0.0*
TREM-1	*0.0*	*0.0*	*0.0*	*211.1*	*185.9*	*0.0*
VEGF-C	*0.0*	*0.0*	*0.0*	*10.5*	*0.0*	*62.9*
VEGF R1	865.1	1597.9	1195.9	6149.9	7503.6	9027.8

Table 7.9 RayBiotech Cytokine Array 2
2102Ep Vs 2102Ep 3day Cisplatin Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 Cis 1	2102 Cis 2	2102 Cis 3
Adiponectin	13.1	0.0	0.0	0.0	0.0	0.0
Adipsin	46.1	0.0	13.0	0.0	365.0	0.0
AFP	0.0	0.0	0.0	0.0	0.0	0.0
ANGPTL4	76082.8	50848.3	52577.4	41656.6	60375.4	0.0
B2M	5474.7	6463.1	6615.4	4980.8	5431.3	4311.3
BCAM	597.8	760.3	1641.3	100.6	2517.1	250.2
CA125	0.0	0.0	10.6	0.0	0.0	0.0
CA15-3	0.0	659.8	0.0	0.0	0.0	0.0
CEA	0.0	26.3	6.5	0.0	10.0	0.0
CRP	0.0	0.0	0.0	0.0	0.0	0.0
ErbB2	0.0	0.0	0.0	0.0	0.0	0.0
Ferritin	0.0	0.0	0.0	0.0	0.0	0.0
FSH	0.0	0.4	0.0	0.0	0.0	0.0
GROa	1999.8	6878.8	9381.0	0.0	326.8	0.0
hCGb	16.0	42.2	88.9	21.9	219.2	0.0
IGF-I SR	0.0	0.0	0.0	0.0	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	0.0	0.0	0.0
IL-18 Rb	0.0	31.7	28.7	0.0	0.0	0.0
IL-21	271.5	0.0	0.0	45.1	125.1	0.0
Leptin	0.0	0.0	0.0	0.0	0.0	0.0
MMP-1	0.0	16.5	12.0	17.4	0.0	0.0
MMP-2	0.0	0.0	0.0	0.0	0.0	0.0
MMP-3	174.3	317.5	460.7	1456.1	524.7	47.0
MMP-8	0.0	6.8	2.9	0.0	0.0	0.0
MMP-9	90.6	79.4	131.8	117.5	71.0	5.2
MMP-10	78.7	79.6	114.6	555.9	219.7	257.7
MMP-13	0.0	2.2	1.4	9.4	0.0	0.0
NCAM-1	16560.0	20872.5	22554.2	19108.4	19246.5	18335.6
Nidogen-1	17374.8	19889.6	20901.8	21097.9	18471.5	20056.0
NSE	32187.8	36130.1	60173.2	66957.0	82327.3	7480.2
OSM	85.0	0.0	0.0	0.0	0.0	0.0
Procalcitonin	0.0	372.8	436.5	68.5	388.3	461.5
Prolactin	0.0	166.7	227.3	0.0	263.7	0.0
PSA	0.0	0.0	0.0	0.0	0.0	0.0

Siglec-9	*18.3*	*72.4*	*52.0*	*0.0*	*5.0*	*0.0*
TACE	*0.0*	*0.0*	*0.0*	*0.0*	*20.9*	*0.0*
Thyroglobulin	*70.8*	*0.0*	*242.1*	*0.0*	*0.0*	*0.0*
TIMP-4	18490.8	21808.5	22602.7	16847.3	13736.2	8286.0
TSH	*0.0*	*3.0*	*0.0*	*7.0*	*22.4*	*21.5*

Table 7.10 RayBiotech Growth Factor Array
NTera2 Vs NTera2 3day Cisplatin Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera Cis 1	NTera Cis 2	NTera Cis 3
AR	416.7	519.8	1118.1	*13.8*	*13.8*	273.6
BDNF	*0.0*	*0.0*	*0.0*	*2.5*	*2.5*	37.9
bFGF	862.4	*10.6*	*0.0*	*99.1*	*99.1*	*0.0*
BMP-4	*0.0*	*0.0*	2973.0	*0.0*	*0.0*	*0.0*
BMP-5	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	1146.6
BMP-7	1419.3	669.5	643.7	11343.4	11343.4	647.9
b-NGF	*0.0*	*1.3*	553.1	339.4	339.4	*0.0*
EGF	12.4	3.6	3.2	7.3	7.3	9.6
EGF R	*0.0*	*0.0*	*0.0*	93.0	93.0	*0.0*
EG-VEGF	2369.1	2373.4	2078.5	**26330.7**	**26330.7**	13019.8
FGF-4	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	888.5
FGF-7	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
GDF-15	*0.0*	*22.1*	59.9	*0.0*	*0.0*	*0.0*
GDNF	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*12.9*
GH	*0.0*	*17.4*	*0.0*	*0.0*	*0.0*	*0.0*
HB-EGF	*0.0*	*1.1*	*0.0*	*0.0*	*0.0*	*0.0*
HGF	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	112.1
IGFBP-1	*7.1*	*8.3*	*0.0*	*3.7*	*3.7*	*0.6*
IGFBP-2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IGFBP-3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*1388.7*
IGFBP-4	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IGFBP-6	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IGF-I	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*42.8*
Insulin	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
MCF R	*0.0*	*0.0*	*27.5*	*35.7*	*35.7*	*20.6*
NGF R	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*28.2*
NT-3	6577.8	4869.8	6181.8	3469.6	3469.6	3079.7
NT-4	1150.6	427.5	1166.5	3279.6	3279.6	3664.5
OPG	*0.0*	*0.0*	*0.0*	*34.8*	*34.8*	*7.2*
PDGF-AA	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
PIGF	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*1.7*
SCF	*0.0*	*11.5*	*0.0*	*0.0*	*0.0*	*16.1*
SCF R	*0.0*	*12.4*	*0.0*	*10.2*	*10.2*	*33.7*

TGFa	3673.2	2533.7	3051.2	5724.4	5724.4	5142.9
TGFb1	*0.0*	*71.6*	*0.0*	*0.0*	*0.0*	401.0
TGFb3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF	*0.0*	1695.0	*41.6*	54.5	54.5	*0.0*
VEGF R2	*0.0*	*0.0*	*0.0*	*24.2*	*24.2*	*287.5*
VEGF R3	*0.0*	*15.0*	*0.0*	41.5	41.5	*0.0*
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.11 RayBiotech Cytokine Array 1
NTera2 Vs NTera2 3day Cisplatin Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera Cis 1	NTera Cis 2	NTera Cis 3
Activin A	0.0	0.0	0.0	0.0	128.4	0.0
AgRP	0.4	0.0	0.0	0.0	1.2	5.5
ANG	217.6	151.4	201.1	755.5	364.9	328.0
ANG-1	49.2	33.4	0.0	0.0	637.3	587.5
Angiostatin	889.4	500.6	995.1	689.3	1870.2	1185.9
Catheprin S	0.0	0.0	3.6	13.6	25.6	14.2
CD 40	0.0	0.0	0.0	8.5	39.7	34.5
Cripto-1	9960.4	8783.1	9855.1	6787.9	7328.4	7806.9
DAN	0.0	0.0	0.0	0.0	84.8	26.1
DKK-1	0.0	250.3	255.5	95.8	254.8	0.0
E-Cadherin	360.6	277.1	97.8	729.6	0.0	0.0
EpCAM	5.1	0.0	0.0	106.1	12.2	13.3
FAS L	0.0	0.0	0.0	0.0	5.2	4.0
Fcr RIIB/C	3.7	0.0	0.0	1.3	7.9	7.4
Follistatin	47034.9	39047.3	46796.4	45320.1	60412.7	61409.6
Galectin-7	4623.3	3984.8	4919.0	7680.8	7001.7	6990.2
ICAM-2	172.1	0.0	0.0	123.8	729.5	661.9
IL-13 R1	0.0	0.0	0.0	49.7	949.2	1011.3
IL-13 R2	0.0	0.0	0.0	8.3	411.9	434.0
IL-17B	43.0	0.0	0.0	59.3	53.4	149.3
IL-2 Ra	31.0	24.6	46.6	0.0	49.7	65.6
IL-2 Rb	0.0	0.0	87.4	0.0	269.1	446.3
IL-23	0.0	0.0	0.0	0.0	0.0	0.0
LAP	591.1	430.8	497.1	689.0	963.0	1451.6
NrCAM	0.0	0.0	0.0	0.0	35.3	29.2
PAI-I	25733.8	25049.7	28631.9	23309.4	34741.2	36452.5
PDGF-AB	1324.0	1178.1	1043.8	3508.7	4108.9	4444.9
Resistin	131.8	115.7	267.7	0.0	107.2	212.9
SDF-1b	79.6	36.2	40.1	0.0	32.0	28.4
sgp130	0.0	0.0	0.0	97.0	448.9	453.9
Shh N	0.0	0.0	0.0	0.0	31.1	100.1
Siglec-5	71.3	47.3	62.8	1315.4	647.6	792.5
ST2	0.0	0.0	0.0	0.0	3.8	19.3
TGF-b2	0.0	0.0	0.0	0.0	79.7	57.6
Tie-2	0.0	0.0	0.0	0.0	100.9	78.6

TPO	*0.0*	*0.0*	*0.0*	*0.0*	*103.7*	*152.4*
TRAIL-R4	*0.0*	*0.0*	*0.0*	*0.0*	*21.4*	*37.0*
TREM-1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*143.3*
VEGF-C	*0.0*	*0.0*	*0.0*	*0.0*	*8.0*	*14.8*
VEGF R1	24892.9	21547.6	27441.9	14972.0	21967.8	11119.0

Table 7.12 RayBiotech Cytokine Array 2
NTera2 Vs NTera2 3day Cisplatin Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera Cis 1	NTera Cis 2	NTera Cis 3
Adiponectin	0.0	0.0	0.0	0.0	0.0	0.0
Adipsin	0.0	0.0	0.0	78.0	0.0	0.0
AFP	0.0	0.0	0.0	0.0	2.8	0.0
ANGPTL4	0.0	1273.5	0.0	0.0	0.0	0.0
B2M	6552.7	6164.9	5707.6	4797.7	5055.6	4909.6
BCAM	725.2	341.5	307.6	350.2	1081.1	800.7
CA125	0.0	0.0	0.0	0.0	0.0	0.0
CA15-3	0.0	0.0	0.0	0.0	0.0	0.0
CEA	0.0	0.0	0.0	64.0	0.0	0.0
CRP	0.0	0.0	0.0	0.0	0.0	0.0
ErbB2	0.0	0.0	0.0	0.0	2923.1	0.0
Ferritin	0.0	0.0	0.0	0.0	0.0	0.0
FSH	0.0	0.0	0.0	0.4	0.0	0.0
GROa	1414.6	0.0	671.9	1531.6	2372.6	983.8
hCGb	38.4	38.8	25.4	7.0	292.9	368.7
IGF-I SR	0.0	0.0	0.0	0.0	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	21.4	83.9	0.0
IL-18 Rb	0.0	121.2	8.1	0.0	0.0	0.0
IL-21	0.0	0.0	0.0	0.0	259.7	0.0
Leptin	0.0	0.0	0.0	0.0	0.0	0.0
MMP-1	0.0	11.1	0.0	0.0	7.5	0.0
MMP-2	0.0	1073.6	2310.0	0.0	54.5	0.0
MMP-3	0.0	0.0	0.0	99.7	0.0	0.0
MMP-8	0.0	0.0	0.0	17.4	0.0	0.0
MMP-9	26.9	33.1	25.5	13.4	29.4	29.4
MMP-10	327.5	297.4	273.5	321.9	173.2	163.9
MMP-13	0.0	0.0	0.0	0.0	0.0	0.0
NCAM-1	22970.7	23578.0	21297.5	20831.4	17843.2	18274.0
Nidogen-1	27264.2	25867.1	23986.1	22567.3	26026.1	25868.7
NSE	17533.4	11250.5	13830.9	7846.4	89526.2	112863.5
OSM	0.0	0.0	0.0	0.0	123.9	0.0
Procalcitonin	617.1	651.4	322.7	876.9	2961.9	3174.6
Prolactin	52.1	164.7	0.0	0.0	543.5	0.0
PSA	0.0	11.2	0.0	0.0	0.0	0.0

Siglec-9	*35.0*	*51.2*	*16.0*	*0.0*	*0.0*	*0.0*
TACE	*0.0*	*0.0*	*0.0*	*0.0*	*147.9*	*0.0*
Thyroglobulin	836.9	*279.3*	*0.0*	*0.0*	*0.0*	*0.0*
TIMP-4	19671.8	16470.2	16659.1	7770.6	9235.3	9871.8
TSH	*0.0*	*0.0*	*0.0*	*41.7*	*0.0*	*0.0*

Table 7.13 RayBiotech Growth Factor Array
2102Ep Vs 2102Ep 3 Day Hypoxia Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 Hyp 1	2102 Hyp 2	2102 Hyp 3
AR	348.9	437.8	586.9	400.4	486.4	361.5
BDNF	0.0	0.0	0.0	20.2	40.4	10.3
bFGF	5.7	324.3	0.0	0.0	2155.0	1129.3
BMP-4	0.0	0.0	0.0	0.0	0.0	0.0
BMP-5	0.0	0.0	0.0	1678.1	2432.1	0.0
BMP-7	14148.8	11138.0	10313.5	7221.2	7701.2	18553.4
b-NGF	1263.1	935.7	701.1	184.4	178.9	352.9
EGF	13.4	6.3	5.7	12.6	21.2	9.9
EGF R	0.0	0.0	0.0	0.0	652.2	0.0
EG-VEGF	22092.9	18248.3	14501.9	14644.6	14166.9	10903.8
FGF-4	0.0	0.0	0.0	0.0	877.4	324.1
FGF-7	0.0	0.0	0.0	0.0	0.0	0.0
GDF-15	0.0	0.0	0.0	0.0	0.0	0.0
GDNF	0.0	0.0	0.0	6.1	23.6	3.3
GH	0.0	0.0	0.0	0.0	0.0	0.0
HB-EGF	0.0	0.0	0.0	0.0	0.0	0.0
HGF	0.0	0.0	0.0	153.6	161.3	84.4
IGFBP-1	0.0	0.0	0.0	3.8	0.0	2.7
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-3	0.0	0.0	0.0	4248.3	6040.2	983.0
IGFBP-4	0.0	0.0	0.0	0.0	1955.3	0.0
IGFBP-6	0.0	0.0	0.0	0.0	0.0	0.0
IGF-I	0.0	0.0	0.0	100.8	123.8	44.6
Insulin	0.0	0.0	0.0	16.5	0.0	0.0
MCF R	40.9	40.6	21.0	12.5	0.0	154.3
NGF R	0.0	0.0	0.0	75.9	61.0	0.0
NT-3	2612.8	1867.1	2324.1	5198.7	4843.0	4047.4
NT-4	1953.5	670.9	1524.2	4352.6	4061.4	3558.0
OPG	1.8	0.0	0.0	29.0	25.7	0.0
PDGF-AA	0.0	0.0	0.0	20.9	0.0	0.0
PIGF	0.0	0.0	0.0	0.0	0.0	0.0
SCF	0.0	0.0	0.0	111.5	70.3	14.6
SCF R	25.4	0.0	1.2	37.8	12.7	8.1
TGFa	6474.8	5758.7	6308.2	6697.7	4110.4	2731.9

TGFb1	*0.0*	*0.0*	*0.0*	1218.7	3157.9	*0.0*
TGFb3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF	*14.6*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF R2	*0.0*	*0.0*	*111.4*	*177.4*	453.7	*0.0*
VEGF R3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.14 RayBiotech Cytokine Array 1
2102Ep Vs 2102Ep 3 Day Hypoxia Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 Hyp 1	2102 Hyp 2	2102 Hyp 3
Activin A	46.9	0.0	0.0	0.0	26.8	270.9
AgRP	0.0	0.0	7.2	0.0	0.0	0.0
ANG	458.5	606.7	655.7	874.5	1057.5	1190.2
ANG-1	201.9	521.9	117.4	0.0	55.8	130.4
Angiostatin	559.4	774.9	641.6	1734.1	1835.3	1422.6
Catheprin S	11.3	20.4	4.9	11.5	3.7	13.5
CD 40	0.0	11.9	0.0	0.0	20.1	2.9
Cripto-1	7600.7	9804.7	10216.9	8520.7	8319.2	7899.1
DAN	26.6	24.3	0.0	0.0	0.0	0.0
DKK-1	0.0	527.2	183.3	0.0	409.4	0.0
E-Cadherin	187.8	595.1	317.3	0.0	0.0	0.0
EpCAM	321.8	283.2	375.1	488.6	343.6	352.0
FAS L	4.1	4.5	2.1	0.6	0.0	0.0
Fcr RIIB/C	4.0	5.9	6.5	11.1	0.0	0.0
Follistatin	4397.6	4704.4	5252.7	4373.2	3965.3	3630.3
Galectin-7	1184.5	3101.7	2211.7	1234.4	799.3	1249.1
ICAM-2	273.7	282.6	324.0	405.9	400.9	227.1
IL-13 R1	219.3	391.3	173.4	446.6	195.7	262.6
IL-13 R2	37.5	235.1	85.1	255.3	229.5	286.7
IL-17B	112.4	128.3	0.0	0.0	0.0	0.0
IL-2 Ra	17.4	74.7	44.5	50.4	65.6	68.5
IL-2 Rb	121.6	697.0	217.5	337.8	590.8	655.9
IL-23	0.0	0.0	0.0	0.0	0.0	0.0
LAP	165.9	218.3	210.1	445.1	471.3	404.4
NrCAM	17.8	18.5	3.0	9.6	6.0	11.9
PAI-I	19631.2	25745.0	31629.7	32376.4	33916.4	25273.4
PDGF-AB	349.3	505.4	509.3	754.7	882.1	517.1
Resistin	21.3	154.3	114.5	203.4	209.5	197.9
SDF-1b	0.0	17.8	0.0	0.0	55.6	6.0
sgp130	0.0	136.0	0.0	113.6	175.2	314.7
Shh N	0.0	0.0	0.0	8.7	0.0	8.2
Siglec-5	456.6	535.2	756.8	356.5	422.7	3146.5
ST2	0.0	0.0	0.0	0.0	2.5	0.0
TGF-b2	0.0	31.0	0.0	0.0	0.0	30.3

Tie-2	*0.0*	*0.0*	*0.0*	*0.0*	*20.6*	*0.0*
TPO	*31.3*	*50.4*	*30.4*	*100.4*	*91.5*	*0.0*
TRAIL-R4	*0.0*	*10.6*	*0.0*	*21.4*	*0.0*	*0.0*
TREM-1	*0.0*	*0.0*	*0.0*	*82.8*	*0.0*	*0.0*
VEGF-C	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF R1	865.1	1597.9	1195.9	1655.7	1979.1	908.0

Table 7.15 RayBiotech Cytokine Array 2
2102Ep Vs 2102Ep 3 Day Hypoxia Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 Hyp 1	2102 Hyp 2	2102 Hyp 3
Adiponectin	13.1	0.0	0.0	24609.6	0.0	0.0
Adipsin	46.1	0.0	13.0	20113.7	0.0	408.0
AFP	0.0	0.0	0.0	2405.4	0.0	0.0
ANGPTL4	76082.8	50848.3	52577.4	5438476.5	0.0	136233.3
B2M	5474.7	6463.1	6615.4	1782909.9	6093.6	6057.3
BCAM	597.8	760.3	1641.3	68314.3	294.9	6124.8
CA125	0.0	0.0	10.6	135854.1	0.0	963.9
CA15-3	0.0	659.8	0.0	#########	0.0	0.0
CEA	0.0	26.3	6.5	52531.7	0.0	56.6
CRP	0.0	0.0	0.0	22239.5	0.0	0.0
ErbB2	0.0	0.0	0.0	188834.4	0.0	0.0
Ferritin	0.0	0.0	0.0	461383.8	0.0	315.8
FSH	0.0	0.4	0.0	1958.7	0.0	1.8
GROa	1999.8	6878.8	9381.0	867872.3	105.5	3872.8
hCGb	16.0	42.2	88.9	6875.1	31.2	1460.3
IGF-I SR	0.0	0.0	0.0	121692.9	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	7740.6	0.0	0.0
IL-3	0.0	0.0	0.0	6708.8	5.0	16.1
IL-18 Rb	0.0	31.7	28.7	11478.9	0.0	0.0
IL-21	271.5	0.0	0.0	42751.4	18.2	44.0
Leptin	0.0	0.0	0.0	9414.2	1.6	0.0
MMP-1	0.0	16.5	12.0	16572.6	0.0	0.0
MMP-2	0.0	0.0	0.0	165907.1	0.0	0.0
MMP-3	174.3	317.5	460.7	24251.8	0.0	0.0
MMP-8	0.0	6.8	2.9	14840.2	0.0	0.0
MMP-9	90.6	79.4	131.8	7962.8	48.3	162.9
MMP-10	78.7	79.6	114.6	2439.3	149.2	132.7
MMP-13	0.0	2.2	1.4	4898.6	0.0	0.0
NCAM-1	16560.0	20872.5	22554.2	208045.9	22556.7	23934.9
Nidogen-1	17374.8	19889.6	20901.8	15205.1	25175.4	26822.5
NSE	32187.8	36130.1	60173.2	43995.9	22198.7	151675.7
OSM	85.0	0.0	0.0	132768.3	0.0	0.0
Procalcitonin	0.0	372.8	436.5	75036.8	344.5	1580.2
Prolactin	0.0	166.7	227.3	146366.9	0.0	127.1
PSA	0.0	0.0	0.0	17518.5	0.0	0.0

Siglec-9	*18.3*	*72.4*	*52.0*	19462.0	*9.3*	*24.6*
TACE	*0.0*	*0.0*	*0.0*	126120.3	*69.8*	*0.0*
Thyroglobulin	*70.8*	*0.0*	*242.1*	**2160073.0**	*0.0*	974.5
TIMP-4	18490.8	21808.5	22602.7	5582.9	11080.8	28312.0
TSH	*0.0*	*3.0*	*0.0*	4565.3	*30.0*	71.1

Table 7.16 RayBiotech Growth Factor Array
NTera2 Vs NTera2 3 Day Hypoxia Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera Hyp 1	NTera Hyp 2	NTera Hyp 3
AR	416.7	519.8	1118.1	162.7	342.4	208.3
BDNF	0.0	0.0	0.0	0.0	0.0	0.0
bFGF	862.4	10.6	0.0	0.0	0.0	13.7
BMP-4	0.0	0.0	2973.0	0.0	0.0	0.0
BMP-5	0.0	0.0	0.0	0.0	0.0	0.0
BMP-7	1419.3	669.5	643.7	0.0	0.0	0.0
b-NGF	0.0	1.3	553.1	0.0	0.0	18.9
EGF	12.4	3.6	3.2	0.8	2.1	0.8
EGF R	0.0	0.0	0.0	0.0	0.0	0.0
EG-VEGF	2369.1	2373.4	2078.5	2583.7	2877.2	3127.9
FGF-4	0.0	0.0	0.0	0.0	0.0	0.0
FGF-7	0.0	0.0	0.0	0.0	0.0	0.0
GDF-15	0.0	22.1	59.9	0.0	0.0	16.5
GDNF	0.0	0.0	0.0	0.0	0.0	0.0
GH	0.0	17.4	0.0	0.0	0.0	0.0
HB-EGF	0.0	1.1	0.0	0.0	0.0	0.0
HGF	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-1	7.1	8.3	0.0	0.0	0.0	0.0
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-3	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-4	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-6	0.0	0.0	0.0	0.0	0.0	0.0
IGF-I	0.0	0.0	0.0	0.0	0.0	0.0
Insulin	0.0	0.0	0.0	0.0	0.0	0.0
MCF R	0.0	0.0	27.5	0.0	0.0	27.3
NGF R	0.0	0.0	0.0	0.0	0.0	0.0
NT-3	6577.8	4869.8	6181.8	1396.2	1442.7	1776.2
NT-4	1150.6	427.5	1166.5	2202.2	2358.6	2459.3
OPG	0.0	0.0	0.0	0.0	0.0	0.0
PDGF-AA	0.0	0.0	0.0	0.0	0.0	0.0
PIGF	0.0	0.0	0.0	0.0	0.0	0.0
SCF	0.0	11.5	0.0	0.0	0.0	0.0
SCF R	0.0	12.4	0.0	0.0	0.0	0.0
TGFa	3673.2	2533.7	3051.2	1308.8	1307.9	1332.9
TGFb1	0.0	71.6	0.0	0.0	0.0	0.0

TGFb3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF	*0.0*	1695.0	*41.6*	*0.0*	*0.0*	*0.0*
VEGF R2	*0.0*	*0.0*	*0.0*	*7.7*	*0.0*	*0.0*
VEGF R3	*0.0*	*15.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.17 RayBiotech Cytokine Array 1
NTera2 Vs NTera2 3 Day Hypoxia Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera Hyp 1	NTera Hyp 2	NTera Hyp 3
Activin A	0.0	0.0	0.0	0.0	0.0	0.0
AgRP	0.4	0.0	0.0	0.0	0.0	0.0
ANG	217.6	151.4	201.1	106.3	128.6	132.5
ANG-1	49.2	33.4	0.0	0.0	0.0	0.0
Angiostatin	889.4	500.6	995.1	163.6	1218.5	1089.8
Catheprin S	0.0	0.0	3.6	0.0	0.0	8.4
CD 40	0.0	0.0	0.0	0.0	0.0	0.0
Cripto-1	9960.4	8783.1	9855.1	6201.2	5370.2	4886.6
DAN	0.0	0.0	0.0	0.0	0.0	0.0
DKK-1	0.0	250.3	255.5	0.0	128.3	0.0
E-Cadherin	360.6	277.1	97.8	0.0	1129.3	0.0
EpCAM	5.1	0.0	0.0	0.0	0.0	0.0
FAS L	0.0	0.0	0.0	0.0	0.0	0.0
Fcr RIIB/C	3.7	0.0	0.0	0.0	0.0	0.0
Follistatin	47034.9	39047.3	46796.4	19919.6	22575.5	22460.8
Galectin-7	4623.3	3984.8	4919.0	1582.8	2219.8	2506.3
ICAM-2	172.1	0.0	0.0	377.2	0.0	0.0
IL-13 R1	0.0	0.0	0.0	19.6	41.9	24.9
IL-13 R2	0.0	0.0	0.0	257.7	107.7	103.0
IL-17B	43.0	0.0	0.0	0.0	0.0	0.0
IL-2 Ra	31.0	24.6	46.6	57.0	43.4	0.0
IL-2 Rb	0.0	0.0	87.4	0.0	0.0	0.0
IL-23	0.0	0.0	0.0	15.9	0.0	0.0
LAP	591.1	430.8	497.1	368.4	352.6	306.1
NrCAM	0.0	0.0	0.0	0.0	0.0	0.0
PAI-I	25733.8	25049.7	28631.9	31048.1	25749.6	19249.8
PDGF-AB	1324.0	1178.1	1043.8	278.5	313.2	211.2
Resistin	131.8	115.7	267.7	163.8	242.5	165.8
SDF-1b	79.6	36.2	40.1	0.0	0.0	0.0
sgp130	0.0	0.0	0.0	0.0	0.0	0.0
Shh N	0.0	0.0	0.0	16.3	0.0	0.0
Siglec-5	71.3	47.3	62.8	30.4	19.3	3.8
ST2	0.0	0.0	0.0	3.3	0.0	0.0
TGF-b2	0.0	0.0	0.0	0.0	0.0	0.0
Tie-2	0.0	0.0	0.0	0.0	0.0	0.0

TPO	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
TRAIL-R4	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	44.9
TREM-1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	1169.8
VEGF-C	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF R1	24892.9	21547.6	27441.9	16467.5	19547.1	13716.9

Table 7.18 RayBiotech Cytokine Array 2
NTera2 Vs NTera2 3 Day Hypoxia Treatment

(pg/ml)	NTera 1	NTera 2	NTera 3	NTera Hyp 1	NTera Hyp 2	NTera Hyp 3
Adiponectin	0.0	0.0	0.0	0.0	0.0	0.0
Adipsin	0.0	0.0	0.0	370.1	347.2	58.2
AFP	0.0	0.0	0.0	0.0	0.0	0.0
ANGPTL4	0.0	1273.5	0.0	124894.5	115253.0	32992.2
B2M	6552.7	6164.9	5707.6	5941.7	5679.3	6762.4
BCAM	725.2	341.5	307.6	5264.0	4630.0	3614.6
CA125	0.0	0.0	0.0	818.4	436.9	0.0
CA15-3	0.0	0.0	0.0	0.0	0.0	0.0
CEA	0.0	0.0	0.0	13.4	66.8	0.0
CRP	0.0	0.0	0.0	0.0	0.0	0.0
ErbB2	0.0	0.0	0.0	0.0	0.0	0.0
Ferritin	0.0	0.0	0.0	0.0	204.6	446.3
FSH	0.0	0.0	0.0	0.0	0.0	0.0
GROa	1414.6	0.0	671.9	2963.2	2643.0	2509.1
hCGb	38.4	38.8	25.4	1461.3	1090.6	879.4
IGF-I SR	0.0	0.0	0.0	9.3	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	4.0	51.9	16.6
IL-18 Rb	0.0	121.2	8.1	0.0	0.0	0.0
IL-21	0.0	0.0	0.0	291.8	130.8	579.6
Leptin	0.0	0.0	0.0	0.0	2.5	0.0
MMP-1	0.0	11.1	0.0	7.1	0.0	0.0
MMP-2	0.0	1073.6	2310.0	0.0	0.0	762.0
MMP-3	0.0	0.0	0.0	0.0	0.0	34.6
MMP-8	0.0	0.0	0.0	0.0	0.0	0.0
MMP-9	26.9	33.1	25.5	166.7	143.5	55.3
MMP-10	327.5	297.4	273.5	118.9	139.9	224.2
MMP-13	0.0	0.0	0.0	0.0	0.0	0.0
NCAM-1	22970.7	23578.0	21297.5	21798.3	19761.3	20709.5
Nidogen-1	27264.2	25867.1	23986.1	26740.3	26273.5	27808.3
NSE	17533.4	11250.5	13830.9	143627.2	152368.1	139488.0
OSM	0.0	0.0	0.0	0.0	59.2	120.8
Procalcitonin	617.1	651.4	322.7	3696.6	3888.1	4414.8
Prolactin	52.1	164.7	0.0	51.1	375.4	0.0
PSA	0.0	11.2	0.0	0.0	6.5	217.0

Siglec-9	*35.0*	*51.2*	*16.0*	*48.8*	*8.9*	*36.4*
TACE	*0.0*	*0.0*	*0.0*	*95.0*	*0.0*	*0.0*
Thyroglobulin	836.9	*279.3*	*0.0*	*100.5*	*0.0*	*0.0*
TIMP-4	19671.8	16470.2	16659.1	26219.7	25614.3	25061.3
TSH	*0.0*	*0.0*	*0.0*	*21.8*	*26.2*	*48.2*

Table 7.19 RayBiotech Growth Factor Array
2102Ep Vs 2102Ep 3 Day RA, 3 Day Cisplatin

(pg/ml)	2102 1	2101 2	2102 3	2102 RA,Cis	2102 RA, Cis, 2	2102 RA, Cis 3
AR	348.9	437.8	586.9	255.7	543.5	428.8
BDNF	0.0	0.0	0.0	14.3	1.8	87.2
bFGF	5.7	324.3	0.0	222.3	52.9	78.2
BMP-4	0.0	0.0	0.0	17.9	45.4	0.0
BMP-5	0.0	0.0	0.0	0.0	0.0	0.0
BMP-7	14148.8	11138.0	10313.5	28261.3	27513.5	29349.6
b-NGF	1263.1	935.7	701.1	850.8	711.7	1279.9
EGF	13.4	6.3	5.7	17.7	10.2	27.5
EGF R	0.0	0.0	0.0	0.0	0.0	0.0
EG-VEGF	22092.9	18248.3	14501.9	7917.6	6969.7	6521.3
FGF-4	0.0	0.0	0.0	0.0	0.0	0.0
FGF-7	0.0	0.0	0.0	0.0	0.0	0.0
GDF-15	0.0	0.0	0.0	0.0	0.0	0.0
GDNF	0.0	0.0	0.0	0.0	0.0	0.0
GH	0.0	0.0	0.0	0.0	0.0	0.0
HB-EGF	0.0	0.0	0.0	0.0	0.0	0.0
HGF	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-1	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-3	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-4	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-6	0.0	0.0	0.0	14.8	166.9	0.0
IGF-I	0.0	0.0	0.0	0.0	0.0	0.0
Insulin	0.0	0.0	0.0	0.0	0.0	0.0
MCF R	40.9	40.6	21.0	132.4	24.3	74.2
NGF R	0.0	0.0	0.0	0.0	0.0	0.0
NT-3	2612.8	1867.1	2324.1	2551.5	2254.9	1818.0
NT-4	1953.5	670.9	1524.2	3402.1	4096.5	4258.9
OPG	1.8	0.0	0.0	1.0	6.3	18.7
PDGF-AA	0.0	0.0	0.0	0.0	0.0	0.0
PIGF	0.0	0.0	0.0	0.0	0.0	0.0
SCF	0.0	0.0	0.0	0.0	0.0	0.0
SCF R	25.4	0.0	1.2	5.9	19.8	11.5
TGFa	6474.8	5758.7	6308.2	2190.9	2967.1	3055.1
TGFb1	0.0	0.0	0.0	0.0	0.0	0.0

TGFb3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*60.6*
VEGF	*14.6*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF R2	*0.0*	*0.0*	*111.4*	*0.0*	*9.7*	*42.0*
VEGF R3	*0.0*	*0.0*	*0.0*	*14.2*	*7.6*	101.2
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.20 RayBiotech Cytokine Array 1
2102Ep Vs 2102Ep 3 Day RA, 3 Day Cisplatin

(pg/ml)	2102 1	2102 2	2102 3	2102 RA Cis 1	2102 RA Cis 2	2102 RA Cis 3
Activin A	3768.3	4077.0	4583.0	3500.8	4434.2	4039.4
AgRP	*0.0*	*0.0*	*0.0*	*21.9*	*1.1*	136.1
ANG	14.9	18.9	11.3	17.9	15.4	15.8
ANG-1	3350.2	2799.8	2806.1	4236.3	4299.5	3095.4
Angiostatin	*329.7*	*0.0*	*0.0*	*705.4*	*75.8*	*75.7*
Catheprin S	3158.6	2502.4	2316.9	6318.6	6054.3	6458.8
CD 40	1279.7	989.2	779.2	927.7	789.0	1292.6
Cripto-1	125.3	71.2	66.2	159.5	100.5	231.5
DAN	569.5	447.8	528.7	563.1	346.2	356.3
DKK-1	**319473.9**	**265857.1**	**213140.2**	123754.1	108571.1	102430.4
E-Cadherin	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
EpCAM	149.6	98.8	72.2	156.1	125.8	105.1
FAS L	87.0	86.8	88.1	98.3	93.7	93.5
Fcr RIIB/C	60.3	40.3	67.0	67.5	60.4	49.2
Follistatin	*121.4*	*109.2*	*119.8*	*127.0*	*117.6*	*169.1*
Galectin-7	1195.1	991.3	932.1	785.5	1060.6	1204.8
ICAM-2	*352.8*	*0.0*	*0.0*	*424.2*	*257.1*	*375.1*
IL-13 R1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IL-13 R2	*38.0*	*0.0*	*33.9*	*0.0*	*0.0*	*11.0*
IL-17B	2052.4	1597.3	1714.6	1578.7	2069.9	1305.8
IL-2 Ra	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IL-2 Rb	1457.0	1457.0	1056.0	2527.7	3555.7	1729.1
IL-23	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
LAP	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
NrCAM	141.2	140.9	127.3	206.1	129.6	163.4
PAI-I	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
PDGF-AB	1186.1	869.6	1060.4	1177.8	1031.6	847.6
Resistin	14575.7	4964.6	11326.6	25786.7	30559.6	31800.5
SDF-1b	272.4	225.4	221.9	264.0	339.0	525.7
sgp130	744.3	*580.7*	*609.2*	1268.3	1211.4	945.5
Shh N	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
Siglec-5	49.2	48.7	49.7	*24.7*	39.2	45.5
ST2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
TGF-b2	42704.2	37971.5	41488.7	14653.4	19522.1	20118.9
Tie-2	168.1	119.7	159.2	66.9	118.9	212.6

TPO	*0.0*	*0.0*	*0.0*	*0.0*	*14.4*	*95.1*
TRAIL-R4	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
TREM-1	739.9	455.6	3136.2	590.0	1080.3	1735.6
VEGF-C	*30.3*	*1.4*	*10.8*	*58.1*	*48.1*	161.2
VEGF R1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*93.6*

Table 7.21 RayBiotech Cytokine Array 2
2102Ep Vs 2102Ep 3 Day RA, 3 Day Cisplatin

(pg/ml)	2102 1	2102 2	2102 3	2102 RA Cis 1	2102 RA Cis 2	2102 RA Cis 3
Adiponectin	1302.1	1394.6	1549.0	1208.5	1503.9	1384.8
Adipsin	58.9	22.9	19.3	226.4	123.4	895.9
AFP	0.0	2.0	0.0	0.0	0.0	0.0
ANGPTL4	54098.1	45786.8	45987.3	66692.0	68591.0	50312.9
B2M	1351.1	1120.6	881.8	1493.4	1245.8	1244.9
BCAM	16984.7	13215.1	12176.1	34751.2	33697.2	36001.1
CA125	9593.4	7198.5	5479.4	6600.8	5557.7	9713.0
CA15-3	33272.1	7843.6	5571.6	48452.9	21720.4	94750.6
CEA	0.0	0.0	0.0	0.0	0.0	0.0
CRP	22154.9	18344.1	14623.8	8132.6	7164.8	6722.0
ErbB2	101.5	73.8	84.5	100.0	90.4	83.4
Ferritin	0.0	0.0	0.0	0.0	0.0	0.0
FSH	0.0	0.0	0.0	0.0	0.0	0.0
GROa	0.0	0.0	0.0	0.0	0.0	0.0
hCGb	27.7	22.8	27.4	28.3	26.4	46.9
IGF-I SR	149.8	113.1	0.0	0.0	76.1	156.2
IL-1 sRII	0.0	0.0	0.0	5.7	0.0	0.0
IL-3	0.0	0.0	0.0	0.0	0.0	0.0
IL-18 Rb	0.0	0.0	0.0	0.0	0.0	0.0
IL-21	0.0	0.0	0.0	0.0	0.0	0.0
Leptin	15.2	1.6	0.0	26.9	3.7	25.0
MMP-1	98.1	98.2	71.2	168.3	241.7	116.9
MMP-2	0.0	0.0	0.0	0.0	0.0	0.0
MMP-3	0.0	0.0	0.0	0.0	0.0	0.0
MMP-8	0.0	0.0	0.0	0.0	0.0	0.0
MMP-9	12.9	4.3	9.5	26.2	2.1	9.3
MMP-10	167.1	120.6	149.0	163.8	144.7	117.6
MMP-13	3020.1	1061.5	2363.2	5246.8	6289.5	6538.4
NCAM-1	60.4	0.0	0.0	31.6	220.0	661.7
Nidogen-1	0.0	0.0	0.0	0.0	0.0	0.0
NSE	0.0	0.0	0.0	0.0	0.0	0.0
OSM	516.1	508.9	527.9	85.1	346.8	454.1
Procalcitonin	0.0	0.0	0.0	0.0	0.0	0.0
Prolactin	120600.9	107057.3	117383.7	39730.3	54258.8	55932.7
PSA	0.0	0.0	0.0	0.0	0.0	0.0

Siglec-9	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
TACE	*118.8*	*20.9*	*40.8*	*0.0*	*0.0*	*0.0*
Thyroglobulin	*0.0*	*0.0*	3309.9	*0.0*	*0.0*	929.8
TIMP-4	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*9.3*
TSH	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.22 RayBiotech Growth Factor Array
NTera2 Vs NTera2 3 Day RA, 3 Day Cisplatin

(pg/ml)	Ntera 1	NTera 2	nTera 3	Ntera RA, cis 1	NTera RA, Cis 2	NTera Ra, Cis 2
AR	416.7	519.8	1118.1	255.3	282.6	246.1
BDNF	0.0	0.0	0.0	0.0	0.0	0.0
bFGF	862.4	10.6	0.0	136.3	108.2	0.0
BMP-4	0.0	0.0	2973.0	0.0	5.1	0.0
BMP-5	0.0	0.0	0.0	0.0	0.0	0.0
BMP-7	1419.3	669.5	643.7	1012.3	950.0	942.7
b-NGF	0.0	1.3	553.1	4.0	13.2	1.4
EGF	12.4	3.6	3.2	2.5	1.3	0.1
EGF R	0.0	0.0	0.0	75.1	133.9	167.3
EG-VEGF	2369.1	2373.4	2078.5	6327.5	7600.3	9748.9
FGF-4	0.0	0.0	0.0	0.0	0.0	0.0
FGF-7	0.0	0.0	0.0	0.0	0.0	0.0
GDF-15	0.0	22.1	59.9	0.0	13.5	2.8
GDNF	0.0	0.0	0.0	0.0	0.0	0.0
GH	0.0	17.4	0.0	0.0	0.0	0.0
HB-EGF	0.0	1.1	0.0	0.0	0.0	0.0
HGF	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-1	7.1	8.3	0.0	0.0	0.0	0.0
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-3	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-4	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-6	0.0	0.0	0.0	0.0	0.0	0.0
IGF-I	0.0	0.0	0.0	0.0	0.0	0.0
Insulin	0.0	0.0	0.0	0.0	0.0	0.0
MCF R	0.0	0.0	27.5	20.6	23.8	0.0
NGF R	0.0	0.0	0.0	0.0	0.0	0.0
NT-3	6577.8	4869.8	6181.8	0.0	0.0	424.4
NT-4	1150.6	427.5	1166.5	1125.6	1212.7	1353.2
OPG	0.0	0.0	0.0	8.8	4.0	0.1
PDGF-AA	0.0	0.0	0.0	0.0	0.0	0.0
PIGF	0.0	0.0	0.0	0.0	0.0	0.0
SCF	0.0	11.5	0.0	0.0	0.0	0.0
SCF R	0.0	12.4	0.0	0.0	0.0	0.0
TGFa	3673.2	2533.7	3051.2	3866.5	3743.7	3854.9
TGFb1	0.0	71.6	0.0	0.0	0.0	0.0

TGFb3	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF	*0.0*	1695.0	*41.6*	*0.0*	*0.0*	*0.0*
VEGF R2	*0.0*	*0.0*	*0.0*	*16.6*	*0.0*	*48.2*
VEGF R3	*0.0*	*15.0*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.23 RayBiotech Cytokine Array 1
NTera2 Vs NTera2 3 Day RA, 3 Day Cisplatin

(pg/ml)	NTera 1	NTera 2	NTera 3	Nter RA Cis 1	Nter RA Cis 2	NTera RA Cis 3
Activin A	4006.8	4368.0	6461.7	3431.8	3530.6	3405.4
AgRP	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
ANG	25.6	15.0	12.5	16.5	16.1	13.6
ANG-1	3185.5	3185.1	19397.5	2672.5	4096.5	3953.1
Angiostatin	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
Catheprin S	387.0	223.7	218.3	297.3	284.0	282.6
CD 40	147.9	161.5	651.7	163.2	171.6	161.2
Cripto-1	117.8	50.9	47.6	42.5	33.1	24.4
DAN	205.7	509.4	380.6	917.4	1111.9	1222.6
DKK-1	44778.2	44853.1	40784.5	99634.5	117429.3	147373.3
E-Cadherin	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
EpCAM	93.0	138.2	113.9	117.7	112.0	83.8
FAS L	86.1	113.1	138.2	96.7	107.1	100.2
Fcr RIIB/C	63.7	37.1	44.1	36.8	28.2	40.8
Follistatin	*129.6*	264.5	*156.3*	*158.0*	*174.0*	*168.2*
Galectin-7	995.6	1540.0	982.3	1023.9	1411.1	1058.8
ICAM-2	*0.0*	*62.2*	*0.0*	*0.0*	*0.0*	*0.0*
IL-13 R1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IL-13 R2	*0.0*	*12.5*	*0.0*	*0.0*	*0.0*	*0.0*
IL-17B	1445.8	1915.5	2026.3	1273.2	1666.2	1177.0
IL-2 Ra	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IL-2 Rb	1065.9	1195.9	1336.2	1643.8	1957.1	1724.6
IL-23	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
LAP	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
NrCAM	90.1	96.4	132.4	127.1	129.4	102.5
PAI-I	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
PDGF-AB	2869.3	2145.3	2705.0	*0.0*	*0.0*	257.6
Resistin	8564.2	3144.5	8695.6	8351.2	9010.9	10065.9
SDF-1b	*140.5*	*168.5*	*154.3*	376.5	304.6	245.8
sgp130	705.2	*265.9*	*657.5*	969.3	961.3	*609.7*
Shh N	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
Siglec-5	*24.4*	83.2	51.4	38.3	49.8	41.8
ST2	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
TGF-b2	24238.8	16724.6	20160.7	25436.7	24652.7	25395.8

Tie-2	77.0	262.2	160.2	175.4	*0.0*	173.8
TPO	*0.0*	*5.0*	*0.0*	*0.0*	*0.0*	*0.0*
TRAIL-R4	*0.0*	542.5	*0.0*	*0.0*	*0.0*	*0.0*
TREM-1	*0.0*	844.7	528.3	1219.8	582.0	1862.3
VEGF-C	*0.0*	*57.3*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF R1	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*23.4*

Table 7.24 RayBiotech Cytokine Array 2
NTera2 Vs NTera2 3 Day RA, 3 Day Cisplatin

(pg/ml)	NTera 1	NTera 2	NTera 3	Nter RA Cis 1	Nter RA Cis 2	NTera RA Cis 3
Adiponectin	1372.8	1480.3	2103.8	1204.0	1232.6	1194.7
Adipsin	38.4	22.6	16.8	43.5	40.8	6.8
AFP	47.4	0.0	0.0	0.0	0.0	0.0
ANGPTL4	51584.1	46565.6	296288.8	43950.3	65471.4	63276.8
B2M	1069.5	1078.6	1146.3	1136.8	1156.7	1054.3
BCAM	1050.6	112.0	80.0	539.9	462.2	453.4
CA125	114.7	366.3	3796.4	385.0	453.0	366.5
CA15-3	29724.7	0.0	0.0	0.0	0.0	0.0
CEA	0.0	0.0	0.0	0.0	0.0	0.0
CRP	2610.1	2614.6	2322.9	6528.6	7790.8	9920.2
ErbB2	56.0	70.7	72.7	70.9	70.2	70.8
Ferritin	0.0	0.0	0.0	0.0	0.0	0.0
FSH	0.0	0.0	8.4	0.0	0.0	0.0
GROa	0.0	0.0	0.0	0.0	0.0	0.0
hCGb	30.9	84.6	41.4	42.6	48.8	46.5
IGF-I SR	38.1	341.7	30.0	55.7	271.6	74.4
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	0.0	0.0	0.0
IL-18 Rb	0.0	0.0	0.0	0.0	0.0	0.0
IL-21	0.0	0.0	0.0	0.0	0.0	0.0
Leptin	4.4	21.0	6.7	7.4	6.1	0.0
MMP-1	71.4	80.2	73.7	111.2	132.5	116.5
MMP-2	0.0	0.0	0.0	0.0	0.0	0.0
MMP-3	0.0	0.0	0.0	0.0	0.0	0.0
MMP-8	0.0	0.0	0.0	0.0	0.0	0.0
MMP-9	4.3	0.0	0.0	0.0	0.0	1.0
MMP-10	413.8	307.6	389.3	0.0	0.0	30.1
MMP-13	1794.2	689.9	1819.1	1755.0	1888.3	2102.9
NCAM-1	0.0	0.0	0.0	309.1	137.9	0.0
Nidogen-1	0.0	0.0	0.0	0.0	0.0	0.0
NSE	0.0	0.0	0.0	0.0	0.0	0.0
OSM	90.8	1099.4	553.0	332.1	528.2	390.9
Procalcitonin	0.0	0.0	0.0	0.0	0.0	0.0
Prolactin	67643.9	46101.5	55898.0	71252.1	68947.1	71054.7

PSA	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
Siglec-9	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
TACE	*58.6*	3423.2	*129.8*	*0.0*	*0.0*	*0.0*
Thyroglobulin	*0.0*	*0.0*	*0.0*	*486.9*	*0.0*	2480.8
TIMP-4	*0.0*	*0.0*	*0.0*	294.5	*0.0*	*0.0*
TSH	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.25 RayBiotech Growth Factor Array
2102Ep Vs 2102Ep 3 Day siMyD88 Treatment

(pg/ml)	2102 1	2102 2	2102 3	2102 MyKD 1	2102 MyKD 2	2102 MyKD 3	2102 Neg 1	2102 Neg 2	2102Neg 3
AR	348.9	437.8	586.9	79.3	219.9	150.4	465.2	154.5	487.2
BDNF	0.0	0.0	0.0	0.0	4.3	0.0	0.0	0.0	0.0
bFGF	5.7	324.3	0.0	0.0	223.5	492.5	211.3	0.0	0.0
BMP-4	0.0	0.0	0.0	0.0	150.1	470.7	2.7	0.0	22.1
BMP-5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BMP-7	14148.8	11138.0	10313.5	17943.5	19903.0	16526.6	16313.1	14930.4	15340.0
b-NGF	1263.1	935.7	701.1	6433.1	6867.5	5558.8	4758.8	4668.3	4850.3
EGF	13.4	6.3	5.7	53.2	34.9	39.3	59.7	43.7	4.4
EGF R	0.0	0.0	0.0	0.0	6.6	0.0	0.0	0.0	0.0
EG-VEGF	22092.9	18248.3	14501.9	21002.2	22396.9	15524.0	10199.8	9690.8	12395.2
FGF-4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FGF-7	0.0	0.0	0.0	0.0	9.1	15.3	0.0	0.0	0.0
GDF-15	0.0	0.0	0.0	5.1	1.9	0.0	6.7	0.0	9.7
GDNF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GH	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
HB-EGF	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
HGF	0.0	0.0	0.0	0.0	1.8	45.5	0.0	0.0	0.0
IGFBP-1	0.0	0.0	0.0	0.8	6.0	0.0	0.0	0.0	0.0
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

IGFBP-3	*0.0*	*0.0*	*0.0*	*0.0*	*402.2*	*0.0*	*66.7*	*0.0*	*0.0*
IGFBP-4	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
IGFBP-6	*0.0*	*0.0*	*0.0*	*0.0*	*32.9*	*0.0*	*63.1*	*0.0*	*0.0*
IGF-I	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
Insulin	*0.0*	*0.0*	*0.0*	*0.0*	*5.0*	*0.0*	*0.0*	*0.0*	*0.0*
MCF R	*40.9*	*40.6*	*21.0*	*68.5*	*89.7*	*58.7*	*103.6*	*48.2*	*43.7*
NGF R	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
NT-3	2612.8	1867.1	2324.1	3152.5	2922.5	2407.1	2793.7	3207.6	1685.1
NT-4	1953.5	670.9	1524.2	497.1	2116.5	1670.6	525.4	1907.5	2146.0
OPG	*1.8*	*0.0*	*0.0*	*6.2*	*11.3*	*3.2*	*6.1*	*4.5*	*3.6*
PDGF-AA	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
PIGF	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
SCF	*0.0*	*0.0*	*0.0*	*0.0*	*3.9*	*0.0*	*0.0*	*0.0*	*0.0*
SCF R	*25.4*	*0.0*	*1.2*	*38.6*	*46.7*	*22.8*	*16.5*	*10.3*	*11.1*
TGFa	6474.8	5758.7	6308.2	7995.9	10293.1	6352.1	7276.8	7518.3	7862.9
TGFb1	*0.0*	*0.0*	*0.0*	*0.0*	*115.2*	*0.0*	*0.0*	*0.0*	*0.0*
TGFb3	*0.0*	*0.0*	*0.0*	*150.4*	*372.3*	*0.0*	*0.0*	*0.0*	*0.0*
VEGF	*14.6*	*0.0*	*0.0*	72.3	195.8	*0.0*	*0.0*	*0.0*	95.8
VEGF R2	*0.0*	*0.0*	*111.4*	*56.6*	*115.1*	*0.0*	*22.2*	*3.6*	*35.2*
VEGF R3	*0.0*	*0.0*	*0.0*	1442.2	1460.2	445.8	470.4	752.2	1190.5
VEGF-D	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*

Table 7.26 RayBiotech Cytokine Array 1
2102Ep Vs 2102Ep 3 Day siMyD88 Treatment

(pg/ml)	2102 1	2102 2	2102 3	MyD88 KD 1	MyD88 KD 2	MyD88 KD 3	Neg Si 1	Neg Si 2	NegSi 3
Activin A	*46.9*	*0.0*	*0.0*	1209.3	1570.8	1736.8	2109.0	1115.8	1662.7
AgRP	*0.0*	*0.0*	*7.2*	63.9	70.7	59.1	89.9	81.3	77.5
ANG	458.5	606.7	655.7	1309.5	1360.3	1272.9	1120.7	1062.7	959.4
ANG-1	*201.9*	521.9	*117.4*	3111.8	2188.0	2620.7	2637.5	1444.4	1577.7
Angiostatin	*559.4*	*774.9*	*641.6*	*4188.5*	*4034.7*	*3486.7*	*4081.2*	*3829.1*	*4752.5*
Catheprin S	*11.3*	*20.4*	*4.9*	*36.8*	102.8	71.5	116.4	56.9	68.5
CD 40	*0.0*	*11.9*	*0.0*	201.8	217.3	172.5	261.0	191.5	140.5
Cripto-1	7600.7	9804.7	10216.9	9467.4	8086.0	10222.6	9867.0	10034.8	8749.7
DAN	*26.6*	*24.3*	*0.0*	1456.8	893.7	640.5	884.8	455.3	490.8
DKK-1	*0.0*	*527.2*	*183.3*	4588.1	4996.0	4819.4	4417.1	3660.3	3116.8
E-Cadherin	*187.8*	*595.1*	*317.3*	4181.4	2578.7	1514.8	7783.3	3430.7	5539.4
EpCAM	321.8	283.2	375.1	448.6	369.0	578.1	390.9	371.3	332.4
FAS L	*4.1*	*4.5*	*2.1*	59.9	52.0	40.8	60.0	33.4	28.5
Fcr RIIB/C	*4.0*	*5.9*	*6.5*	150.9	56.9	53.1	127.8	55.2	112.2
Follistatin	4397.6	4704.4	5252.7	14253.6	13890.7	12616.3	12639.8	11642.9	10706.5
Galectin-7	1184.5	3101.7	2211.7	3985.1	5008.1	5064.4	3791.9	2267.7	2536.5
ICAM-2	*273.7*	*282.6*	*324.0*	4526.0	4287.9	2223.4	4296.6	2515.6	2203.0
IL-13 R1	219.3	391.3	*173.4*	5764.3	5905.3	7199.6	8636.9	5530.8	5427.5
IL-13 R2	*37.5*	*235.1*	*85.1*	1222.0	1034.4	1400.6	1649.3	1494.4	1038.4

IL-17B	*112.4*	*128.3*	*0.0*	1944.7	1827.6	828.7	1488.7	774.7	815.5
IL-2 Ra	*17.4*	*74.7*	*44.5*	278.4	134.4	147.9	294.8	152.7	297.8
IL-2 Rb	*121.6*	*697.0*	*217.5*	3051.2	2424.0	2723.0	4467.0	1908.7	2135.9
IL-23	*0.0*	*0.0*	*0.0*	717.0	1178.2	467.0	826.5	560.1	600.1
LAP	165.9	218.3	210.1	609.6	503.9	535.8	578.5	408.8	331.7
NrCAM	*17.8*	*18.5*	*3.0*	124.8	133.1	101.5	130.3	72.3	66.1
PAI-I	19631.2	25745.0	31629.7	37014.5	37573.8	34569.1	36249.7	39718.7	34583.9
PDGF-AB	349.3	505.4	509.3	935.5	877.1	957.7	1067.1	939.7	874.9
Resistin	*21.3*	154.3	*114.5*	919.9	2499.4	1024.3	1726.5	961.8	541.3
SDF-1b	*0.0*	*17.8*	*0.0*	744.0	536.8	453.5	882.3	590.6	455.5
sgp130	*0.0*	*136.0*	*0.0*	914.8	795.7	975.3	1542.9	*361.7*	704.2
Shh N	*0.0*	*0.0*	*0.0*	150.8	129.9	102.7	191.4	*58.2*	*0.0*
Siglec-5	456.6	535.2	756.8	1956.8	1499.8	1556.7	1805.7	1875.4	1494.1
ST2	*0.0*	*0.0*	*0.0*	68.9	80.3	*28.1*	65.6	*27.1*	*15.4*
TGF-b2	*0.0*	*31.0*	*0.0*	788.4	1660.6	454.4	940.8	575.2	703.5
Tie-2	*0.0*	*0.0*	*0.0*	431.9	398.4	192.8	429.2	246.3	112.9
TPO	*31.3*	*50.4*	*30.4*	1412.4	1545.5	955.1	1144.0	888.9	612.9
TRAIL-R4	*0.0*	*10.6*	*0.0*	312.4	292.0	134.7	307.9	157.6	58.8
TREM-1	*0.0*	*0.0*	*0.0*	1024.0	1622.6	*0.0*	*0.0*	*88.6*	541.9
VEGF-C	*0.0*	*0.0*	*0.0*	209.8	224.1	*90.5*	186.5	*118.7*	*123.3*
VEGF R1	865.1	1597.9	1195.9	3187.7	4200.0	3384.1	3991.9	4267.3	3110.4

Table 7.27 RayBiotech Cytokine Array 2
2102Ep Vs 2102Ep 3 Day siMyD88 Treatment

(pg/ml)	2102 1	2102 2	2102 3	MyD88 KD 1	MyD88 KD 2	MyD88 KD 3	siNeg 1	siNeg 2	siNeg3
Adiponectin	13.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Adipsin	46.1	0.0	13.0	145.7	134.4	231.2	261.7	195.7	204.5
AFP	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ANGPTL4	76082.8	50848.3	52577.4	134888.7	136948.1	163700.2	131344.6	118520.0	127522.3
B2M	5474.7	6463.1	6615.4	15035.7	16670.5	15384.2	13980.7	12874.5	15299.2
BCAM	597.8	760.3	1641.3	1895.6	2886.6	2324.4	2639.9	2396.9	2659.6
CA125	0.0	0.0	10.6	205.7	0.0	0.0	0.0	797.3	0.0
CA15-3	0.0	659.8	0.0	314.0	0.0	0.0	0.0	0.0	0.0
CEA	0.0	26.3	6.5	630.5	240.6	0.0	297.8	50.8	398.9
CRP	0.0	0.0	0.0	722.2	509.3	429.8	457.7	473.2	602.4
ErbB2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ferritin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	560.9
FSH	0.0	0.4	0.0	0.0	0.0	9.3	0.0	2.8	0.0
GROa	1999.8	6878.8	9381.0	1994.1	3981.2	3630.9	5388.6	3805.5	4818.4
hCGb	16.0	42.2	88.9	115.9	103.3	91.5	101.4	132.6	138.3
IGF-I SR	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	9.7	0.0	0.0	0.0	0.0	0.0

IL-18 Rb	*0.0*	*31.7*	*28.7*	*0.0*	*0.0*	*0.0*	*0.0*	*96.7*	*0.0*
IL-21	*271.5*	*0.0*	*0.0*	459.0	433.9	*11.9*	673.7	*0.0*	*310.4*
Leptin	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	80.8	*0.0*
MMP-1	*0.0*	*16.5*	*12.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
MMP-2	*0.0*	*0.0*	*0.0*	*0.0*	1163.0	1251.7	2182.1	*824.0*	1054.2
MMP-3	*174.3*	*317.5*	460.7	*8.9*	*156.1*	*0.0*	*2.3*	*0.0*	*37.8*
MMP-8	*0.0*	*6.8*	*2.9*	951.6	845.2	882.9	770.4	731.4	928.9
MMP-9	90.6	79.4	131.8	2345.0	2172.3	2381.6	2300.6	1984.9	2569.7
MMP-10	78.7	79.6	114.6	84.7	103.6	67.5	81.6	69.0	90.7
MMP-13	*0.0*	*2.2*	*1.4*	*0.0*	*0.0*	*4.2*	*0.0*	*0.0*	*0.0*
NCAM-1	16560.0	20872.5	22554.2	20284.2	24717.8	21446.2	21975.2	20485.7	24838.3
Nidogen-1	17374.8	19889.6	20901.8	25021.1	26619.3	23262.0	24377.7	23754.7	27123.1
NSE	32187.8	36130.1	60173.2	99541.9	93645.3	116247.4	95033.7	92345.9	105282.3
OSM	*85.0*	*0.0*	*0.0*	*0.0*	625.2	*0.0*	*0.0*	*0.0*	*0.0*
Procalcitonin	*0.0*	*372.8*	*436.5*	1192.3	2323.3	*796.0*	*488.3*	*318.2*	*441.1*
Prolactin	*0.0*	*166.7*	*227.3*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
PSA	*0.0*	*0.0*	*0.0*	*0.0*	*321.1*	*0.0*	*0.0*	*0.0*	*0.0*
Siglec-9	*18.3*	*72.4*	*52.0*	*0.0*	*121.1*	363.3	*0.0*	*36.4*	*0.0*
TACE	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*
Thyroglobulin	*70.8*	*0.0*	*242.1*	*0.0*	*424.8*	1327.7	*0.0*	*0.0*	*0.0*
TIMP-4	18490.8	21808.5	22602.7	25039.2	23783.2	22320.1	21808.5	21243.8	23843.5
TSH	*0.0*	*3.0*	*0.0*	*0.0*	*0.0*	*0.0*	*0.0*	*6.6*	92.4

Table 7.28 RayBiotech Growth Factor Array
2102Ep Vs 2102Ep 3 Day siMyD88 Treatment +/-RA

(pg/ml)	Neg RA 1	Neg RA 2	Neg RA 3	MyD88 RA 1	MyD88 RA 2	MyD88 RA 2	Neg -RA 1	Neg -RA 2	Neg-RA 3	MyD88 - RA 1	MyD88-RA 2	MyD88 - RA3
AR	266.1	138.5	188.7	281.3	358.1	543.3	156.9	189.4	143.6	303.4	206.3	209.7
BDNF	0.0	0.0	0.0	6.5	0.0	0.0	0.0	1.3	0.0	3.9	4.2	12.6
bFGF	73.8	0.0	0.0	0.0	0.0	0.0	17.0	0.0	0.0	0.0	28.2	723.3
BMP-4	0.0	0.0	0.0	0.0	0.0	21.5	0.0	0.0	0.0	0.0	0.0	152.8
BMP-5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	41.6
BMP-7	5491.7	6852.3	11173.1	8771.6	7175.7	8043.4	10497.5	8513.3	11495.2	9898.3	7085.6	4346.2
b-NGF	0.0	0.0	0.0	0.0	0.0	4.2	0.0	0.0	22.5	0.0	21.9	57.6
EGF	0.4	0.6	0.5	0.4	0.0	0.6	0.9	0.8	0.4	1.2	0.5	1.6
EGF R	0.0	0.0	0.0	0.0	0.0	8.9	0.0	0.0	0.0	0.0	29.8	101.7
EG-VEGF	0.0	0.0	21.9	16.9	0.0	70.9	12.9	0.0	67.8	0.0	0.0	87.3
FGF-4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.4
FGF-7	0.0	0.0	0.0	0.0	0.0	4.0	4.7	5.4	0.0	9.6	6.0	26.2
GDF-15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	16.6
GDNF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
GH	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3
HB-EGF	0.0	0.0	0.0	0.0	0.0	1.1	0.2	1.9	0.0	4.1	2.2	5.1
HGF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15.3
IGFBP-1	0.0	0.0	0.0	1.2	0.0	0.0	1.3	3.3	0.0	1.8	4.7	14.2
IGFBP-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	833.0

IGFBP-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	64.0
IGFBP-4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IGFBP-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	17.6
IGF-I	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Insulin	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	0.0	10.9
MCF R	14.3	0.0	2.0	42.3	0.0	16.5	0.0	0.0	0.0	0.0	16.7	126.7
NGF R	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	1.1	2.8
NT-3	57.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	294.9
NT-4	453.6	568.1	489.2	738.9	507.3	390.3	726.7	667.4	696.2	833.7	642.6	49.3
OPG	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
PDGF-AA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7
PIGF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	6.7
SCF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SCF R	0.0	0.0	1.6	2.7	0.0	14.4	5.7	5.8	1.9	18.2	8.8	25.4
TGFa	213.7	281.6	327.6	335.0	270.9	441.8	362.8	339.0	339.4	390.0	380.9	50.1
TGFb1	0.0	0.0	0.0	16.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1046.3
TGFb3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	96.3	0.0	574.4
VEGF	73.4	56.8	57.3	23.2	0.0	115.8	38.1	27.2	64.8	76.1	209.5	206.5
VEGF R2	2.3	9.8	0.0	0.0	6.9	0.0	0.0	0.0	0.0	0.0	0.0	80.9
VEGF R3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1
VEGF-D	0.0	0.0	0.0	68.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 7.29 RayBiotech Cytokine Array 1
2102Ep Vs 2102Ep 3 Day siMyD88 Treatment +/-RA

(pg/ml)	Neg RA 1	Neg RA 2	Neg RA 3	MyD88 RA 1	MyD88 RA 2	MyD88 RA 2	Neg -RA 1	Neg -RA 2	Neg-RA 3	MyD88 - RA 1	MyD88-RA 2	MyD88 - RA3
Activin A	37.3	138.1	0.0	0.0	0.0	0.0	6.2	0.0	131.8	52.7	0.0	68.5
AgRP	4.1	11.4	5.0	0.7	0.8	1.4	6.3	6.2	3.9	2.5	0.0	0.0
ANG	626.8	852.0	907.7	868.7	1034.9	422.2	603.3	630.2	881.4	624.0	438.0	368.5
ANG-1	287.7	259.0	186.2	86.9	272.4	280.9	258.8	340.6	238.8	229.7	80.1	86.5
Angiostatin	1286.8	1308.6	838.0	1225.7	1252.8	893.3	1480.5	1464.6	519.7	1197.9	751.9	988.4
Catheprin S	32.9	22.4	9.0	21.7	18.5	11.9	8.6	11.6	23.3	13.2	3.8	3.4
CD 40	109.9	39.2	0.0	17.0	15.5	121.1	36.0	11.6	28.0	13.5	19.5	0.0
Cripto-1	29.5	17.7	20.0	27.7	24.1	39.3	29.2	29.4	38.3	29.4	41.9	33.2
DAN	66.6	37.3	23.8	80.7	67.5	35.5	31.3	0.0	130.1	15.4	52.5	0.0
DKK-1	1985.4	2358.6	202.3	135.7	401.5	170.9	2067.1	2534.7	791.2	548.1	716.9	65.0
E-Cadherin	342.5	81.4	0.0	973.7	0.0	0.0	0.0	1123.6	263.6	62.5	615.8	0.0
EpCAM	668.7	859.7	862.7	1109.9	890.7	335.1	535.1	580.7	871.3	834.7	453.4	263.0
FAS L	7.6	8.7	4.0	3.8	4.0	3.6	9.5	9.6	2.6	1.7	2.7	3.8
Fcr RIIB/C	13.1	7.3	9.2	10.1	7.2	5.0	9.2	10.5	8.5	9.0	10.2	8.6
Follistatin	8193.7	8040.0	7543.6	10494.4	7683.3	6434.6	6437.4	7770.2	10689.5	6772.5	5900.5	4038.3
Galectin-7	502.6	619.5	653.0	682.6	517.3	383.7	492.2	518.9	736.4	356.2	231.4	85.3
ICAM-2	753.2	700.4	569.4	352.1	449.3	503.1	406.4	580.7	857.9	475.7	287.3	313.1
IL-13 R1	889.7	1306.8	810.0	530.1	310.4	0.0	592.1	591.3	491.2	166.2	0.0	261.9
IL-13 R2	507.3	334.6	349.3	288.0	249.2	23.6	457.8	185.8	246.9	250.2	110.0	451.0
IL-17B	178.9	183.3	82.5	0.0	70.7	47.9	82.5	109.5	154.4	32.8	16.2	151.2

IL-2 Ra	30.7	78.2	83.4	64.8	65.4	75.4	96.1	92.4	77.2	68.2	40.1	59.0
IL-2 Rb	383.7	626.5	513.1	530.4	502.9	364.7	365.3	358.7	531.1	392.0	217.1	297.7
IL-23	0.0	33.6	39.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LAP	154.0	349.1	326.4	395.2	268.9	177.7	280.7	362.0	456.9	578.1	251.5	198.0
NrCAM	13.5	16.0	23.2	7.1	7.7	7.3	12.9	6.3	10.7	8.4	0.0	0.0
PAI-I	24461.1	28399.4	28200.8	28394.5	27494.5	30891.5	28544.0	34792.3	37277.4	31024.0	26548.5	19318.0
PDGF-AB	121.9	147.6	149.2	155.5	171.7	179.2	125.0	141.3	143.3	107.8	92.8	78.6
Resistin	87.9	104.1	160.4	146.0	103.0	45.0	191.1	162.5	40.3	81.4	74.1	359.2
SDF-1b	2.2	20.7	0.0	16.3	7.7	86.1	0.0	13.9	27.9	5.8	0.0	4.4
sgp130	775.4	1299.6	1053.9	1081.5	1134.9	521.3	69.2	583.4	958.9	548.9	0.0	374.1
Shh N	0.0	0.0	0.0	48.3	0.0	0.0	17.0	0.0	2.7	30.6	0.0	0.0
Siglec-5	936.8	1316.2	1444.4	1371.8	1621.5	896.9	1120.8	1077.6	1296.0	974.5	577.6	350.0
ST2	0.0	14.4	8.6	2.1	0.0	0.0	0.0	6.5	7.3	0.0	0.0	0.0
TGF-b2	36.0	105.3	78.2	69.8	35.3	32.2	63.2	98.5	81.7	51.6	0.0	0.0
Tie-2	0.0	43.5	0.0	59.5	30.5	0.0	0.0	0.0	129.4	20.9	8.9	0.0
TPO	106.0	148.0	128.6	73.7	87.2	60.8	11.5	107.4	126.0	149.0	98.6	0.0
TRAIL-R4	3.4	7.3	36.5	0.0	0.0	0.0	8.7	160.4	0.0	14.2	21.1	0.0
TREM-1	0.0	40.3	109.7	26.9	59.8	0.0	0.0	79.7	0.0	115.1	147.9	0.0
VEGF-C	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VEGF R1	4688.0	6431.8	5968.0	5406.1	5646.6	#REF!	3256.7	3636.9	3347.2	2987.6	3123.1	1434.7

Table 7.30 RayBiotech Cytokine Array 2
2102Ep Vs 2102Ep 3 Day siMyD88 Treatment +/-RA

(pg/ml)	Neg RA 1	Neg RA 2	Neg RA 3	MyD88 RA 1	MyD88 RA 2	MyD88 RA 2	Neg -RA 1	Neg -RA 2	Neg-RA 3	MyD88 - RA 1	MyD88-RA 2	MyD88 - RA3
Adiponectin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Adipsin	215.1	267.7	383.5	313.5	318.9	93.1	227.9	222.2	262.8	80.2	59.0	13.7
AFP	2.6	0.0	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	0.0
ANGPTL4	6640.2	3227.2	20788.0	0.0	4492.3	7133.9	18820.2	14912.9	16066.9	3622.4	2477.6	758.9
B2M	6448.9	6555.9	6957.6	6057.6	7194.8	7341.3	6964.0	6834.8	7122.9	6605.0	7852.5	7812.0
BCAM	2269.1	2101.1	4338.7	2225.9	3117.3	2370.6	2813.4	3347.1	3854.4	1464.4	1922.7	1035.2
CA125	392.2	230.6	625.6	458.5	401.3	0.0	589.4	716.9	696.2	0.0	0.0	0.0
CA15-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2097.2	2065.0
CEA	0.0	0.0	0.0	73.7	194.0	22.0	0.0	0.0	0.0	0.0	30.3	0.0
CRP	0.0	0.0	0.0	46.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
ErbB2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ferritin	0.0	0.0	0.0	2505.9	2289.2	3328.2	198.2	611.7	1647.6	2989.1	3350.1	2966.0
FSH	0.0	0.3	0.8	0.0	8.7	3.4	0.6	0.0	0.0	1.2	1.6	0.0
GROa	517.8	0.0	462.9	1266.3	1958.0	948.3	0.0	0.0	626.6	991.6	272.3	657.4
hCGb	80.3	142.2	211.4	122.4	144.1	92.5	154.3	211.5	230.4	128.5	113.8	13.6
IGF-I SR	20.6	237.5	201.0	0.0	129.2	0.0	153.9	165.2	29.0	0.0	0.0	0.0
IL-1 sRII	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-3	0.0	0.0	0.0	0.0	230.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IL-18 Rb	0.0	0.0	36.5	0.0	0.0	0.0	0.0	19.5	85.3	0.0	0.0	0.0
IL-21	379.8	85.4	0.0	261.7	284.7	568.7	0.0	0.0	42.2	455.5	392.6	983.8

Leptin	0.0	12.4	0.0	0.0	0.0	0.0	4.7	26.7	0.0	0.0	0.0	0.0
MMP-1	16.4	43.7	19.1	0.0	0.0	0.0	33.0	0.0	0.0	15.0	0.0	0.0
MMP-2	24.3	446.0	844.0	0.0	0.0	890.9	707.9	1027.1	1143.6	0.0	1075.8	0.0
MMP-3	0.0	0.0	0.0	0.0	51.7	56.8	3.1	0.0	0.0	0.0	76.0	0.0
MMP-8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MMP-9	151.3	133.5	168.3	198.6	159.0	97.9	232.6	237.7	245.0	219.6	212.7	143.3
MMP-10	32.8	18.8	23.8	33.0	70.7	11.7	100.0	101.7	116.3	111.7	82.9	28.2
MMP-13	10.4	20.9	20.6	0.0	0.0	0.0	51.7	43.5	8.3	2.2	2.2	0.0
NCAM-1	17716.2	14760.3	19163.7	19027.5	17552.8	20073.9	18162.7	17403.9	18480.2	17799.8	17331.3	19709.9
Nidogen-1	17006.7	17824.9	19944.4	21159.3	18301.5	17334.5	18832.2	19317.6	20395.0	19990.9	18663.7	18701.5
NSE	37544.5	42674.7	78206.9	55418.6	57856.2	40391.2	70403.5	84561.7	77314.2	63562.5	39120.3	14890.6
OSM	130.1	0.0	0.0	239.7	0.0	0.0	70.8	0.0	0.0	0.0	0.0	36.5
Procalcitonin	0.0	0.0	166.7	365.7	208.9	491.7	14.2	172.9	0.0	655.2	297.5	1854.3
Prolactin	0.0	0.0	0.0	170.4	1579.3	0.0	394.1	0.0	188.7	700.0	0.0	75.5
PSA	0.0	0.0	46.9	202.6	70.4	28.2	22.3	0.0	0.0	112.6	89.8	404.6
Siglec-9	56.2	15.4	44.8	0.0	47.8	0.0	72.1	14.5	0.0	23.9	0.0	0.0
TACE	64.9	24.0	0.0	0.0	76.2	0.0	10.6	0.0	0.0	0.0	0.0	861.5
Thyroglobulin	1122.8	0.0	0.0	5859.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TIMP-4	3701.9	3703.3	8353.1	8839.1	22347.3	23266.3	3958.3	21447.3	22283.6	24464.4	24530.0	23757.9
TSH	0.0	33.2	35.9	0.0	7.9	25.3	25.9	12.1	20.1	14.0	28.8	49.4