REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN ENDOMETRIAL CANCER CELLS BY FOOD COMPOUNDS

JAMES DANN

A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science in Cellular and Molecular Biology at the University of Canterbury

June 2008

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Associate Professor John Evans, for all his supervision with my experimental work, feedback on my writing, and development of ideas in the course of this project. Many thanks also my associate supervisors, Dr Drusilla Mason, for her help with guiding me in the right direction, and Dr Peter Sykes for his invaluable support and opinion, as well as helping to provide the endometrial cancer samples that are such a key part of the study. I would also like to thank Dianne Harker from the Department of Obstetrics and Gynaecology, Christchurch Women's Hospital, for helping with processing and logging the samples. Helen Morrin from the Cancer Society Tissue Bank

Thanks must also be given to Junko Nagase, as without her assistance in showing me how to perform many of the laboratory methods necessary for the project, I would have had a more difficult time. The assistance of two Dutch students who worked in our lab, Ilona Molenaar and Gaby Posthumus, was a great help in working on much of the cell culture. I would also like to think Katya Volkova and Michelle Hunt, from the Angiogenesis Research Group at the University of Otago, Christchurch, for their assistance and advice on laboratory techniques.

Finally, I would like to thank Gloria Evans for her coffee, baking, company, and motherly advice around the lab.

ABSTRACT

Endometrial cancer is one of the most significant gynaecological malignancies that affect women from New Zealand and the rest of the world. One of the critical stages in the development of a tumour is the onset of hypoxia. The malignancy responds by having raised levels of Hypoxia Inducible Factor (HIF) that in turn induces increased production of Vascular Endothelial Growth Factor (VEGF). VEGF is a potent angiogenic factor that will mediate vascular supply of nutrients and oxygen to the developing tumour. The aim of this study was to investigate whether two compounds found in extracts of plant materials, Resveratrol (Resveratrol) and Epigallocatechin gallate **(**EGCG), altered the levels of VEGF in the supernatant of cultured endometrial cancer cells.

Resveratrol is a phytoalexin that is found in many foods, such as grapes, nuts and berries, as well as in high concentrations in some red wines. 100 μM of resveratrol was added to cell cultures for 24 hours. VEGF levels in the supernatant were then analysed using ELISA. Resveratrol was found to have significant inhibitory effects in both primary endometrial cancer cell cultures and immortalised endometrial cancer cell cultures. Resveratrol was also shown to reverse the increase in VEGF caused by the hypoxia mimic cobalt chloride ($CoCl_2$).

Epigallocatechin gallate **(**EGCG) is an antioxidant catechin extracted from green tea. The effect of EGCG was analysed using the same method as for resveratrol. 100 μM of EGCG was also shown to have a significant inhibitory

effect on the level of VEGF in the supernatant of cultured endometrial cancer cells, as well as reducing the effect of $CoCl_2$.

These results suggest that selected food compounds, resveratrol and EGCG, can reduce VEGF levels by inhibiting HIF. Further investigation This may have anti-tumour effects in women with endometrial cancer.

ABBREVIATIONS

ARNT	aryl hydrocarbon receptor nuclear translocator protein
BCA	bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
$CoCl_2$	cobalt chloride
DMSO	dimethyl sulfoxide
EGCG	(-)-epigallocatechin-3-gallate
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FIGO	Federation Internationale de Gynecologie et d'Obstetrique
HIF	hypoxia inducible factor
HRP	horse radish peroxidase
MEM	minimum essential medium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MVD	microvessel density
NS	not significant
PBS	phosphate buffered saline
PHD	prolyl hydroxylase
PRF-MEM	phenol red free minimal essential medium
RIPA	radioimmuno precipitation assay
RT-PCR	real-time polymerase chain reaction

si-RNA	small interfering ribonucleic acid
TMB+	tetramethylbenzidine
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von-Hippel Lindau

1

INTRODUCTION

1.1 Endometrial Cancer

Endometrial cancer is the cancer found in the endometrium, the lining of the uterus. It is one of the most common cancers occurring in women (Bakkum-Gamez et al., 2008). The incidence and mortality rates for this disease are increasing in the western world. Treatment for endometrial cancer, though invasive, is very effective for most women. However, recurrent endometrial cancer is very difficult to treat, and survival rates for these patients are very low. Hence, a non-invasive, non-toxic treatment remains a clinical need.

1.1.1 Incidence & Epidemiology

Endometrial cancer is the most common invasive tumour of the female genital tract (Ozbudak et al., 2008). Epidemiological studies show that the incidence of the disease is increasing. Understanding the controlling factors is key to successful treatment and intervention in patients with endometrial cancer.

There are a number of significant risk factors for endometrial cancer, which contribute to the increase in incidence in western countries. Obesity is a very strong risk factor for endometrial cancer. Women with a Body Mass Index (BMI)

of over 25 kg.m^2 have twice the risk of developing the disease; for women with a BMI of over 30 kg.m^2, the risk is triple. As obesity is widely acknowledged to be an epidemic in western countries, the rate of endometrial cancer in these countries is forecast to increase further. For a further comparison, the rate of endometrial cancer in the US, where around 40% of adult women are obese, is 7 times that in Japan, where 3% of adult women are obese. A link has also been established between the excessive consumption of fat, and an increase in the rate of endometrial cancer. One of the most marked risk factors is post-menopausal status. Approximately 75% of endometrial cancers are identified in post-menopausal women. With an aging populating, the number of post-menopausal women in society will also increase, and with it, the rates of endometrial cancer.

Breast cancer has also been linked to increased rates of endometrial cancer. This may be in part due to shared risk factors for the two diseases. However, it has also been noted that Tamoxifen, a drug frequently used in treatment of breast cancer, is linked to an increased incidence of endometrial cancer (Pukkala et al., 2002). Without an effective treatment for endometrial cancer, dietary and demographic pressures will ensure that the incidence of this disease continues to increase (Wright et al., 2007).

In New Zealand endometrial cancer is the 6th most common cancer in women, with an incidence of 15 cases per 100,000 women per year. This is predicted to increase as the population of New Zealand ages, and becomes more obese.

Endometrial cancer is more prevalent in the New Zealand Maori population than in the New Zealand non-Maori population. This reflects socio-economic status as well as genetics. Mortality is also higher within this population.

1.1.3 Prognosis

Prognosis for women with endometrial cancer is largely dependent on the stage of the disease when it is first diagnosed. If detected early, removal of the uterus will remove both the cancer and the risk for most patients. A total abdominal hysterectomy (surgical removal of the uterus) with removal of both ovaries (bilateral salpingo-oophorectomy) is the most common therapeutic approach (Bakkum-Gamez et al., 2008). The chances of a patient's survival are correlated with the stage of the cancer. The International Federation for Gynaecology and Obstetrics (FIGO) classification system rates endometrial cancers from stage I (least progressed) to stage IV (highly progressed). The 5-year survival rate for women with stage 1 endometrial cancer is between 75% and 95%, while for women with stage 4 tumours a less than 5% chance of 5-year survival.

There is a small group of patients who will have recurrent cancer. As the surgical approach can only be undertaken once, treating patients with systemic endometrial cancer becomes problematic. Chemotherapy is not ideal, as it has a low chance of success, coupled with a high level of toxicity (Oehler et al., 2005). Hypoxia has been shown to be a significant contributor to the development of recurrent endometrial cancers (Pijnenborg et al., 2007).

1.2 Hypoxia and Angiogenesis

1.2.1 Hypoxia

Hypoxia occurs when the oxygen tension within a tissue becomes low. Cells require constant access to the vasculature which provides nutrients and oxygen. If access to the vasculature becomes restricted, then the tissue can only acquire limited amounts of oxygen and nutrients. Thus, hypoxia limits the ability of cells to grow. Tissue hypoxia is a characteristic of most rapidly developing tumours.

The oxygen tension in normal tissue is approximately 7%. Hypoxia is generally associated with oxygen tension dropping below 5% (Gordan and Simon, 2007). The average oxygen tension of tumours is 1.5% (Kimbro and Simons, 2006; Vaupel, 2004; Vaupel et al., 2001). As the cells cannot survive with oxygen levels as low as this, hypoxic tumours can respond with either programmed cell death (apoptosis) or the development of new vasculature (angiogenesis). While much research is conducted into apoptosis,the study reported in this thesis is concerned only with angiogenesis.

Hypoxia is a common feature of solid tumours, frequently occurring in the cells of developing tumours (Melillo, 2006). As cancerous cells grow without the cellular controls of normal tissues, tumours lack the cellular architecture of normal tissues (Garcia, 2006). While cells on the outer extremities of a growing tumour can exchange oxygen and nutrients with cells in close proximity, the cells at the centre of the tumour will lack this possibility. Blood vessels are required to carry

nutrients and oxygen to these cells. They are also needed to remove waste products from the cell. Thus, without blood vessels, the core of a tumour can become necrotic. Without the development of new vasculature the growth of a tumour will be self-limiting. Angiogenesis is the process by which tumours develop new vasculature, thus promoting the transition from limited to rapid growth in tumours (Wong et al., 2003).

1.2.2 Angiogenesis

Angiogenesis is the process by which cells develop new blood vessels. This occurs normally in growing tissues, as well as in tissues that are repairing any damage to blood vessels caused by a wound. It also occurs routinely as part of a woman's menstrual cycle, but is uncommon in normal adult tissue. The process is utilised by tumour cells to provide the vasculature required for continued cell growth. As angiogenesis is a critical stage in the development of cancers it is a subject of much interest.

Though angiogenesis is linked with the growth of cancers it is a process that occurs normally in growth and wound healing. Synthesis of new blood vessels begins when endothelial cells, which line the walls of existing blood vessels, receive a stimulus to create new vessels. This signal includes the common growth factor, Vascular Endothelial Growth Factor (VEGF). On receiving this signal the endothelial cells release protease enzymes, which break down the surrounding extracellular matrix. Endothelial cells then migrate to and proliferate in the newly

created space within the extracellular matrix. As this process continues, several millimetres of new blood vessels can be created per day.

Unlike most other mature tissues, angiogenesis is part of the normal physiological process in the endometrium. Once the endometrial lining is shed as part of the menstrual cycle, angiogenesis is required to grow a new endometrium, ready for implantation. As the menstrual cycle repeats monthly, the endometrium is almost always undergoing angiogenesis, and hence the control of angiogenesis in endometrial cancers may differ from that in other tumour tissues. Because of this the role of angiogenesis in endometrial cancer is of particular interest.

When a tumour reaches a size where it becomes hypoxic (see previous section) it then needs to create new blood vessels. Tumours can trigger an angiogenic response to achieve this. With new blood vessels, the central cells of the tumour can be prevented from becoming necrotic, and the tumour can continue growth. The microvessel density (MVD) of a tumour, which reflects the size and number of blood vessels in the tumour, is a marker of angiogenesis. Without angiogenesis the tumour would only be able to grow to a small size, and it has been shown that increased angiogenesis is linked to poor prognosis in women with endometrial cancer (Sivridis, 2001). Hence preventing tumour angiogenesis is the focus of much research and drug development. One of the key molecules of the hypoxic response leading to angiogenesis is Hypoxia Inducible Factor (HIF), which will be discussed in the next section.

1.3 Molecular responses to hypoxia

1.3.1 HIF-1 α and β

Hypoxia Inducible Factor 1 alpha (HIF1-α) is a transcription factor that has a critical role in mediating the hypoxic response. It is a heterodimeric molecule, consisting of an α and β subunit. HIF1-α binds to a specific gene sequence, leading to the expression of target genes. Because of the key role it plays in mediating the response to hypoxia, HIF1-α is a target of much interest for agents that may inhibit it. As direct measurement of tumour oxygen levels is difficult ,HIF1-α is often used as a marker of tumour hypoxia (Vaupel and Mayer, 2007).

The molecule was first identified as a regulator of erythropoietin expression in response to low oxygen. It is part of the helix-loop-helix family of transcription factors. HIF1-α is coded by a 15 exon gene, the product of which is a 120-kD HIF1-α subunit that complexes with a 91 to 94 kD HIF1-β subunit to form the active heterodimer (Iyer et al., 1998). While it requires two subunits to be active, the α subunit is of most interest. HIF1-β is a constitutively expressed protein (Stroka et al., 2001), also known as ARNT - Aryl Hydrocarbon Receptor Nuclear Translocator protein (Wang et al., 1995). The expression of HIF1-β is not affected by the amount of cellular oxygen. The protein is found mainly in the nucleus of the cell.

Although HIF1-α is transcribed by the cell at a constant rate, the stability of HIF1-α is dependent on oxygen availability. Under normoxic conditions, it is readily degraded. Two proline residues are hydroxylated by a family of oxygen-dependent Proline Hydroxylases (PHD1-3) Bruick and McKnight, 2001; Jaakkola et al., 2001). This leads to the hydroxylated HIF1-α being bound to and ubiquitinylated by the von Hippel-Lindau (VHL) tumour suppressor (Cockman et al., 2000; Epstein et al., 2001; Maxwell et al., 1999; Yu et al., 2001a, b). When the cell is hypoxic, hydroxylation by the PHD enzymes is reduced, leading to an increase in the amount of HIF1-α present in the cytoplasm. Stable HIF1-α then translocates to the nucleus, where it forms a heterodimer with HIF1-β. The active HIF1 dimer can then bind to, and induce transcription, of target genes. (Kong et al., 2005). More than 100 HIF1-α target genes have been identified. One of the genes which HIF1-α binds to encodes Vascular Endothelial Growth Factor (VEGF) (see next section).

1.3.2 Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factor (VEGF) is a potent angiogenic factor and one of the primary effectors of hypoxia (Wong et al., 2003). It is a 40 - 45 kDa disulphide linked homodimeric glycoprotein, responsible for the recruitment of proteases and endothelial cells that begin growing new blood vessels (Ozbudak et al., 2008). Expression of VEGF is stimulated by HIF (see previous section). VEGF is secreted by endothelial cells, and acts by binding to the VEGF receptor. Measurement of VEGF levels in the supernatant of cells cultured in vitro can be

quantified using the ELISA assay, This shows the effects of various treatment conditions on the level of VEGF.

As mentioned previously, VEGF is a key molecule in the angiogenic process. Once its production has been stimulated by hypoxia, as well as by other growth factors, cytokines, or oncogenes, VEGF leads the recruitment of proteases to the site where the new blood vessel is required (Ozbudak et al., 2008). VEGF binds to and activates Vascular Endothelial Growth Factor Receptor (VEGFR), which is a tyrosine kinase transmembrane protein that specifically binds VEGF (Meunier-Carpentier et al., 2005; Yokoyama et al., 2003). The activation of VEGFR induces a signal within the cell, leading to the release of proteases. These proteases enzymatically break down the extracellular matrix, allowing for endothelial cells to migrate into the space and create new vessels.

While VEGF has been shown to have a key role in tumour angiogenesis, it is also involved in angiogenesis of the normoxic endometrium. It is essential for the cyclic growth of the endometrium, which requires the growth of new vasculature each time the endometrium is replaced. Studies have also shown that VEGF is required for successful implantation of the ovum (Hastings et al., 2003; Rockwell et al., 2002).

1.3.3 HIF1-α, VEGF and Endometrial Cancer

Hypoxia, HIF1-α and VEGF are all strongly implicated in endometrial cancer. Elevated levels of HIF1-α, as well as mutations in the gene which codes for the transcription factor, have been shown to be increased risk factors for cancer (Zhong et al., 1999). As increased HIF1-α leads to increased VEGF, elevated levels of VEGF would be expected to occur with greater prevalence in patients with cancer. Studies in a number of cancer types have shown this to be the case. Such studies have also shown that there is a significant correlation between HIF1-α levels and VEGF levels.

Elevated HIF1-α levels have been shown in the cells of many types of cancer (Kimbro and Simons, 2006). Breast cancer is one example where the over-expression of HIF1-α is strongly correlated with the disease (Alkhalaf et al., 2008; Zhong et al., 1999). High levels of HIF1-α at diagnosis have been shown to be an accurate predictor for early relapses and metastatic disease in breast cancer patients (Dales et al., 2005). As HIF1-α is elevated in these examples, we would expect VEGF to also be raised. Studies have shown that there is a strong correlation between HIF1-α expression and VEGF levels (Wong et al., 2003). In transgenic mice, designed to over-express the HIF1-α gene in skin and squamous epithelial cells, researchers found that there was a 13-fold elevation in the levels of VEGF expressed (Elson et al., 2001).

As both HIF1-α and VEGF are molecules which have important roles in the hypoxic response, they have also been shown to have important roles in human cancers (Melillo, 2007). Elevated levels of the two molecules may indicate a

disease state. A number of studies have shown that HIF1-α, VEGF, or both, are important in endometrial cancer. A DNA microarray analysis showed that the level of HIF1-α gene expression was higher in the endometrial cells of cancer patients than in the equivalent cells from a non-cancer control. The up-regulation of HIF1-α has been shown to be a common event in endometrial cancers (Sivridis et al., 2002). A more recent study has shown that HIF1-α expression was observed in 73% of the primary tumours of patients with endometrial carcinoma. (Pijnenborg et al., 2007) In a study which analysed endometrial cancers by grade, 90% of stage III + IV tumours showed high expression of VEGF, while 100% of that group showed high HIF1-α levels (Ozbudak et al., 2008). Associations have been shown between mutations in the HIF1-α gene and endometrial cancer. An epidemiological study in Turkish women showed that HIF1-α mutations were a significant risk factor for endometrial cancer. High MVD has been shown to be associated with HIF1-α gene mutations in endometrial cancer (Horree et al., 2008), as well as being a risk factor for the recurrence of the disease (Pijnenborg et al., 2007)

Clinical studies have shown that VEGF can be an important predictor of endometrial carcinoma. The level of VEGF can be used as independent prognostic factor for women with stage I endometrial cancer ((Pijnenborg et al., 2007), A combination of histological grade and VEGF expression can also be used as independent predictors of clinical outcome (Chen et al., 2001). However, some studies have failed to find a link between VEGF and prognosis. In a study of

Finnish women, no significant correlation was seen between VEGF expression and the rate of survival of patients with endometrial cancer. Also, while HIF1-α is strongly linked to the disease, it's expression has not been shown to correlate with tumour grade, stage, or myometrial invasion.

1.4 Food and Cancer

It has been shown that populations in some countries have significantly lower levels of diseases, including heart disease and cancer, and been it has hypothesised that these differences may in part be due to the diets in these countries. One such observation is called the "French Paradox", where the population of France has significantly lower levels of a range of medical conditions, including cardiovascular disease and cancer, despite a diet that is richer in fats and alcohol than most other Western nations. It has been hypothesised that this may be an effect of the French diet, which includes a high amount of oils such as olive oil, as well as regular consumption of red wine. As red wine is fermented with the skin of the grape, unlike most white wine where the skins are separated, red wine contains a higher concentration of phenols and flavonoids that may be linked to health benefits seen in epidemiological studies (Saiko et al., 2008).

The North East Asian region also has a population with lower than expected rates of cancer and cardiovascular diseases. Japan in particular has a population with significantly lower rates of cancer than would be expected from a nation that

consumes large amounts of protein and is relatively sedentary. Again, this may be in part attributable to consumption of relatively large amounts of omega-3 rich fish oil, as well as regular consumption of green tea. During processing of green tea, most of the green leaf phenols remain unoxidised (unlike black tea). Hence extracts of green tea include polyphenols. One such polyphenol is (-)-epigallocatechin-3-gallate (EGCG). The beneficial effects of green tea and its active components have been documented in the literature and include cancer chemoprevention, inhibition of tumour cell growth, invasion, and metastasis, and antiviral and anti-inflammatory activities.

1.4.1 Resveratrol

Resveratrol (3,4',5-trihydroxystilbene) is a potential anti-angiogenic molecule that is found in significant concentrations in red wine (Baur and Sinclair, 2006). It was first isolated in 1940 (Takaoka, 1940), but investigations into the potential utility of the substance as a nutraceutical were increased after it was identified at high concentrations in red wine in 1992 (Siemann and Creasy, 1992). In 1997 resveratrol was shown to have dramatic anti-carcinogenic effects in mice, Jang et al., 1997) and subsequent studies have supported the notion that resveratrol has chemoprotective activities ((Bhat and Pezzuto, 2002). It has also been shown to have anti-inflammatory, neuroprotective and antiviral properties (Bhat and Pezzuto, 2001). It is not yet known whether resveratrol has any effect at concentrations found in a normal human diet.

While resveratrol has been shown to have marked effects *in vivo*, it's action is not clearly understood (de la Lastra and Villegas, 2007). It has been demonstrated to have chemoprotective activities in each of the three stages of cancer - initiation, promotion and progression (Athar et al., 2007). It has been shown that resveratrol enhances the degradation of HIF1-α protein, although exact mechanisms have not yet been elucidated. Resveratrol also inhibits the accumulation of HIF1-α protein, which leads to a reduction of VEGF transcriptional activity. We have chosen to study resveratrol in this project because in the few studies that investigated HIF1-α, it was reported that resveratrol efficiently blocked HIF1-α and VEGF expression in ovarian cancer cells (Park et al., 2007), human papillomavirus-transfected cervical cancer cells (Tang et al., 2007), human tongue squamous cell carcinoma and hepatoma cells (Zhang et al., 2005). Given the efficient inhibition of HIF1-α and VEGF seen in these few studies we hypothesise that resveratrol will also inhibit VEGF in endometrial cancer cells.

1.4.2 EGCG

EGCG is a catechin that is found in green tea *Camellia sinensis* (Yance 2006). It has recently be shown that EGCG can have a wide range of health benefits. At the cellular level, it has been shown that EGCG interferes with HIF1-α expression. A prospective cohort study from Saitama Prefecture, Japan reported that green tea is preventative against cancers in many organs including stomach, lung, colorectum and liver (Imai et al., 1997). Another cohort study (Iowa Women's Health Study)

found that food-derived catechin intake was inversely associated with rectal cancer incidence in postmenopausal women (Arts et al., 2002).

Both *in vitro* and *in vivo* studies have shown that EGCG has antiangiogenic properties (Jung and Ellis, 2001). The substance has been shown to inhibit angiogenesis and restrain growth in tumour models (Fassina et al., 2004). Evidence of the mechanisms of EGCG action is incomplete, however it has been shown to reduce levels of VEGF by decreasing mRNA levels (Sartippour et al., 2002), inhibiting gene expression (Zhang et al., 2006) and decreasing the levels of VEGF secreted (Sartippour *et al.* 2002). HIF1-α protein accumulation is inhibited in the presence of EGCG (Zhang et al., 2006).

Although the mechanisms through which EGCG reduces angiogenesis are not completely elucidated, evidence of the potential health benefits have led to the introduction of a strategy of cancer prevention with green tea in Japan (Fujiki, 2005). Drinking green tea has been reported to be protective against oesophageal cancer (Gao et al., 1994), colorectal cancers in females from Hebei Province, China (Zhang et al., 2002), stomach cancers in Nagoya, Japan (Inoue et al., 1998), gastric cancer in Kyushu, Japan (Kono et al., 1988), pancreatic and colorectal cancers in Shanghai, China (Ji et al., 1997) and breast cancer in Saitama, Japan (Nakachi et al., 1998). The success of these trials encourages the investigation of the effect of EGCG on the regulation of VEGF secretion by endometrial cancer cells.

1.4.3 Aims of this study

As hypoxia causes an increase in VEGF in cells, which leads to angiogenesis in developing tumours, it is important to investigate whether an inhibition of HIF1-α can reduce the levels of VEGF in endometrial cancer cells, and prevent tumour development.

The overall aim of this project was to investigate whether increases in VEGF secretion from cultured endometrial cancers could be inhibited by naturally occurring compounds. Specifically, the first objective was to investigate whether $CoCl_2$, a hypoxia mimic reported to increase levels of HIF1-α, elicits an increase in VEGF secretion from endometrial cancer cells. The second objective was to observe whether the secretion of VEGF in basal culture conditions in vitro, could be reduced or prevented by treating the cells with the food compounds, resveratrol and EGCG. The third aim of this study was to investigate whether the $CoCl_2$ stimulated increase in VEGF could be reversed by the addition of resveratrol and EGCG.

2

MATERIALS AND METHODS

2.1 Materials, solutions and media

For details of materials used in this study, see Appendix A

For details of media and solutions used in this study, see Appendix B

2.2 Preparation of Primary Culture

2.2.1 Collection of Endometrial Tumour Samples

Endometrial cancer tissue samples were collected from consenting women (under Ethics Approval CTB/04/02/005) with endometrial cancer undergoing hysterectomy operation to removed the uterus. A sample of the tumour was removed by the duty pathologist, and placed in cell culture media in preparation for dispersion.

2.2.2 Cell Dispersion

All cell culture work was undertaken in a sterile biological safety cabinet (EMAIL Air Handling Biological Safety Cabinet Class II), unless otherwise indicated. All glassware, solutions and instruments used for cell preparation were sterilised by autoclaving or 70% ethanol spray before use. Tumour tissue was washed briefly three times in α-MEM pre-warmed to 37°C. After washing, the

tissue was placed onto a glass slide, where it was minced into small pieces (less than 3 mm^3) using a size 24 surgical blade. The minced tissue was transferred into a 15 ml Falcon tube, and 10 ml of α-MEM were added. It was then centrifuged at 1500 rpm (Centrifuge 5702, Eppendorf, Germany) for 5 minutes at room temperature. The supernatant was removed by aspiration with a Pasteur pipette. The tissue pellet was then resuspended in 10 ml of α-MEM, and centrifuged as before. The supernatant was removed by aspiration as before The tissue pellet was then resuspended in 4 ml of collagenase solution. and placed into an incubator (Forma Scientific) at 37ºC with 5% Co_2 for 30-45 minutes. The tube was shaken manually at ten-minute intervals.

The cells were then mechanically disrupted using an 18-gauge needle to triturate the solution repeatedly. Next, the suspension was centrifuged for 5 minutes at 1500 rpm. The supernatant was removed by aspiration, and the pellet was washed by resuspension in α-MEM, followed by centrifugation for 5 minutes at 1500 rpm. The supernatant was removed, and centrifuged as above. The supernatant was removed by aspiration. Pellet was then resuspended in α-MEM. The resuspended cells were then passed through a 70 μm filter. The filtrate was collected and centrifuged as above. The cells were then resuspended in α-MEM supplemented with 10% Fetal Bovine Serum (α-MEM with FBS; see appendix B), and plated into a 25 cm^2 flask.

Maintenance of Cell Culture

Cell culture media was changed when required, which was generally every second or third day. Medium was aspirated and α-MEM with FBS, which had been pre-warmed to 37°C, was then added to the flask, using a 10 ml transfer pipette. The volume of α-MEM with FBS added was dependent on the size of the flask: 25 cm^2 – 10 ml; 75 cm^2 – 20 ml; 175 cm^2 – 30 ml

Splitting Confluent Cell Culture

When cells reached a confluent state, they were split in preparation for further use. Confluence of cells was determined by observing the flask under the confocal microscope (Olympus CK40). Once the cultures had become confluent the cell culture medium was removed by aspiration. 10 ml of Phosphate Buffered Saline , (PBS; see Appendix B) pre-warmed to 37°C, was then used to wash the cells. This PBS was removed, and the wash step was repeated twice more. Following the final aspiration of the PBS from the flask, 2.5% trypsin in PBS was added to the cells, with the volume of trypsin added dependent on the size of the flask: 25 cm^2 flask – 0.5 ml; 75 cm^2 flask – 1 ml; 175 cm^2 flask – 2 ml. Cells were dislodged by light tapping on the side of the flask. Split cells were used for four purposes:

I. Transfer into a larger sized flask for further growth

10 ml of α-MEM with FBS was added to the flask with the cells in trypsin. The cells were then resuspended in the α-MEM with FBS, and transferred into a sterile

flask. Further α-MEM with FBS was then added to bring the total volume of medium in each flask to 20 ml (for 75 cm^2 flask) or 30 ml (175 cm^2 flask).

II. Transfer into a multi-welled plate for an experiment

10 ml of α-MEM with FBS was added to the cell suspension in the flask, and the cells and media were then transferred into a 15 ml Falcon tube. Cells were evenly dispersed through the media by light tapping on the outside of the tube. A small sample of the cell suspension was then removed, and used to perform a cell count with a haemocytometer. Cells were then centrifuged at 1500 rpm for 5 minutes at room temperature and the supernatant was aspirated. α-MEM with FBS was then added, with the volume added dependent on the cell count performed earlier, and the concentration of cells required. 0.5 ml of cell suspension was added to each well of a 24 well plate. Cells were plated at a concentration of $2.4x10^5$ cells/ml, giving approximately 120,000 cells per well in each well of a 24 well plate.

III. Frozen for storage

10 ml of α-MEM with FBS was added to the cell suspension in the flask, and the cells and media were then transferred into a 15 ml Falcon tube. Cells were evenly dispersed through the media by light tapping on the outside of the tube. A small sample of the cell suspension was then removed, and used to perform a cell count with a haemocytometer. Cells were then centrifuged at 1500 rpm for 5 minutes at room temperature. The tube was brought back to the culture hood, and the supernatant was aspirated. α-MEM with FBS was then added, to give a final

concentration of $2x10^6$ cells per ml. To each cryovial, 400 µl of FBS, 100 µl of DMSO, and 500 µl of the cell suspension was added. The cryovial was then placed into a Mr Frosty container, and stored at -80ºC for 24 hours. Vials are then removed from Mr Frosty and placed into a labelled box in the -80ºC freezer until required.

IV. Fixed for histological staining

10 ml of α-MEM with FBS was added to the cell suspension in the flask, and the cells and media were then transferred into a 15 ml Falcon tube. Cells were then centrifuged at 1500 rpm for 5 minutes at room temperature. The supernatant was then removed by aspiration. The cell pellet was then resuspended in 10 ml of fixative solution and passed on to the pathology department who fixed the cells, then stained them with the required immunologic stains.

Thawing Cells

For culturing, vials were removed from the freezer and thawed at room temperature. Next, they were transferred into a 25 cm^2 flask containing 10 ml of pre-warmed α-MEM with FBS using a sterile 1 ml pipette. Cells were then incubated overnight, and the medium was replaced with fresh α-MEM with FBS the following day.

Stimulation of Cell Culture

Once confluent, cells grown in Phenol-Red Free Minimum Essential Medium (PRF-MEM; see Appendix B) for 24 hours. Each of the wells of a 24 well plate contained 500 µl of PRF-MEM. After being incubated for 24 hours, the medium was removed by aspiration and 500 µl of fresh PRF-MEM was added to each well. Plates were then incubated for a further 1 hour. Following this incubation, the medium was then aspirated and replaced with 500 µl of the treatment solution for stimulation. The plate was then returned to the incubator for the course of the experiment (24 hours unless otherwise specified).

Experimental treatments were prepared in the biological safety cabinet to give the concentrations required for the various experiments detailed in the results section. Appropriate volumes of PRF-MEM were measured out into Falcon tubes or 1.7 ml Eppendorf tubes, dependent on the total volume required. Tubes were then made up to the required total volume with the appropriate treatment solutions. The details of the different concentrations and treatment regimes used in the individual experiments can be found in the following 'Results' chapter. Finally, the tubes were inverted repeatedly to mix before being used in the experiments.

On the completion of the experimental incubation the supernatants were collected from the plate. 500 µl of supernatant from each well was placed in an individually labelled 0.5 ml or 1.7 ml Eppendorf tube. These were then stored in the freezer until required for assay.

2.3 ASSAYS

2.3.1 MTT Viability Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) tests were performed as a measure of cell viability and cell proliferation after cells had been exposed to experimental treatment solutions. After the supernatants have been removed and stored, as above, each well of the plate was washed with PBS. 500 µl of PBS was added to each well, then removed by aspiration. This wash step was then repeated. 900 µl of PRF-MEM was then added to each well of the plate. Next, 100 µl of MTT (5 ng/ml) was added, and the plate was placed in the 37ºC incubator for 2 hours for the colour to develop.

Following the incubation, 1 ml of 10% DMSO in isopropanol was added to each well. Crystals produced during the incubation were then dislodged from the plate using a pipette. The contents of each well was transferred into an Eppendorf tube and then centrifuged for 5 minutes at 10,000 rpm. An aliquot of the supernatant from each tube was next transferred into a 96-well plate. Absorbance was measured at 570 nm (wavelength reduction at 690 nm), using water as a blank.

2.3.2 Protein Assay

Protein assay experiments were performed using the bicinchoninic acid (BCA) protein assay kit from Pierce Biotechnology. This kit contains the working reagent

and albumin standards used in the assay. 100 µl of RIPA Lysis buffer was added to each well on the plate. Cells with RIPA were left for ~10 minutes for lysis to occur. 900 µl 1X PBS was added to each well, bringing the total volume of solution in each well to 1 ml. 150 µl of this diluted protein solution was then transferred in duplicate to a 96-well plate. A standard curve was created by adding 8 albumin standards in duplicate. The range of standards was created by serial dilution from a high concentration of 200g/ml, down to 0g/ml. 150 µl of working reagent was added to each of the sample and standard wells on the plate. The plate was then covered, and incubated at 37ºC for two hours. Following incubation, the plate was then left to cool to room temperature. Finally, the absorbance of each well was measured at 562 nm using a plate reader.

2.3.3 VEGF ELISA

Solutions

ELISA was performed according to the methods described in the R&D Systems DuoSet ELISA Development System kit. The kit contains:

Capture antibody

Detection antibody

Streptavidin / HRP

VEGF Standard

Reagents also required:

Reagent Diluent (see Appendix B)

PBS

TMB+ Substrate (see Appendix B)

2N H_2SO_4 – Stop Solution

ELISA Wash buffer

Standards

The VEGF standard contained within the DuoSet kit, when reconstituted with 0.5 ml of Reagent Diluent , was at a concentration of 120 ng/ml. This was diluted further to give aliquots at a concentration of 10,000 ρg/ml, which were stored in the freezer until required. Standards for each ELISA experiment were prepared not less than 15 minutes before the assay was begun. Serial dilution of a 10,000 ρg/ml aliquot with Reagent Diluent, was then used to create a standard curve. The concentrations on this curve were: 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 ρg/ml. If a sample set had been previously assayed and shown VEGF values outside the 2,000 ρg/ml range, then further experiments were performed using a 10,000 ρg/ml as the highest point on the standard curve.

Assay Procedure

On the first day of the assay, 100 µl of Capture Antibody, diluted in PBS, was added to each of 96-wells on a Nunc ELISA plate. This plate was then covered, and left to incubate overnight at room temperature.

The following day, the plate was washed with ELISA wash buffer (Wash Buffer; see Appendix B). Each well on the plate was filled with Wash Buffer, dispensed by a squirt bottle. The plate was then inverted and tapped repeatedly, to remove all the Wash Buffer. This step was repeated a further two times. After the final wash, the plate was inverted and blotted on a clean paper towel to remove all remaining liquid from the plate. The plate was then "blocked" by adding 300 µl of Reagent Diluent, covering it and incubating it at room temperature for a minimum of one hour.

Following this the plate was washed three times with Wash Buffer, as above. 100 µl of sample or standard was then added to each well on the plate in duplicate. The origin of the samples was then recorded on a diagram showing the plate layout. When all the samples had been added, the plate was again covered, and incubated at room temperature for 2 hours. Next, the plate was washed three times with Wash Buffer, as above. 100 µl of Detection Antibody (see Appendix B) were then added to each well. The plate was covered and incubated at room temperature for 2 hours.

Next, the plate was washed three times with Wash Buffer. 100 µl of Streptavidin / HRP (see Appendix B) was added to each well. The plate was covered and incubated at room temperature for 20 minutes. The plate was then washed with Wash Buffer a further three times. 100 µl of Substrate Solution (TMB+; see Appendix B) was then added to each of the wells. The plate was covered and

incubated at room temperature for 20 minutes. Next, 50 µl of Stop Solution (2N H_2SO_4; see Appendix B) was added to each well. The plate was then gently tapped to mix the Stop Solution and the Substrate Solutions.

Reading Absorbances

The optical density of each well was determined using a microplate reader. (Spectra Max 190, Molecular Devices). Optical density was recorded at 450 nm, with wavelength correction set to 540 nm. A standard curve was created from the data-set using a 4 parameter logistic (4-PL) curve-fit. The software (Soft Max Pro, version 2.6.1, Molecular Devices) then calculated the amount of VEGF in each sample by comparing the optical density to that on the standard curve.

Data Analysis and statistics

Graphing and data analysis and statistics were performed using GraphPad Prism 5 for Mac. Data were statistically analysed using paired or unpaired Student's *t*-test, and one-way or two-way ANOVA. $P < 0.05$ was considered significant. All data were reported as mean ± SEM.

3

RESULTS

3.1 Effects of Cobalt Chloride on VEGF Secretion in endometrial cancer cells

Cobalt Chloride ($CoCl_2$) is routinely used as an experimental mimic of hypoxia (Dai et al., 2008; Shu et al., 2008). It interferes with the breakdown of HIF1-α, which leads to the transcription factor accumulating within the cell. This increase in HIF1-α results in an induction of the hypoxic response, as outlined in the previous section. $CoCl_2$ induces the hypoxic response by preventing the breakdown of the HIF1-α molecule. $CoCl_2$ competitively inhibits the PHD molecules, which hydroxylate HIF1-α under normoxic conditions. By preventing HIF1-α from becoming hydroxylated, $CoCl_2$ stops HIF1-α from being broken down by VHL. This leads to the accumulation of HIF1-α, which then combines with HIF1-β, resulting in an increased level of the dimer. This leads to an up-regulation of the genes which the HIF1 heterodimer modulates. In this project, we looked at the effect that two variables, duration and concentration of exposure to $CoCl_2$, have on cell viability and VEGF production of endometrial cancer cells. The effects of the duration of $CoCl_2$ exposure are presented in 3.1.1, while the effects of $CoCl_2$ concentration are presented in 3.1.2.

3.1.1 Effect of Duration of $CoCl_2$ Treatment on VEGF Release from RL952 cells

The length of time the cells are exposed to $CoCl_2$ is an important factor in the subsequent expression of VEGF. Studies reported in the literature, together with prior studies on endometrial cancer from this laboratory, have incubated cells with $CoCl_2$ for 24 hours. A 100 μM concentration of $CoCl_2$ was incubated for 24 hours to induce VEGF expression in cultured cancer cells (Dai et al., 2008). To confirm the robustness of this protocol, a preliminary experiment was undertaken to establish a pattern of VEGF expression. An exposure time was then selected to be used for all subsequent experiments.

Initial experiments were undertaken in the endometrial adenocarcinoma cell line RL952. Cells were grown to confluence in a 175 cm^2 flask, then split into a 24 well plate, following the protocol outlined in the methods section. Cells were then incubated with 100 μM of $CoCl_2$ for the duration indicated. Four wells were incubated with $CoCl_2$ for the time indicated. The supernatant from each set of samples was collected from the plate and immediately frozen.

It was found that the amount of VEGF in the supernatant of RL952 cells, measured by VEGF ELISA, did not change significantly during the first 6 hours of incubation (Figure 3.1.1). Between the 6 and 12 hour time points there was a dramatic increase in the amount of VEGF found in the supernatant. This response was observed to continue to increase over the next 12 hours. The response at 24 hours was roughly double that at 12 hours. The variation in VEGF levels, as represented by the standard deviation of each data point on the graph, was much

larger at the 12 hour time point than at the 24 hour time point. As the 24 hour time of incubation with $CoCl_2$ caused a significant increase in VEGF activity, and with less variability than the 12 hour incubation, 24 hour incubations were used in all subsequent assays.

3.1.2 Effect of concentration of $CoCl_2$ exposure on VEGF release from RL952 Cells

Whilst $CoCl_2$ is a potent mimic of hypoxia, and elicits VEGF secretion, it can also have cytotoxic effects on the cell. Experiments were undertaken, first in the immortalised endometrial cancer cell line, RL952, followed with experiments using primary cultured endometrial cancer cells, to find a dose of $CoCl_2$ that increased VEGF release without having an excessively cytotoxic effect on cells. Levels of VEGF secretion were measured by ELISA of supernatant from cultured cells. The viability of the cells was determined using the MTT assay of cell viability. Initial experiments were undertaken in the endometrial adenocarcinoma cell line RL952. Cells were grown to confluence in a 175 cm^2 flask, then split into a 24 well plate, following the protocol outlined in the methods section. Cells were then incubated with concentrations of $CoCl_2$, from 0 μM to 1600 μM. Four wells were incubated for each concentration indicated. After 24 hours, the supernatant from each set of samples was collected from the plate and immediately frozen.

In RL952 cells, the level of VEGF was increased markedly by 100 μM $CoCl_2$ dose. The highest level of secretion was attained at 400 μM $CoCl_2$, and above this dose the levels declined. The variation in VEGF secretion in response to 100 μM

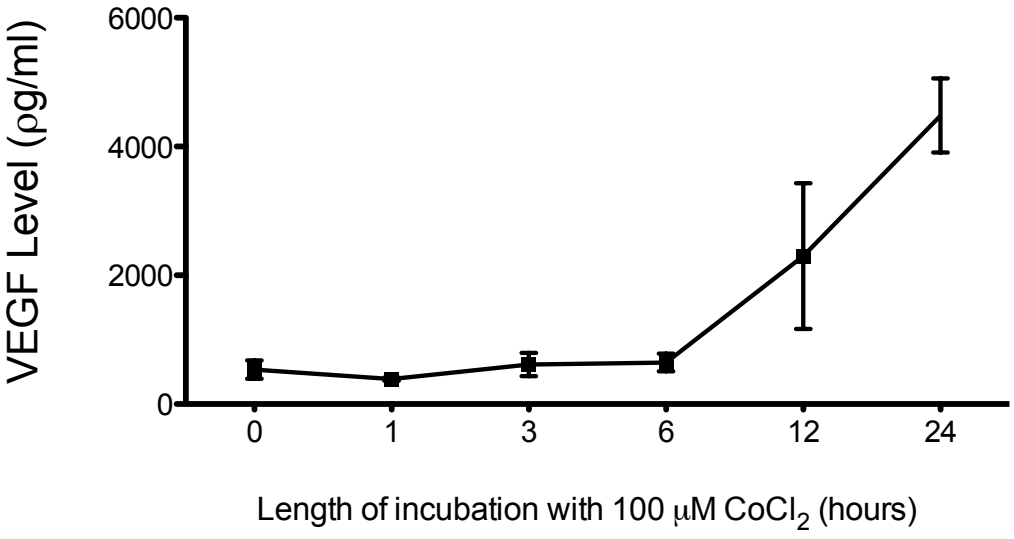

Figure 3.1.1 VEGF levels in the supernatant in response to duration of exposure to $CoCl_2$. RL952 endometrial cancer cells were incubated with 100 μM of $CoCl_2$ for the times indicated. Supernatants were removed from the cells at these points. Data is plotted as mean of 4 samples ± SEM.

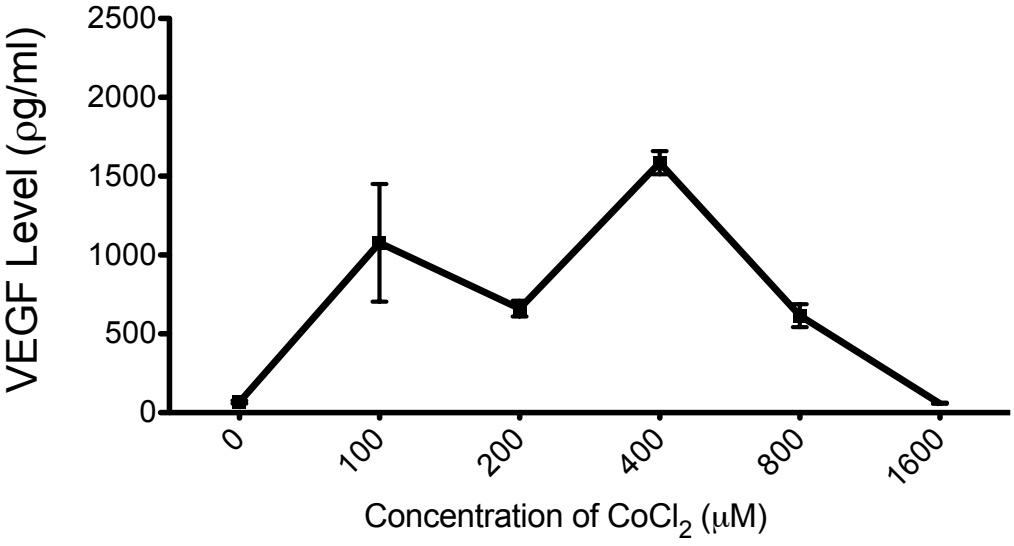

Figure 3.1.2a VEGF levels in supernatant in response to concentration of $CoCl_2$. RL952 endometrial cancer cells were incubated with $CoCl_2$ at the concentrations shown for 24 hours. Supernatants were removed from the cells at this point. Data is plotted as mean of 4 wells ± SEM.

$CoCl_2$ was relatively large, however the graph (figure 3.1.2.a) depicts the results of a small number of samples. The effect of 100 μM $CoCl_2$ on the viability of the cells was measured using the MTT test. Higher absorbance values showed that the cells were converting more of the MTT into the coloured product that was measured in the assay. A higher absorbance indicates more active cells in the well, and thus higher viability. As can be seen in figure 3.1.2.b, the mean absorbance of the groups of cells exposed to $CoCl_2$ is significantly lower than the control group ($p<0.0001$, $n=6$), showing that at 100 μM $CoCl_2$ increases the level of VEGF in cultured RL952 endometrial cancer cells, whilst also having a small but significant negative effect on the viability these cells.

3.1.3 Effect of $CoCl_2$ concentration on VEGF release from Endometrial Cancer Cells

The effects of $CoCl_2$ on VEGF and cell viability that were seen in RL952 cells were also seen in primary cultured cells. The basal VEGF levels in the cultured primary endometrial cancer cells were lower than that of the the RL952 cells. This occurred consistently throughout the subsequent experiments, with the immortalised cell line showing higher activity when compared to the primary cells. This was not unexpected, as the RL952 cells had been specifically selected to be a fast growing, robust cancer cell line. The cultured primary tissues, however, are samples from patients, which had been randomly selected, not

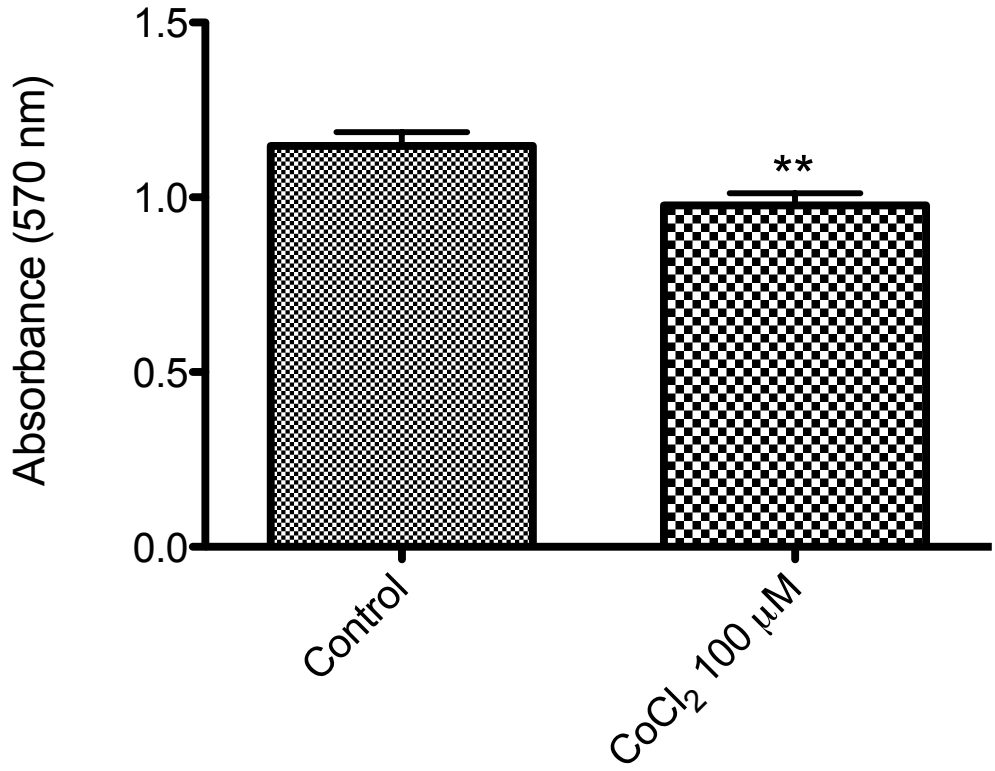

Figure 3.1.2b Cell Viability in RL952 Endometrial Cancer Cells when exposed to 100 μM $CoCl_2$ for 24 hours. Cell viability of adherent cells was measure using the MTT assay. After supernatant had been removed, MTT was added and cells left to incubate as described in methods section 2.3.1. Data is plotted as mean of 15 samples ± SEM.

selected for on the basis of the rate at which they grow or express certain molecules.

Concentrations of $CoCl_2$ less than 100 μM did not appear to significantly increase the amount of VEGF released (figure 3.1.3a). 200 μM increased VEGF levels further than 100 μM, but with a larger standard deviation. The 400 μM dose did not increase VEGF any more than 200 μM, which suggesting a maximum effect. As figure 3.1.3b shows, 100 μM $CoCl_2$ would appear to reduce the MTT product formed by cultured primary cells. This shows that a 100 μM dose of $CoCl_2$ increased the level of VEGF released from cultured primary endometrial cancer cells, whilst also having a small but significant negative effect on the viability these cells.

A $CoCl_2$ concentration of 100 μM incubated for 24 hours resulted in an increase in VEGF in both RL952 and primary endometrial cancer cells. This increase is considered to reflect the ability of $CoCl_2$ to accurately mimic an effect of hypoxia. Though this dose of $CoCl_2$ would appear to result in a small but significant decrease in the viability of the cultured cells, the magnitude of the change is such that the system can still be considered a viable model in which to study the effects of HIF1-α during hypoxia. As both the 100 μM concentration of $CoCl_2$ and the 24 hour incubation time are routinely used in examples in the literature, these two variables were fixed for the remainder of experiments.

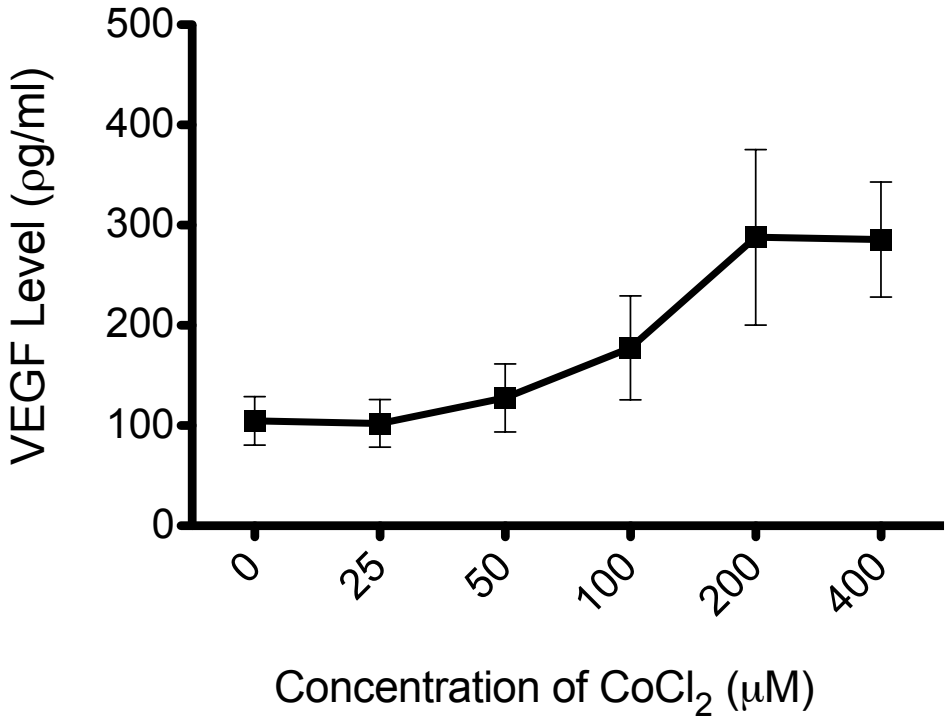

Figure 3.1.3a VEGF levels in primary endometrial cancer cells when exposed to $CoCl_2$. VEGF level was measured using ELISA. Cells were incubated with the relevant dose of $CoCl_2$ for 24 hours. Supernatants were removed from the cells at this point. Data is plotted as mean of n samples ± SD

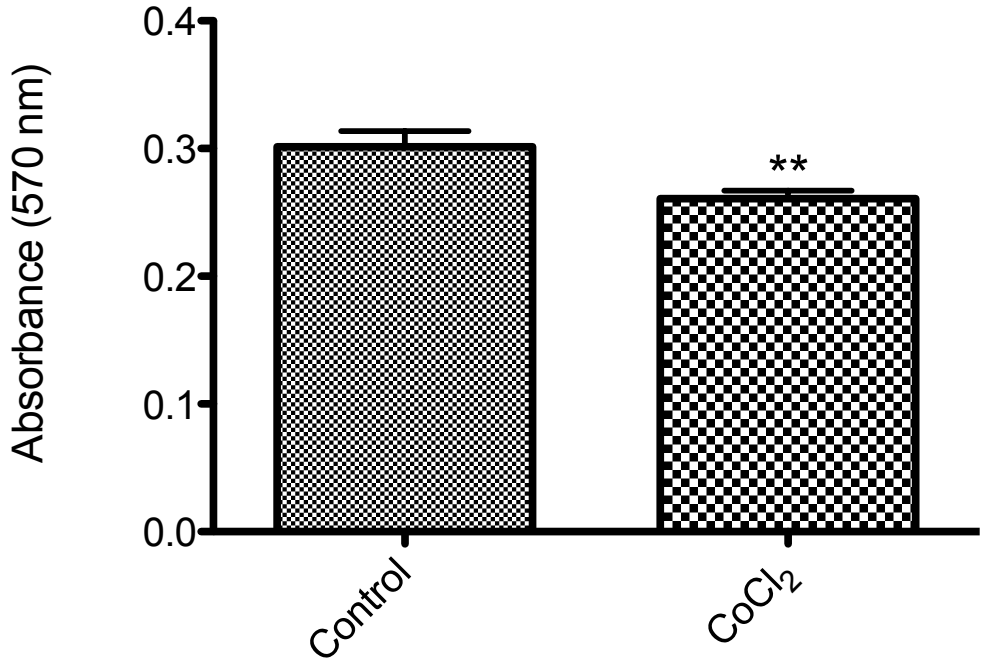

Figure 3.1.3b Cell Viability in Primary Endometrial Cancer Cells when exposed to $CoCl_2$. Cell viability of adherent cells was measure using the MTT assay. After supernatant had been removed, MTT was added and cells left to incubate. Data is plotted as mean of n samples ± SD

3.2 Effects of Resveratrol on VEGF Release from in Endometrial Cancer Cells

3.2.1 Effects of Resveratrol Concentration on Cell Viability

The viability of cells in the presence of resveratrol is an important consideration in determining the utility of the substance. Experiments were undertaken on both RL952 and primary cultures of tumour cells. Resveratrol was added to the cells at a concentration of 100 μM, and cells were incubated for 24 hours. The supernatant was then collected for VEGF assay (see section 3.2.3), and also an MTT assay was performed on the adhesive cells. When performed on RL952 cells (Figure 3.2.1a), the MTT assay revealed that the absorbance of the cells treated with resveratrol was significantly lower than those without (mean (0 μM) =1.147 ± 0.04; mean (100 μM) = 0.929 ± 0.038). A similar decrease in viability was seen in primary cultures (figure 3.2.1b). Three doses of resveratrol were used in this assay (Figure 3.2.1b). 50 μM resveratrol was seen to reduce the viability by approximately the same amount as 100 μM but the viability levels were not significantly different from control incubations. 200 μM appears to reduce the viability further, giving an absorbance value that is significantly less than the control.

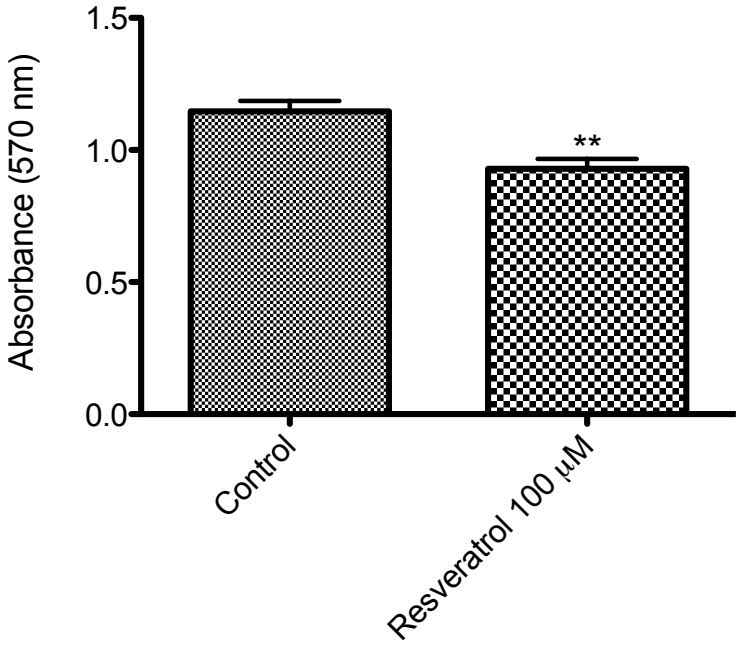

Figure 3.2.1a Cell Viability in RL952 Endometrial Cancer Cells when exposed to resveratrol. Cell viability of adherent cells was measured using the MTT assay. Cells were exposed to either 0 μM resveratrol or 100 μM resveratrol. After supernatant had been removed, MTT was added and cells left to incubate. Data is plotted as mean of 6 samples ± SEM

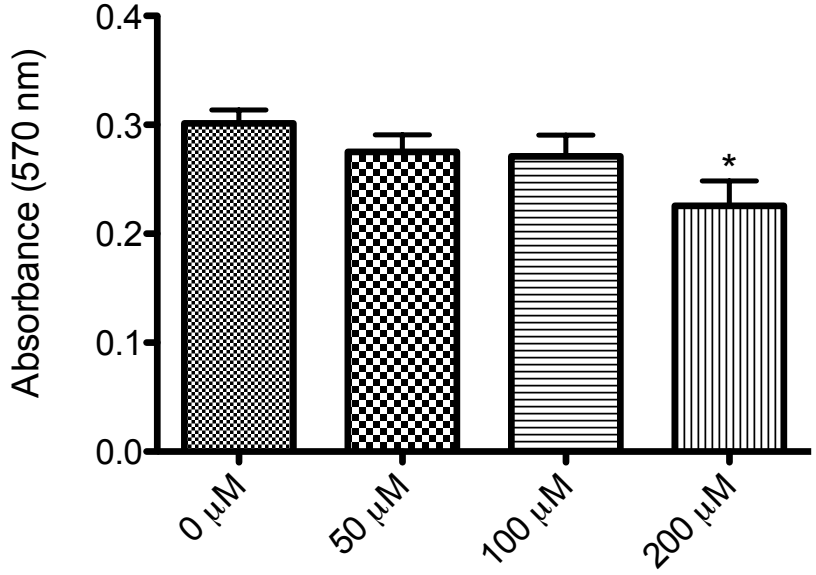

Figure 3.2.1b Cell Viability in primary endometrial cancer cells when exposed to resveratrol. Cell viability of adherent cells was measure using the MTT assay. Cells were exposed to a dose of resveratrol as indicated on the X axis. After supernatant had been removed, MTT was added and cells left to incubate. Data is plotted as mean of 15 samples ± SEM

3.2.2 Effects of Resveratrol in combination with $CoCl_2$ on cell viability

As a key aim of this research was to investigate whether resveratrol inhibits the effect of hypoxia in endometrial cancer cells, the effect it has on cell viability when combined with $CoCl_2$ is relevant. As both resveratrol and $CoCl_2$ have negative effects on the viability of cultured cells, the aim of these experiments was to investigate whether the two substances in combination had an additive effect on the viability of cells.

As was seen in the prior experiment, 100 μM $CoCl_2$ and 100 μM resveratrol both significantly reduced the viability of cells when used separately. When given together as a treatment in RL952 cells, a reduction in viability was not observed (Figure 3.2.2a). Cells with 0 μM resveratrol plus 100 $CoCl_2$ or 100 μM resveratrol plus 0 μM $CoCl_2$ had comparable levels of cell viability. There was no significant decrease in viability in cells which were exposed to 100 μM resveratrol plus 100 μM $CoCl_2$. This suggests that 100 μM resveratrol plus 100 μM $CoCl_2$ in combination have less effect on cell viability than resveratrol or $CoCl_2$ in isolation.

The pattern shown in RL952 cells was again seen with primary endometrial cancer cells. Figure 3.2.2b shows cells exposed to four doses of resveratrol (0 μM, 50 μM, 100 μM, 200 μM) with or without the presence of $CoCl_2$. While $CoCl_2$ reduces cell viability when given to cells without resveratrol, there was an increase in the viability when the two agents were added together. As this effect is

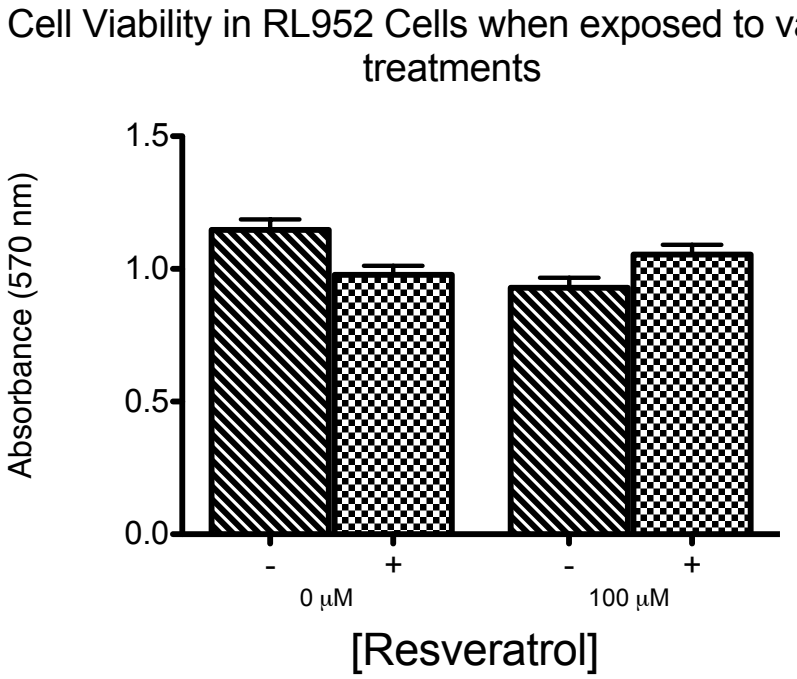

Figure 3.2.2a Cell Viability in RL952 cells when exposed to resveratrol and $CoCl_2$. Cell viability of adherent cells was measure using the MTT assay. Cells were exposed to a dose of resveratrol as indicated on the X axis. + and - indicates whether the cells were incubated with $CoCl_2$ (+) or without $CoCl_2$ (-). After supernatant had been removed, MTT was added and product was measured as described in methods. Data is plotted as mean of 6 samples ± SEM

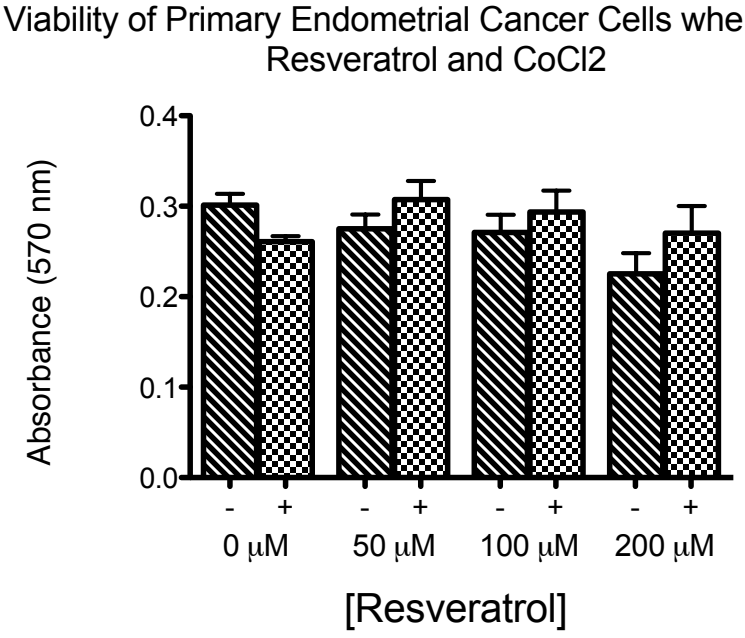

Figure 3.2.2b Cell Viability in primary endometrial cancer cells when exposed to resveratrol and $CoCl_2$. Cell viability of adherent cells was measure using the MTT assay. Cells were exposed to a dose of resveratrol as indicated on the X axis. + and - indicates whether the cells were incubated with $CoCl_2$ (+) or without $CoCl_2$. After supernatant had been removed, MTT was added and product was measured as described in methods. Data is plotted as mean of 15 samples ± SD

seen across both RL952 and primary cultured cells, it can be concluded that whilst resveratrol and $CoCl_2$ each have negative effects on cell viability, the combination of the two results in nonsignificant decrease in cell viability. Given this result, any effects which resveratrol may have on reducing VEGF levels in the presence of $CoCl_2$ cannot be attributed to the substance having a cytotoxic effect on the cells.

3.2.3 Effects of Resveratrol on VEGF Levels

The effect of resveratrol on VEGF Expression was investigated. It was hypothesised that adding resveratrol to cultured endometrial cancer cells would lead to a reduction in VEGF released. VEGF is a readily assayed marker of angiogenic stimulation. As was shown in the previous sections, $CoCl_2$ increased the expression of VEGF, and can thus be considered a hypoxia mimic. Since angiogenesis is induced by increased levels of HIF1-α, the aim of the following experiments was to investigate whether resveratrol could reduce the hypoxic-like behaviour of cells occurring as a consequence of the effects of $CoCl_2$. This hypothesis was tested in both RL952 immortalised cells and primary endometrial cancer cell cultures.

RL952 Endometrial Cell Line

The effects of resveratrol 100 μM on basal and $CoCl_2$-stimulated VEGF release were measured in RL952 cells. RL952 cells were grown on 24 well culture plates, and incubated in the presence or absence of resveratrol or $CoCl_2$; control, $CoCl_2$ only, resveratrol only and resveratrol with $CoCl_2$. In the absence of resveratrol,

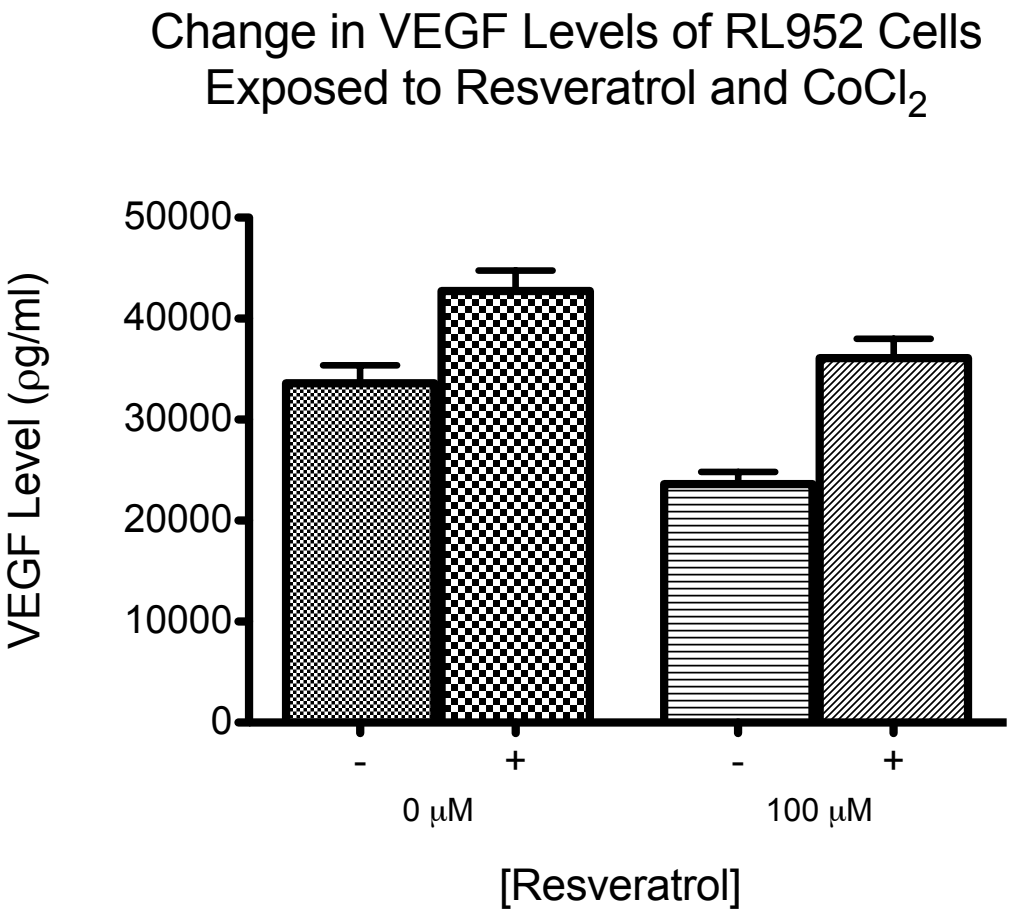

Figure 3.2.3a VEGF levels supernatant of primary endometrial cancer cells after exposure to resveratrol and $CoCl_2$. VEGF Level was measured using ELISA. Cells were incubated with the relevant concentration of $CoCl_2$ for 24 hours. Cells were exposed to a dose of resveratrol as indicated on the X axis. + and - indicates whether the cells were incubated in the presence of $CoCl_2$ (+) or in the absence $CoCl_2$ (-). Supernatants were removed from the cells at this point. Data is plotted as mean of 26 samples ± SEM

$CoCl_2$ caused a significant increase in the amount of VEGF released (figure 3.2.3a). This would suggest that the hypoxic effect of $CoCl_2$ leads to an increase in the amount of VEGF in the supernatant of cells. The level of VEGF released by cells incubated with resveratrol alone was significantly lower than the control treatment. ($p<0.0001$, n=15, paired t-test), indicating that resveratrol reduces VEGF secretion. When resveratrol and $CoCl_2$ were combined, the mean VEGF level was not significantly different from the control group (36050 ρg/ml and 33602 ρg/ml respectively, n=15, NS, paired t-test). It was, however, significantly less than the VEGF release seen with the $CoCl_2$ only treatment ($p<0.0006$, n=15, paired t-test), indicating that resveratrol significantly reduced the hypoxic effect of $CoCl_2$ on RL952 cells.

Primary endometrial tumour cells

The effects of resveratrol 100 μM on basal and $CoCl_2$-stimulated VEGF release were measured in primary endometrial cancer cells. The treatments were as described above for RL952 cells. The cells used were from 19 primary tumours that were collected and grown as cell cultures, as indicated in the methods section. $CoCl_2$ caused an increase in VEGF levels (figure 3.2.3b.A) that was significantly above that of the control treatment (mean (control)= 350.4 ρg/ml ± 57.46 cf mean ($CoCl_2$)= 926.3 ρg/ml ± 125.4, n=108, $P<0.0001$, paired t-test). The increase gave a VEGF level that was well over double that of the control. The second graph (figure 3.2.3b.B) showed that resveratrol significantly reduced VEGF to 120.6 ρg/ml ± 30.3 (n=113, $p<0.0001$, t-test)

In the third graph (figure 3.2.3b.C), $CoCl_2$ was compared to resveratrol plus $CoCl_2$. Though the mean of the resveratrol and $CoCl_2$ was lower (mean = 245.6 ρg/ml ± 45.45) than that of the control (mean= 350.4 ρg/ml ± 57.46), this difference was not significant (n=111, NS, t-test). Though the resveratrol and $CoCl_2$ group could not be shown to be statistically lower than the control, this could be interpreted as the resveratrol and $CoCl_2$ group being the same as the control. This shows that the resveratrol treatment reversed the effect of HIF1-α increase induced by $CoCl_2$.

The fourth graph (figure 3.2.3b.D) compared the 0 μM resveratrol plus 100 μM $CoCl_2$ group to the 100 μM resveratrol plus 100 μM $CoCl_2$ group. The mean VEGF level of the resveratrol plus $CoCl_2$ group (mean = 245.6 ρg/ml ± 45.45) was significantly lower (n=111, $p<0.0001$, t-test) than that of the $CoCl_2$ group (mean = 926.3 ρg/ml ± 125.4). These results support those described in RL952 cells earlier in this section, and suggest that the pattern of VEGF response to $CoCl_2$ and resveratrol in both endometrial cancer cell lines and primary cultured endometrial cells is robust. These results are collated in the table below.

Treatment	**0 μM Resveratrol**	**100 μM Resveratrol**
0 μM $CoCl_2$	350.4 ρg/ml ± 57.46	120.6 ρg/ml ± 30.3
100 μM $CoCl_2$	926.3 ρg/ml ± 125.4	245.6 ρg/ml ± 45.45

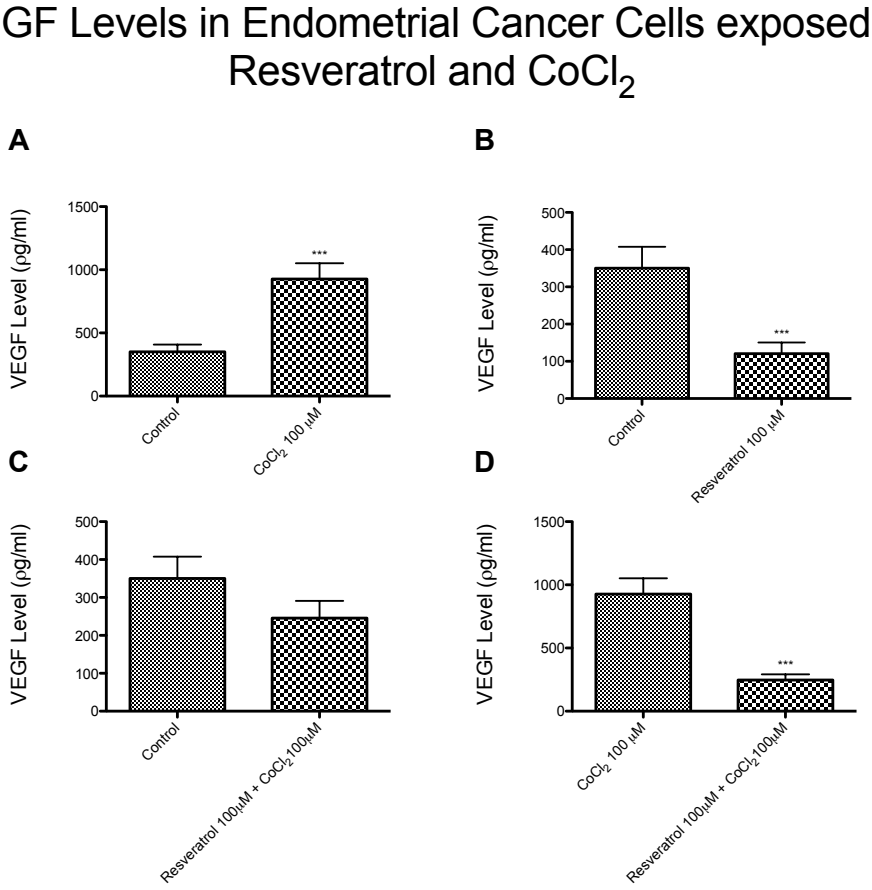

Figure 3.2.3b VEGF levels supernatant of Primary Endometrial Cancer Cells after exposure to resveratrol and $CoCl_2$. VEGF Level was measured using ELISA. Cells were incubated with the relevant treatment for 24 hours. Supernatants were removed from the cells at this point. **(A)** shows mean VEGF from control group (350.4 ρg/ml ± 57.46) is less than mean VEGF from $CoCl_2$ group (926.3 ρg/ml ± 125.4) (n=108, P<0.0001, paired t-test). **(B)** shows that the mean of the resveratrol 100 group (120.6 ρg/ml ± 30.3) is less than the control (n=113, p<0.0001, t-test). **(C)** shows mean VEGF from resveratrol + $CoCl_2$ group (245.6 ρg/ml ± 45.45) is less than the mean VEGF from control group (350.4 ρg/ml ± 57.46), however this does not reach significance (n=111, NS, t-test) **(D)** shows mean VEGF from resveratrol + $CoCl_2$ group (245.6 ρg/ml ± 45.45) is significantly less than mean VEGF from $CoCl_2$ group (926.3 ρg/ml ± 125.4) (n=111, p<0.0001, t-test) Data is plotted as mean of samples ± SEM

3.3 Effect of EGCG on VEGF release from in endometrial cancer cells

The effects of EGCG on VEGF levels in endometrial cancer cells in basal culture conditions and when HIF1-α levels are increased by $CoCl_2$ was examined. The experimental procedure used was identical to that used to test the effects of resveratrol on VEGF levels, except that EGCG was substituted for resveratrol. Primary endometrial cancer cells were grown up to confluence, then transferred into 24 well plates. The cells were then incubated with the treatment agent for 24 hours, before the supernatant was collected for assay.

3.3.1 Effect of EGCG on VEGF Levels

As was seen in previous experiments, 100 μM $CoCl_2$ (figure 3.3.1b.A) caused an increase in the amount of VEGF in the supernatant (mean=537.65 ρg/ml ± 70.26) when compared to the control (436.06 ρg/ml ± 86.74). However, unlike the previous graphs, this result is not significant (n=22, NS, t-test), possibly due to smaller sample size in this experiment (n=22), giving larger standard error.

Figure 3.3.1b.B showed that EGCG strongly inhibited VEGF levels. Cells were treated with 100 μM of EGCG. This led to a significant reduction in the amount of VEGF found in the supernatant (82.39 ρg/ml ± 19.54). This was approximately one fifth of the level of VEGF seen in the control group. The difference between the two means was statistically significant (n= 22, $p<0.0001$, t-test).

The next figure (figure 3.3.1b.C) shows that 100 µM EGCG plus 100 µM $CoCl_2$ reduces VEGF, though not to the same degree as 100 µM EGCG only. The mean VEGF level of the supernatant from cells incubated with 100 µM EGCG plus 100 µM $CoCl_2$ was 235.65 ρg/ml ± 47.42. This was significantly lower than the VEGF level of the Control (n=22, P=0.0315, t-test). The final graph (figure 3.3.1b.D) compares the $CoCl_2$ only group with the 100 µM EGCG plus 100 µM $CoCl_2$ group. The difference between the two means was statistically significant ($p<0.0001$, n=36, t-test), with the 100 µM EGCG plus 100 µM $CoCl_2$ treatment having a mean VEGF level of less than half the $CoCl_2$ only group. This showed that EGCG was a potent inhibitor of VEGF secretion from endometrial cancer cells, and significantly reduced the effects of $CoCl_2$. The results are summarised in the following table.

Treatment	**0 µM EGCG**	**100 µM EGCG**
0 µM $CoCl_2$	436.06 ρg/ml ± 86.74	82.39 ρg/ml ± 19.54
100 µM $CoCl_2$	537.65 ρg/ml ± 70.26	235.65 ρg/ml ± 47.42

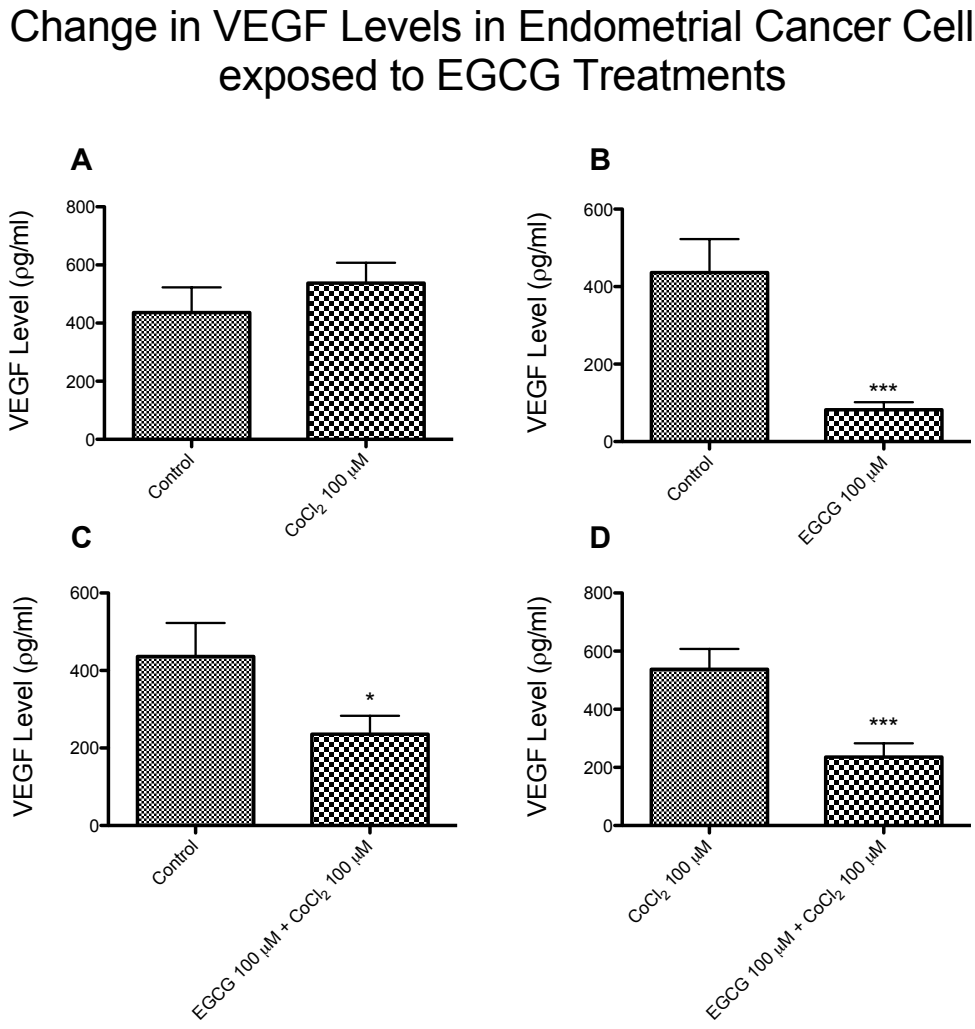

Figure 3.3.1b VEGF levels supernatant of Primary Endometrial Cancer Cells after exposure to EGCG and $CoCl_2$. VEGF Level was measured using ELISA. Cells were incubated with the relevant treatment for 24 hours. Supernatants were removed from the cells at this point. **(A)** shows mean VEGF from control group (436.06 ρg/ml ± 86.74) is less than mean VEGF from $CoCl_2$ group (mean=537.65 ρg/ml ± 70.26) (n=22, NS, t-test) **(B)** shows that the mean of the EGCG 100 group (82.39 ρg/ml ± 19.54) is less than the control (n= ?, p<0.0001, t-test). **(C)** shows mean VEGF from EGCG + $CoCl_2$ group (235.65 ρg/ml ± 47.42) is significantly less than the mean VEGF from control group (350.4 ρg/ml ± 57.46) (n=?, P=0.0315, t-test)**. (D)** shows mean VEGF from EGCG + $CoCl_2$ group (235.65 ρg/ml ± 47.42) is significantly less than mean VEGF from $CoCl_2$ group (537.65 ρg/ml ± 70.26) (p<0.0001, n=36, t-test) Data is plotted as mean of n samples ± SD

3.4 Immunohistochemistry

Immunological staining of endometrial cancer cells used in experimental work was undertaken to establish the type of cells that were experimented on. Cells were fixed and stained by the Pathology Department, Christchurch Hospital. Staining solutions were selected to identify whether cells were endometrial epithelial or endometrial stromal cells. These solutions were stains that were either cytokeratin or vimentin specific. At least 50% of cells stained positive for cyotkeratin, whilst less than 10% stained positive for vimentin. This staining shows that most of the cultured cells are stromal cells. Two further stains were then used, CD10 and CD31. CD31, which stains for endothelial cells, was negative in the cultured cells. CD10, which stains for stromal cells, was positive. This staining confirmed that the cells grown and experimented upon were predominantly endometrial stromal cells. It was expected that most of the cells would have been epithelial cells, as they are cells in which endometrial adenocarcinomas develop. When the tumour was post-operatively dissected by the pathologist, a section of epithelial cells was provided. However, stromal cells appear to grow more readily than epithelial cells using the cell culture methods utilised in this project.

4

DISCUSSION

4.1 Effect of $CoCl_2$ on VEGF release from endometrial cancer cells

This study shows that endometrial cancer cells incubated in the presence of $CoCl_2$ released significantly larger amounts of VEGF than cells that were incubated without $CoCl_2$. As increased VEGF is a major angiogenic factor in endometrial cancers (Sivridis et al., 2002), endometrial cancer cells incubated with $CoCl_2$ can be considered as an *in vitro* model for cells undergoing angiogenesis. Based on previous literature that has used the same concentrations of $CoCl_2$ (Dai et al., 2008; Shu et al., 2008), it is assumed that $CoCl_2$ is working by inducing HIF1-α expression and therefore acting as a hypoxia mimic.

The results of the immunohistochemistry tests identified that the majority of cells cultured cells in this project were endometrial stromal cells. As the cells that were initially cultured were from the epithelial layer of the tumour, it was expected that the cultured cells would also be epithelial cells. However, cell dispersion and repeat passages of the cells may have selected out stromal cells from sample. As the stromal cells appear to grow better in these conditions that epithelial cells, the ratio of stromal to epithelial cells being cultured moves toward the former. Previous work in this laboratory has shown that $CoCl_2$ can trigger an increase in

the amount of VEGF released from endometrial stromal cells (Abbas et al., 2004), which supports the patterns of VEGF release reported in this thesis.

4.2 Effect of Resveratrol on VEGF release from endometrial cancer cells

Resveratrol has been used previously in endometrial cancer cells, however this project is the first to have investigated the effects of resveratrol on VEGF levels in this system. It had previously been shown that resveratrol exerted a tumouricidal effect in uterine cancer cells (Sexton et al., 2006), including endometrial cancer cell lines RL952 and Ishikawa, which were both used in this study. However, the focus of this study was apoptosis, rather than angiogenesis, and studied the COX molecule. Similarly, resveratrol was used in a study which looked at cytostatic and anti-estrogenic markers in the Ishikawa endometrial adenocarcinoma cell line (Bhat and Pezzuto, 2001), but concluded that it had little effect on these markers. The dose used in the study (10 μm) was much lower than used in this work. This study was the first to show that resveratrol reduces the secretion of VEGF in endometrial cancer cells. By using resveratrol at a concentration of 100 μm, the amount of VEGF released into the supernatant of endometrial cancer cells was significantly reduced, when compared to the VEGF levels in the supernatant of endometrial cancer cells without treatment. When compared to cells with had increased VEGF release induced by $CoCl_2$, resveratrol again significantly reduced the amount of VEGF in the supernatant. This suggests that resveratrol can reduce the angiogenic effects induced by hypoxia in endometrial cancer cells. This

supports research previously published that has investigated the effect of resveratrol on VEGF and HIF1-α in other tumour types.

While this study did not measure cellular HIF1-α levels directly, studies reported in the literature suggest that resveratrol reduces VEGF by inhibiting HIF1-α. Increased levels of VEGF and HIF1-α are found in ovarian cancer (Wong et al., 2003) and resveratrol has been shown to inhibit HIF1-α and reduce VEGF levels in this cancer (Cao et al., 2004). As VEGF has been shown to correlate with HIF expression in endometrial cancers (Ozbudak et al., 2008), it would be expected that inhibition of HIF1-α by resveratrol would appear to be the mechanism by which the compound reduces VEGF levels. However, direct measurement of HIF1-α levels in endometrial cancer cells treated with resveratrol is still required to confirm this.

Studies that have investigated the role of VEGF in endometrial cancer have shown that the level of VEGF and HIF1-α was significantly elevated in these cancers (Kazi and Koos, 2007). A large study that looked at rates of breast cancer - another cancer that shows elevated HIF levels (Kimbro and Simons, 2006) - found that women who consumed more resveratrol in their diets had lower risks of developing breast cancer (La Vecchia and Bosetti, 2006). Given the significant reductions in VEGF caused by resveratrol in this study, further investigation into the effects of dietary resveratrol on endometrial cancer rates are warranted.

4.3 Effect of EGCG on VEGF release from endometrial cancer cells

This study was the first to show that EGCG, a catechin extract from green tea, reduces the secretion of VEGF in endometrial cancer cells. By using EGCG at a concentration of 100 μm, the amount of VEGF released into the supernatant of endometrial cancer cells was significantly reduced, when compared to the VEGF levels in the supernatant of endometrial cancer cells without treatment. When compared to cells with had increased VEGF release induced by $CoCl_2$, EGCG again significantly reduced the amount of VEGF in the supernatant. This suggests that EGCG can reduce the angiogenic effects induced by hypoxia in endometrial cancer cells. This result is supported from other worked previously reported that has investigated the effects of EGCG on VEGF release in other cancer cell types, including cervical and hepatoma cells (Zhang et al., 2006). However, replication of this result is required, as it has been reported that EGCG can actually lead to an increase in VEGF (Thomas and Kim, 2005) and in cell proliferation (Zhou et al., 2004).

As seen in figure 3.1.1.b, the Cocl2 group was not significantly higher than the control group, though it was significantly higher in the resveratrol experiments. This is possibly due to smaller sample size in this experiment (n=22), giving larger standard deviation. It could also be attributed, in part, to primary tumours used in this experiment rather than a cell line. As fewer tumours were used than in

the resveratrol study, a disproportionate number of tumours that express low levels of VEGF may be represented in this graph. Further investigation into the effects of EGCG on VEGF levels in cancer cells should be undertaken before it can be recommended that green tea be considered as an anti-cancer agent.

4.4 Suggestions for further research

Though it has been shown that resveratrol and EGCG reduce VEGF, the effects of these substances on the expression of HIF1-α have not been measured directly. An investigation into the effects of these food compounds on HIF1-α protein levels, as well as mRNA levels would contribute to our better understanding of the role with HIF1-α and VEGF have in endometrial cancers. It would also be interesting to see whether there is a correlation between HIF1-α expression and VEGF levels in cultured cancer cells, and if, as we expect, there is, whether the changes in VEGF that are seen with $CoCl_2$, resveratrol and EGCG can be primarily attributed to HIF1-α, or whether there is another factor involved that effects VEGF in these situations. Another area of interest would be investigating the level of basal HIF1-α expression in primary endometrial tumours, to see whether this was correlated with grade. Many of the tumour that have higher basal levels of VEGF secretion have a less dramatic response to $CoCl_2$ than tumours that have lower levels of basal VEGF. It would be interesting to investigate whether this differential response was related to basal HIF1-α levels, as it may be that tumours with higher basal HIF1-α are less affected than tumours with lower basal levels. HIF1-α protein expression could be measured by Western Blotting, or HIF1-α ELISA. The mRNA levels of HIF1-α could be investigated using Real-Time Polymerase Chain

Reaction (RT-PCR). Interfering with the HIF1-α gene with small interfering RNA (si-RNA) could be performed to see what the levels of VEGF secreted by the cell were when there was little or not active HIF1-α inducing expression. By combining these techniques with the results of this paper and work completed previously in the literature, we could construct a more complete picture of the role of HIF1-α and VEGF in the hypoxic response of endometrial cancer cells.

While this research has shown that resveratrol is a strong inhibitor of VEGF secretion in endometrial cancer cells, there may be other compounds in grapes or red wine that have anti-angiogenic activity. Experiments similar to the ones performed in this study could be undertaken, to investigate whether less processed fractions of grape skin extract have similar effects on VEGF and HIF in endometrial cancer. It has been shown that resveratrol and an extract of grape skin act to inhibit prostate cancer growth, but that the two substances act through distinct pathways (Hudson et al., 2007). Further work on the effects of resveratrol and other extracts of grape skin will be necessary for any consideration of red wines as anti-cancer dietary supplements. It would also be interesting to investigate whether there was a syngeristic effect on VEGF levels when resveratrol and EGCG were combined, as has been suggested (Morre and Morre, 2006; Sagar et al., 2006a, b).

While some papers investigating links between mutations in the HIf gene and cancer have failed to establish a link (Apaydin et al., 2008; Kuwai et al., 2004), a

recent paper (Konac et al., 2007) showed that women with mutations in their HIF1-α genes had an increased risk of developing gynaecologic cancers, including endometrial cancer. Using the endometrial cancer samples collected during this study, and those stored in the cancer society tissue bank, gene sequencing could be undertaken to establish whether this pattern was observable in a group of New Zealand women. Basal VEGF expression from the samples could be matched with the gene sequence, to establish whether the amount of VEGF secreted by cultured endometrial cancer cells was directly related to mutation status of the HIF1-α gene. Further to this, mutations in the HIF1-α gene could then be cross referenced with tumour grade to investigate whether there is a correlation between the two.

HIF1-α gene sequence information could also be used to investigate the pharmacogenetic properties of Resveratrol and EGCG. A number of recent papers have looked at the relationship between genes and environmental factors in determining a response to a stimulus (Caspi et al., 2002; Caspi et al., 2003). It would be interesting to investigate whether a tumours response to Resveratrol or EGCG was a function of the mutation state of its HIF1-α gene. The effect of the food compounds on VEGF levels may be affected by whether the specific tumour carries a mutation in the HIF1-α gene.

REFERENCES

Abbas, M.M., Evans, J.J., Sykes, P.H., and Benny, P.S. (2004). Modulation of vascular endothelial growth factor and thymidine phosphorylase in normal human endometrial stromal cells. Fertil Steril *82 Suppl 3*, 1048-1053.

Alkhalaf, M., El-Mowafy, A., Renno, W., Rachid, O., Ali, A., and Al-Attyiah, R. (2008). Resveratrol-induced apoptosis in human breast cancer cells is mediated primarily through the caspase-3-dependent pathway. Arch Med Res *39*, 162-168.

Apaydin, I., Konac, E., Onen, H.I., Akbaba, M., Tekin, E., and Ekmekci, A. (2008). Single nucleotide polymorphisms in the hypoxia-inducible factor-1alpha (HIF-1alpha) gene in human sporadic breast cancer. Arch Med Res *39*, 338-345.

Arts, I.C., Jacobs, D.R., Jr., and Folsom, A.R. (2002). Dietary catechins and cancer incidence: the Iowa Women's Health Study. IARC Sci Publ *156*, 353-355.

Athar, M., Back, J.H., Tang, X., Kim, K.H., Kopelovich, L., Bickers, D.R., and Kim, A.L. (2007). Resveratrol: a review of preclinical studies for human cancer prevention. Toxicol Appl Pharmacol *224*, 274-283.

Bakkum-Gamez, J.N., Gonzalez-Bosquet, J., Laack, N.N., Mariani, A., and Dowdy, S.C. (2008). Current issues in the management of endometrial cancer. Mayo Clin Proc *83*, 97-112.

Baur, J.A., and Sinclair, D.A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov *5*, 493-506.

Bhat, K.P., and Pezzuto, J.M. (2001). Resveratrol exhibits cytostatic and antiestrogenic properties with human endometrial adenocarcinoma (Ishikawa) cells. Cancer Res *61*, 6137-6144.

Bhat, K.P., and Pezzuto, J.M. (2002). Cancer chemopreventive activity of resveratrol. Ann N Y Acad Sci *957*, 210-229.

Bruick, R.K., and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science *294*, 1337-1340.

Cao, Z., Fang, J., Xia, C., Shi, X., and Jiang, B.H. (2004). trans-3,4,5'-Trihydroxystibene inhibits hypoxia-inducible factor 1alpha and vascular endothelial

growth factor expression in human ovarian cancer cells. Clin Cancer Res *10*, 5253-5263.

Caspi, A., McClay, J., Moffitt, T.E., Mill, J., Martin, J., Craig, I.W., Taylor, A., and Poulton, R. (2002). Role of genotype in the cycle of violence in maltreated children. Science *297*, 851-854.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A.*, et al.* (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science *301*, 386-389.

Chen, C.A., Cheng, W.F., Lee, C.N., Wei, L.H., Chu, J.S., Hsieh, F.J., and Hsieh, C.Y. (2001). Cytosol vascular endothelial growth factor in endometrial carcinoma: correlation with disease-free survival. Gynecol Oncol *80*, 207-212.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem *275*, 25733-25741.

Dai, M., Cui, P., Yu, M., Han, J., Li, H., and Xiu, R. (2008). Melatonin modulates the expression of VEGF and HIF-1 alpha induced by CoCl2 in cultured cancer cells. J Pineal Res *44*, 121-126.

Dales, J.P., Garcia, S., Meunier-Carpentier, S., Andrac-Meyer, L., Haddad, O., Lavaut, M.N., Allasia, C., Bonnier, P., and Charpin, C. (2005). Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients. Int J Cancer *116*, 734-739.

de la Lastra, C.A., and Villegas, I. (2007). Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. Biochem Soc Trans *35*, 1156-1160.

Elson, D.A., Thurston, G., Huang, L.E., Ginzinger, D.G., McDonald, D.M., Johnson, R.S., and Arbeit, J.M. (2001). Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1alpha. Genes Dev *15*, 2520-2532.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A.*, et al.* (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43-54.

Fassina, G., Vene, R., Morini, M., Minghelli, S., Benelli, R., Noonan, D.M., and Albini, A. (2004). Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. Clin Cancer Res *10*, 4865-4873.

Fujiki, H. (2005). Green tea: Health benefits as cancer preventive for humans. Chem Rec *5*, 119-132.

Gao, Y.T., McLaughlin, J.K., Blot, W.J., Ji, B.T., Dai, Q., and Fraumeni, J.F., Jr. (1994). Reduced risk of esophageal cancer associated with green tea consumption. J Natl Cancer Inst *86*, 855-858.

Garcia, J.A. (2006). HIFing the brakes: therapeutic opportunities for treatment of human malignancies. Sci STKE *2006*, pe25.

Gordan, J.D., and Simon, M.C. (2007). Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev *17*, 71-77.

Hastings, J.M., Licence, D.R., Burton, G.J., Charnock-Jones, D.S., and Smith, S.K. (2003). Soluble vascular endothelial growth factor receptor 1 inhibits edema and epithelial proliferation induced by 17beta-estradiol in the mouse uterus. Endocrinology *144*, 326-334.

Horree, N., Groot, A.J., van Hattem, W.A., Heintz, A.P., Vooijs, M., and van Diest, P.J. (2008). HIF-1A gene mutations associated with higher microvessel density in endometrial carcinomas. Histopathology *52*, 637-639.

Hudson, T.S., Hartle, D.K., Hursting, S.D., Nunez, N.P., Wang, T.T., Young, H.A., Arany, P., and Green, J.E. (2007). Inhibition of prostate cancer growth by muscadine grape skin extract and resveratrol through distinct mechanisms. Cancer Res *67*, 8396-8405.

Imai, K., Suga, K., and Nakachi, K. (1997). Cancer-preventive effects of drinking green tea among a Japanese population. Prev Med *26*, 769-775.

Inoue, M., Tajima, K., Hirose, K., Hamajima, N., Takezaki, T., Kuroishi, T., and Tominaga, S. (1998). Tea and coffee consumption and the risk of digestive tract cancers: data from a comparative case-referent study in Japan. Cancer Causes Control *9*, 209-216.

Iyer, N.V., Leung, S.W., and Semenza, G.L. (1998). The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation. Genomics *52*, 159-165.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J*., et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G*., et al.* (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science *275*, 218-220.

Ji, B.T., Chow, W.H., Hsing, A.W., McLaughlin, J.K., Dai, Q., Gao, Y.T., Blot, W.J., and Fraumeni, J.F., Jr. (1997). Green tea consumption and the risk of pancreatic and colorectal cancers. Int J Cancer *70*, 255-258.

Jung, Y.D., and Ellis, L.M. (2001). Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. Int J Exp Pathol *82*, 309-316.

Kimbro, K.S., and Simons, J.W. (2006). Hypoxia-inducible factor-1 in human breast and prostate cancer. Endocr Relat Cancer *13*, 739-749.

Konac, E., Onen, H.I., Metindir, J., Alp, E., Biri, A.A., and Ekmekci, A. (2007). An investigation of relationships between hypoxia-inducible factor-1 alpha gene polymorphisms and ovarian, cervical and endometrial cancers. Cancer Detect Prev *31*, 102-109.

Kong, D., Park, E.J., Stephen, A.G., Calvani, M., Cardellina, J.H., Monks, A., Fisher, R.J., Shoemaker, R.H., and Melillo, G. (2005). Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. Cancer Res *65*, 9047-9055.

Kono, S., Ikeda, M., Tokudome, S., and Kuratsune, M. (1988). A case-control study of gastric cancer and diet in northern Kyushu, Japan. Jpn J Cancer Res *79*, 1067-1074.

Kuwai, T., Kitadai, Y., Tanaka, S., Kuroda, T., Ochiumi, T., Matsumura, S., Oue, N., Yasui, W., Kaneyasu, M., Tanimoto, K*., et al.* (2004). Single nucleotide polymorphism in the hypoxia-inducible factor-1alpha gene in colorectal carcinoma. Oncol Rep *12*, 1033-1037.

La Vecchia, C., and Bosetti, C. (2006). Diet and cancer risk in Mediterranean countries: open issues. Public Health Nutr *9*, 1077-1082.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

Melillo, G. (2006). Inhibiting hypoxia-inducible factor 1 for cancer therapy. Mol Cancer Res *4*, 601-605.

Melillo, G. (2007). Targeting hypoxia cell signaling for cancer therapy. Cancer Metastasis Rev *26*, 341-352.

Meunier-Carpentier, S., Dales, J.P., Djemli, A., Garcia, S., Bonnier, P., Andrac-Meyer, L., Lavaut, M.N., Allasia, C., and Charpin, C. (2005). Comparison of the prognosis indication of VEGFR-1 and VEGFR-2 and Tie2 receptor expression in breast carcinoma. Int J Oncol *26*, 977-984.

Morre, D.M., and Morre, D.J. (2006). Anticancer activity of grape and grape skin extracts alone and combined with green tea infusions. Cancer Lett *238*, 202-209.

Nakachi, K., Suemasu, K., Suga, K., Takeo, T., Imai, K., and Higashi, Y. (1998). Influence of drinking green tea on breast cancer malignancy among Japanese patients. Jpn J Cancer Res *89*, 254-261.

Oehler, M.K., Fung, A., and Jobling, T.W. (2005). Advances in the treatment of endometrial cancer. J Br Menopause Soc *11*, 18-22.

Ozbudak, I.H., Karaveli, S., Simsek, T., Erdogan, G., and Pestereli, E. (2008). Neoangiogenesis and expression of hypoxia-inducible factor 1alpha, vascular endothelial growth factor, and glucose transporter-1 in endometrioid type endometrium adenocarcinomas. Gynecol Oncol *108*, 603-608.

Park, S.Y., Jeong, K.J., Lee, J., Yoon, D.S., Choi, W.S., Kim, Y.K., Han, J.W., Kim, Y.M., Kim, B.K., and Lee, H.Y. (2007). Hypoxia enhances LPA-induced HIF-1alpha and VEGF expression: their inhibition by resveratrol. Cancer Lett *258*, 63-69.

Pijnenborg, J.M., Wijnakker, M., Hagelstein, J., Delvoux, B., and Groothuis, P.G. (2007). Hypoxia contributes to development of recurrent endometrial carcinoma. Int J Gynecol Cancer *17*, 897-904.

Pukkala, E., Kyyronen, P., Sankila, R., and Holli, K. (2002). Tamoxifen and toremifene treatment of breast cancer and risk of subsequent endometrial cancer: a population-based case-control study. Int J Cancer *100*, 337-341.

Rockwell, L.C., Pillai, S., Olson, C.E., and Koos, R.D. (2002). Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. Biol Reprod *67*, 1804-1810.

Sagar, S.M., Yance, D., and Wong, R.K. (2006a). Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-Part 1. Curr Oncol *13*, 14-26.

Sagar, S.M., Yance, D., and Wong, R.K. (2006b). Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-Part 2. Curr Oncol *13*, 99-107.

Saiko, P., Szakmary, A., Jaeger, W., and Szekeres, T. (2008). Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutat Res *658*, 68-94.

Sartippour, M.R., Shao, Z.M., Heber, D., Beatty, P., Zhang, L., Liu, C., Ellis, L., Liu, W., Go, V.L., and Brooks, M.N. (2002). Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. J Nutr *132*, 2307-2311.

Sexton, E., Van Themsche, C., LeBlanc, K., Parent, S., Lemoine, P., and Asselin, E. (2006). Resveratrol interferes with AKT activity and triggers apoptosis in human uterine cancer cells. Mol Cancer *5*, 45.

Shu, B., Yang, W.W., and Yang, H.T. (2008). Expression pattern of E2F6 in physical and chemical hypoxia-induced apoptosis. Sheng Li Xue Bao *60*, 1-10.

Siemann, E.H., and Creasy, L.L. (1992). Concentration of the phytoalexin resveratrol in wine. Am J Eno Vitic *43*, 49-52.

Sivridis, E. (2001). Angiogenesis and endometrial cancer. Anticancer Res *21*, 4383-4388.

Sivridis, E., Giatromanolaki, A., Gatter, K.C., Harris, A.L., and Koukourakis, M.I. (2002). Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer *95*, 1055-1063.

Stroka, D.M., Burkhardt, T., Desbaillets, I., Wenger, R.H., Neil, D.A., Bauer, C., Gassmann, M., and Candinas, D. (2001). HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. Faseb J *15*, 2445-2453.

Takaoka, M.J. (1940). Of the phenolic substances of white hellebore (Veratrum grandiflorum). J Faculty Sci Hokkaido Imperial University *3*, 1-16.

Tang, X., Zhang, Q., Nishitani, J., Brown, J., Shi, S., and Le, A.D. (2007). Overexpression of human papillomavirus type 16 oncoproteins enhances hypoxia-inducible factor 1 alpha protein accumulation and vascular endothelial growth factor expression in human cervical carcinoma cells. Clin Cancer Res *13*, 2568-2576.

Thomas, R., and Kim, M.H. (2005). Epigallocatechin gallate inhibits HIF-1alpha degradation in prostate cancer cells. Biochem Biophys Res Commun *334*, 543-548.

Vaupel, P. (2004). The role of hypoxia-induced factors in tumor progression. Oncologist *9 Suppl 5*, 10-17.

Vaupel, P., Kelleher, D.K., and Hockel, M. (2001). Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. Semin Oncol *28*, 29-35.

Vaupel, P., and Mayer, A. (2007). Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev *26*, 225-239.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Wong, C., Wellman, T.L., and Lounsbury, K.M. (2003). VEGF and HIF-1alpha expression are increased in advanced stages of epithelial ovarian cancer. Gynecol Oncol *91*, 513-517.

Wright, J.D., Powell, M.A., Rader, J.S., Mutch, D.G., and Gibb, R.K. (2007). Bevacizumab therapy in patients with recurrent uterine neoplasms. Anticancer Res *27*, 3525-3528.

Yance, D.R., Jr., and Sagar, S.M. (2006). Targeting angiogenesis with integrative cancer therapies. Integr Cancer Ther *5*, 9-29.

Yokoyama, Y., Charnock-Jones, D.S., Licence, D., Yanaihara, A., Hastings, J.M., Holland, C.M., Emoto, M., Sakamoto, A., Sakamoto, T., Maruyama, H.*, et al.* (2003). Expression of vascular endothelial growth factor (VEGF)-D and its receptor, VEGF receptor 3, as a prognostic factor in endometrial carcinoma. Clin Cancer Res *9*, 1361-1369.

Yu, F., White, S.B., Zhao, Q., and Lee, F.S. (2001a). Dynamic, site-specific interaction of hypoxia-inducible factor-1alpha with the von Hippel-Lindau tumor suppressor protein. Cancer Res *61*, 4136-4142.

Yu, F., White, S.B., Zhao, Q., and Lee, F.S. (2001b). HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci U S A *98*, 9630-9635.

Zhang, M., Binns, C.W., and Lee, A.H. (2002). Tea consumption and ovarian cancer risk: a case-control study in China. Cancer Epidemiol Biomarkers Prev *11*, 713-718.

Zhang, Q., Tang, X., Lu, Q., Zhang, Z., Rao, J., and Le, A.D. (2006). Green tea extract and (-)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1alpha protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. Mol Cancer Ther *5*, 1227-1238.

Zhang, Q., Tang, X., Lu, Q.Y., Zhang, Z.F., Brown, J., and Le, A.D. (2005). Resveratrol inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1alpha and VEGF expression in human tongue squamous cell carcinoma and hepatoma cells. Mol Cancer Ther *4*, 1465-1474.

Zhong, H., De Marzo, A.M., Laughner, E., Lim, M., Hilton, D.A., Zagzag, D., Buechler, P., Isaacs, W.B., Semenza, G.L., and Simons, J.W. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res *59*, 5830-5835.

Zhou, Y.D., Kim, Y.P., Li, X.C., Baerson, S.R., Agarwal, A.K., Hodges, T.W., Ferreira, D., and Nagle, D.G. (2004). Hypoxia-inducible factor-1 activation by (-)-epicatechin gallate: potential adverse effects of cancer chemoprevention with high-dose green tea extracts. J Nat Prod *67*, 2063-2069.

APPENDICES

SOLUTIONS

1. Cell Culture

Phosphate Buffered Saline (PBS) 10X

The solution was made up by dissolving the following reagents in milliQ H_2O. The total volume was 1L, and the concentration was 10X that which was generally used in experimental work. After all reagents had been completely dissolved, the pH was adjusted to 7.4 using concentrated NaOH or HCl. The concentrated PBS was then stored at room temperature.

NaCl	80.0g
Na_2HPO_4	11.5g
KCl	2.0g
KH_2PO_4	2.0g
MilliQ H_20	1L

Collagenase Dissolving Buffer

The first six reagents were added together, and dissolved in the MilliQ H_2O. Once dissolved, glucose and sodium bicarbonate were added. Once dissolved, $CaCl_2.2H_20$ was added. 5.0 ml of Penicillin Streptomycin was then added and the pH of the solution was adjusted to 7.4 using concentrated NaOH or HCl. The solution was then passed through a 0.22 µm filter (millexGP) into a sterile bottle, and stored at 4°C.

NaCl	4.0g
KCl	0.2g

Na_2HPO_4	0.19g
KH_2PO_4	0.03g
$MgSO_4.7H_2O$	0.05g
$MgCl_2.6H_2O$	0.05g
MilliQ H_20	495 ml

Then

Glucose	0.5g
$NaHCO_3$	0.175g
$CaCl_2.2H_20$	0.092g
Penicillin / Streptomycin	5.0 ml

Collagenase Solution

The collagenase was added to the dissolving buffer, and well mixed. Then, under aseptic conditions, the solution was passed through a 0.22 µm filter (millexGP) into 50 ml Falcon tubes. These were then stored at 4°C.

Collagenase Type 1A	100 mg
Collagenase dissolving buffer	50 ml

α-Minimum Essential Medium Medium (α-MEM)

950 ml of milliQ water was measured out into a 1L Schott bottle that had been washed and autoclaved. To this, the powdered medium was added. MilliQ water was then used to rinse the packet, and remove any remaining traces of medium powder. NaHCO3 was then added, and Penicillin Streptomycin and fungizone

added. Solution was next made with to 1 litre with MilliQ water. Solution was then stirred with a magnetic stirring bar. pH of the solution was adjusted to 7.4 using concentrated NaOH or HCl, added slowly with stirring. Solution was then filtered into another sterile, autoclaved 1l Schott bottle using a vacuum filter (Vacucap 0.2 μm). This was done under aseptic conditions in the cell culture hood. α-MEM was stored at 4°C until required.

Minimum Essential Medium α Medium	1 Packet
$NaHCO_3$	2.2g
Penicillin Streptomycin	10.0 ml
Fungizone	4.0 ml
MilliQ H_20	1L

Phenol-Red Free Minimum Essential Medium (PRF-MEM)

950ml of milliQ water was measured out into a 1L Schott bottle that had been washed and autoclaved. To this, the powdered medium was added. MilliQ water was then used to rinse the packet, and remove any remaining traces of medium powder. $NaHCO_3$, L-Glutamine, Penicillin Streptomycin and fungizone were added. Solution was next made with to 1 litre with MilliQ water. Solution was then stirred with a magnetic stirring bar. pH of the solution was adjusted to 7.4 using concentrated NaOH or HCl, added slowly with stirring. Solution was then filtered into another sterile, autoclaved 1l Schott bottle using a vacuum filter (Vacucap 0.2 μm). This was done under aseptic conditions in the cell culture hood. PRF-MEM was stored at 4°C until required.

Minimum Essential Medium Eagle	1 Packet
$NaHCO_3$	2.2g

Penicillin Streptomycin	10.0 ml
Fungizone	4.0 ml
L-Glutamine	0.292g
MilliQ H_20	1L

Fungizone

Fungizone (Amphotericin B 250g/ml)	4.0 ml

Stored in freezer at -18°C until required.

2. Experimental Treatments

Resveratrol Stock Solution

DMSO was added to the resveratrol container. The container was then vortexed until all the powder was in solution. The solution was then transferred to a 50 ml Falcon tube and stored in the freezer at -18°C until required. The concentration of this stock solution was 10,000 µg/ml.

Resveratrol	100 mg
DMSO	43.8212 ml

$CoCl_2$ Stock Solution

$CoCl_2$ powder was measured out, and added to sterile bottle. MilliQ H_2O then added and solution was mixed until all powder was dissolved. Filtered through 0.22 µm filter (millexGP) into 15 ml Falcon tubes. Stored at 4°C. Stock at 20,000 µg/ml

$CoCl_2$	237.93 mg

MilliQ H_2O	50 ml

EGCG Stock Solution

EGCG	50 mg
MilliQ H_2O	10.91 ml

EGCG powder was measured out, and added to sterile bottle. MilliQ H_2O then added and solution was mixed until all powder was dissolved. Filtered through 0.22 µm filter (millexGP) into a 15 ml Falcon tube. Stored at at -18°C. Stock at 20,000 µg/ml

3. ELISA Reagents

ELISA Wash Buffer

TWEEN and 10X PBS were added to a 1l Schott bottle. Solution was made up to 1l with MilliQ H2O.

PBS 10X	100 ml
TWEEN 20	0.5 ml
MilliQ H20	900 ml

Reagent Diluent

BSA was added to sterile bottle with PBS. Solution was stirred using a magnetic stirrer until BSA completely dissolved. The solution was then filtered using a 0.22

μm filter (millexGP) into 50 ml Falcon tubes. Solution was stored at 4°C until required.

BSA	1g
PBS	100 ml