The effects of obesity-related hormones on cancer cells

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Abstract

**Introduction:** Obesity is a significant risk factor in the development of endometrial cancer and poses a serious health threat. Furthermore, morbidities related to obesity are associated with a significantly increased risk of mortality for women who develop endometrial cancer. Obesity is associated with altered levels of many hormones including leptin, insulin, insulin-like growth factor-1 (IGF-I) and adiponectin. The role of these hormones in the progression of endometrial cancer is largely unknown. In this study, it was hypothesised that these obesity-related hormones including as leptin, insulin and IGF-I would promote cancer growth, while adiponectin would inhibit cancer growth.

**Objectives:** The aim of this study was to use a number of selected cells lines to determine if obesity-related hormones leptin, insulin, IGF-I and adiponectin have effects on cell proliferation, secretion of vascular endothelial growth factor (VEGF) which is a protein associated with the development of blood vessels used to support the growth of cancers, and the expression of specific proteins associated with different cell signalling cascades. These are the phosphatidylinositol 3-kinase (PI-3K)/ Protein kinase B (Akt)/ mammalian target of rapamycin (mTOR) pathway (PI-3K/Akt/mTOR), Janus kinase family and signal transducers and activators of transcription-3 (JAK/STAT3) and 5' adenosine monophosphate-activated protein kinase (AMPK) protein.

**Materials and Methods:** To examine these hypotheses, two endometrial cell lines, Ishikawa and HEC-1A, a breast cancer cell line, MFC-7 and an ovarian cancer cell line, SKOV-3 were used in this study. Cell lines were cultured and the cells exposed to selected range of leptin, insulin, IGF-I and adiponectin concentrations, both individually and in combinations. In some culture conditions, the addition of pharmacological inhibitors were used to investigate the hormone-stimulated cellular responses including proliferation, secretion of VEGF, and activation of selective signalling molecules using Western blots, flow cytometry and immunofluorescent imaging with an optical sectioning microscope.
Results: In chapter 3, the aim of the experiments was to investigate the effects of leptin, insulin, IGF-I and adiponectin on cell growth and secretion of VEGF in the endometrial cancer cell line, Ishikawa. It was shown that leptin (20-100 ng/ml), insulin (116-2900 ng/ml), and IGF-I (20-500 ng/ml) increased the proliferation of the Ishikawa endometrial cancer cells. On the other hand, adiponectin inhibited cell proliferation at high concentrations (50 - 100 ng/ml) while increasing it at a lower concentration (20 ng/ml). The combinations of leptin, insulin or IGF-I with adiponectin showed different responses, and there was a lack of evidence for additive growth effects in the combinations. For instance, addition of adiponectin (20 ng/ml) to leptin or insulin increased cell numbers. However, the combination of adiponectin (20 ng/ml) and IGF-I reduced cell numbers to the control (no growth factor) levels. These findings suggest that IGF-I uses a different pathway to that used by leptin and insulin to increase cell proliferation.

In chapter 3, the secretion of VEGF was also investigated. Ishikawa cells did not increase VEGF secretion in response to leptin, insulin or IGF-I compared to control cells. In contrast, the VEGF secretion was increased by adiponectin (20-100 ng/ml).

In chapter 4, the aim of the experiments was to compare the effects of these hormones on alternative cancer cell lines with endometrial cancer cell line Ishikawa. The selected cell lines were the endometrial cancer cell line, HEC-1A, a breast cancer cell line MCF-7 and an ovarian cancer cell line, SKOV-3. Cell proliferation, level of apoptosis and VEGF secretion were determined in these cell lines, and the results showed that the effects of the hormones on these parameters were cell line-dependent. For example, cell numbers were increased by leptin, insulin and IGF-I in HEC-1A cells, but insulin did not increase cell numbers in the MCF-7 cell line. In addition, none of these three hormones affected SKOV-3 cell numbers.

In chapter 5, experiments aimed to investigate the mechanism by which leptin stimulates proliferation of Ishikawa cells. Leptin was shown to trigger oncogenic signalling proteins including PI-3K/Akt/mTOR, JAK/STAT3 and AMPK, thus suggesting that leptin stimulated cell proliferation by triggering PI-3K and JAK activation. This was confirmed by the inhibition of these two proteins using specific inhibitors. The results also suggested that there is a cross-talk between JAK/STAT3 and PI-3K/Akt/mTOR pathways, and that JAK is
the upstream protein for these two pathways. Leptin’s ability to reduce the secretion of VEGF was not affected by these two inhibitors. Thus, these results also suggest that cell proliferation and VEGF secretion are independently regulated by leptin because leptin increased cell proliferation using JAK/PI-3K pathways and reduced VEGF secretion by reduction in AMPK pathway. It is interesting to note that levels of AMPK and phosphorylated AMPK (p-AMPK), which are often associated with or have been reported to be responsible for VEGF secretion in cancer cells, did not show a significant change in leptin-treated cells. This may explain the reduction in VEGF secretion in the presence of leptin.

In chapter 6, experimental aims were to investigate the effects of two concentrations (20 ng/ml and 100 ng/ml) of adiponectin on cell proliferation and secretion of VEGF from the Ishikawa cell line. The lower concentration of adiponectin increased cell proliferation and VEGF secretion. Furthermore, it also increased the levels of Akt, p-Akt, STAT3, p-STAT3, AMPK, and p-AMPK as detected by Western blotting. These results suggest that low concentrations of adiponectin stimulated cell proliferation in a similar manner to leptin except for the AMPK protein expressions, and that adiponectin was triggering the PI-3K/Akt and JAK/STAT3 pathways. Moreover, that these low concentrations of adiponectin increased AMPK and p-AMPK proteins expression and also increased the VEGF secretion, which was unexpected. However, this increase in VEGF secretion was produced only in 20 ng/ml adiponectin, stimulating conditions. This may suggest that 20 ng/ml adiponectin is a more highly tumorigenic stimulator than leptin. Higher concentration of adiponectin (100 ng/ml) reduced Ishikawa cell proliferation. The reduction of cell proliferation was not via the inhibition of any of the stimulatory pathways. This suggests that high adiponectin concentration may exert its inhibitory effects via different pathways other than the stimulatory pathways.

In chapter 7, the aim of the experiments was to investigate the stimulatory effects on growth of insulin and IGF-I in Ishikawa cells. Results showed that both insulin and IGF-I increased cell numbers but reduced secretion of VEGF. The level of Akt and p-Akt were elevated but AMPK expression was not changed, and p-AMPK was significantly reduced. Collectively, these results suggest that insulin and IGF-I activate cell proliferation through a
PI-3K/Akt pathway. Again, it was interesting to note that low AMPK was linked to the low secretion of VEGF that was observed in the presence of insulin and IGF-I.

**Conclusions:** Initially, it was hypothesised that leptin, insulin and IGF-I promote cancer growth, while adiponectin inhibits it. The results were generally consistent with the initial hypothesis in some cases. For example, all of the so-called tumorigenic hormones, (that is, leptin, insulin and IGF-I) increased proliferation in Ishikawa cells, however, these hormones reduced the secretion of angiogenic signal VEGF, moreover, responses to these hormones varied between cell lines, with the hormones stimulating proliferation in the endometrial cell line and the breast cell line but not in the ovarian cell line.

On the other hand, in Ishikawa cells it was found that while higher concentrations of adiponectin reduced proliferation of tumour cells and increased VEGF secretion, as expected, lower concentrations of adiponectin resulted in a surprising increase in cell proliferation as well as an increase in VEGF secretion.

Investigation of the expression of certain intracellular mediators in Ishikawa cells would suggest that leptin-induced cell proliferation but not VEGF secretion is stimulated via the PI-3K/Akt/mTOR pathway and that the JAK/STAT3 pathway is an important mediator of this. Leptin, insulin and IGF-I all increased proliferation and decreased VEGF secretion from Ishikawa cancer cells. While it was demonstrated that activation of the PI-3K/Akt/mTOR pathway occurred, there were some differences in response, especially when in combination with adiponectin, that suggested the utilisation of different pathways or receptors.

From these experiments, it is impossible to determine the effect of these hormones in vivo. While it is somewhat unexpected that VEGF secretion was normally reduced with increasing cell proliferation, these findings are consistent with the hypothesis that obesity-related hormones, and particularly the combination of higher levels of leptin combined with lower levels of adiponectin, have an impact on tumour growth in obese women with low grade endometrial cancer.

Further in vivo and clinical studies are required to determine the clinical importance of these relationships and to explore the therapeutic potential of targets within the identified pathways.
New observations in this study: While it is recognised that there is a significant literature of the potential impact of leptin and adiponectin on cancer growth, I have attempted to perform a systematic study of the chosen hormones on Ishikawa cells and to compare these findings with those of other cell lines. The studies performed for this thesis contribute to an understanding of this subject in the following areas.

- Using Ishikawa cells, I have described the contradictory increase in proliferation but decrease in VEGF secretion under the influence of leptin, insulin and IGF-I. Using these cells, I have also described how low concentrations of adiponectin increase cell proliferation while higher concentrations inhibit proliferation but increase VEGF secretion. I have also described how these effects compare with the effects of these hormones in a selection of other cell lines.

- I have documented the presence of the receptors of leptin (Ob-R), insulin (InsR), IGF-I (IGFR) and adiponectin (AdipoR) in the cell membrane, cytoplasm and nucleus.

- I also described serine phosphorylation of STAT3 in Ishikawa cells in the presence of leptin and low adiponectin and provided evidence that cross-talk occurred between PI-3K/Akt/mTOR and JAK/STAT3 pathways in Ishikawa endometrial cancer cells. In addition, an increase in AMPK and p-AMPK in association with increased VEGF secretion with adiponectin was described.
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<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>acetyl-Co enzyme A carboxylase</td>
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<tr>
<td>AdipoR</td>
<td>adiponectin receptor</td>
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<tr>
<td>Adp</td>
<td>adiponectin</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
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<tr>
<td>ASP</td>
<td>acylation-stimulating protein</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>Ctrl</td>
<td>control</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>Erk</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>FIAF</td>
<td>fasting-induced adipose factor</td>
</tr>
<tr>
<td>FoxO1</td>
<td>forkhead box O1</td>
</tr>
<tr>
<td>IARC</td>
<td>The International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IGFR</td>
<td>insulin-like growth factor receptor</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>InsR</td>
<td>insulin receptor</td>
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<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
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<tr>
<td>JAK</td>
<td>janus kinase</td>
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<tr>
<td>JNK</td>
<td>jun terminal kinase</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>Ob-Ra</td>
<td>short isoform of leptin receptor</td>
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<tr>
<td>Ob-Rb</td>
<td>long isoform of leptin receptor</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for Economic Co-operation and Development</td>
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<tr>
<td>PAI-1</td>
<td>plasminogen Activator Inhibitor-1</td>
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</table>
PI-3K  phosphatidylinositol-3 kinase
PKC  protein kinase C
SDS  sodium dodecyl sulphate
SH2  containing protein tyrosine phosphatase 2
SH3  containing protein tyrosine phosphatase 3
STAT  signal transducer and activator of transcription
TGFβ  transforming growth factor β
tNFα  tumour necrosis factor α
Tyrphostin  tyrphostin AG490
VEGF  vascular endothelial growth factor
VMH  ventromedial hypothalamus
WAT  white adipose tissue
WHO  World Health Organisation
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3- Maurice Phyllis and Paykel Trust travel grant award for oral presentation at the 23rd European Congress for Obstetrics and Gynecology, Glasgow, Scotland. May 2014.
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15th Biennial meeting of the International Gynaecological Cancer Society, 8-11 November 2014, Melbourne, NSW, Australia. ‘Leptin and adiponectin modulate proliferation and VEGF secretion in endometrial cancer cells’. (Poster)
Seminars and Meetings


2. Seminar, Journal presentation, Department of Obstetrics and Gynaecology, Christchurch School of Medicine, 26 Jul. 2013

3. 3 minutes competition, Jul. 2013, one slide presentation, University of Otago Christchurch. (Adipokines alter behaviour of endometrial cancer).

4. Seminar, Department of Obstetrics and Gynaecology, University of Otago Christchurch, 12 Jul 2013 (Leptin and adiponectin).


10. Seminar, Department of Obstetrics and Gynaecology, University of Otago Christchurch, 15, 29 Jul. 2016 (Obesity, hormones and cancer, new story).

11. Seminar, Main lecture theatre, Mustansiriyyah University, Iraq, College of Dentistry, 24 Oct. 2016 (Obesity and uterus cancer proliferation).
Chapter One

Introduction
1 INTRODUCTION

1.1. Obesity

1.1.1. Obesity, an overview

Obesity is a major concern in developed and developing societies [1, 2]. Body fatness is usually assessed using body mass index (BMI), which is calculated as a person’s weight in kilograms divided by the square of their height in metres. This provides a simple index of weight relative to height that can be measured easily in population studies. A BMI of 20–24.9 kg/m\(^2\) is considered normal, a BMI of 25–29.9 kg/m\(^2\) is classed as overweight and a BMI of 30 kg/m\(^2\) or more as obese. Waist circumference (WC) is another simple measure of adiposity [3]. However, for a given WC value, overweight and obese subjects and normal-weight subjects have similar health risks. Nevertheless, BMI remains a significant predictor of health risk whether or not WC is normal or high [4].

Obesity is a significant public health problem among children and adults [5, 6], and has adverse impacts on society at large [7]. Rates of obesity have increased three-fold or more since 1980 in many areas including North America, the United Kingdom, Eastern Europe, the Middle East, the Pacific Islands, Australasia and China [5, 6]. Worldwide, more than one billion adults have been categorised as overweight, and 300 million categorised as obese [5, 6]. The number of deaths resulting from obesity were calculated to be between 100,000 to 300,000 per year from a wide variety of causes [3, 8, 9].

The Organisation for Economics, Co-operation and Development (OECD) report in 2014 titled ‘Obesity and the Economics of Prevention: Fit not Fat’ [10], reported that New Zealand has the third highest percentage of overweight and obese children in the OECD after Greece and Italy [10, 11]. One third of New Zealand children are considered overweight or obese, in contrast only one quarter of Australian children are in this category [10, 11]. By comparison, 64% of the population of the United States of America (USA) was overweight or obese in 2000 [5, 8].
Obesity has been associated with the incidence of several types of cancer in both women and men, including cancer of the oesophagus, colon, pancreas, endometrium, and kidney, as well as postmenopausal breast cancer [2, 3, 12]. A World Health Organisation (WHO) 2014 report concluded that obesity is the most important risk factor worldwide for cancer [3]. Alterations of metabolic profiles associated with obesity (e.g. excess circulating free fatty acids), some genetic factors and lifestyle factors may also increase cancer risk [3, 13]. The risk of endometrial cancer progressing to later stages is significantly reduced by an increase in physical activity by obese women [14] and for several cancers, avoidance of weight gain reduces the risk [3, 15, 16]. This thesis, therefore, focuses on the links between obesity and the progression of endometrial cancer.

Although the mechanisms through which obesity increases cancer risk are only partially understood, it is likely that the influence of obesity is partly mediated through hormonal mechanisms, consistent with observations of the effects of exogenous and endogenous hormones such as oestrogen and progesterone [3]. Nevertheless, hormonal effects appear to vary between different cancer types and sites [3]. For breast and endometrial cancers, the increase in cancer risk associated with obesity in postmenopausal women is possibly related to the increase in circulating oestrone, in turn due to increased formation of oestrogens from precursor hormones in the adipose tissue. Obesity also increases the risk of endometrial cancer in premenopausal women, and this may be due to anovulation and reduced production of progesterone [3].

There are several further inter-related and confounding factors connecting obesity to cancer risk, including physical activities, diet, and energy balance, but links between these factors and cancer risk have not been sufficiently investigated from an epidemiological standpoint. The effect of abnormal metabolism, including metabolic disorders within overweight people, might be a key. In one extensive population study [17], improved metabolic health diminished the risk of obesity-related cancer. The cellular and molecular pathways linking weight gain with established [18] hallmarks of cancer [19] remain unclear. These pathways, however, remain under the spotlight of current research [20]. Altered hormonal profiles, notably oestrogen and other growth factors including leptin, insulin,
insulin-like growth factor and adiponectin, are also differentially controlled in the obese state [20].

Obesity is recognized as a chronic inflammatory condition that influences cardiovascular disease, and has been linked to approximately 20% of all cancers [3, 4]. Obesity’s influences vary with gender and cancer-type [2]. One aspect of obesity’s influence involves the accumulation of macrophages in adipose tissue which establishes pro-inflammatory feedback loops among macrophages, pre-adipocytes and adipocytes, leading to the generation of inflammatory cytokines and free radicals [3, 21]. The size of the adipose tissue stores, referred to as adiposity, increases in periods of food intake and relaxation, and declines when energy expenditure is in excess of intake [22]. This increased adiposity bestows a higher risk of developing certain cancers in obese individuals such as endometrial and breast cancers [23-25] (Figure 1.1). Notably, the risk of endometrial cancer increases threefold with an increase in BMI of 10.
Figure 1.1 The relationship between body mass index and the risk of developing cancers of the endometrium, ovary and breast. The dashed line indicates a relative risk of 1 which means that at that level, obesity does not significantly alter the risk. Data are from reference [3]. (Illustrated with permission from the copyright holder).

There are direct effects of obesity and adiposity on the secretion of pro-inflammatory cytokines and adipokines, and there are also indirect effects such as insulin resistance and high insulinaemia (an excessive increase in insulin concentration in the blood) that are commonly associated with obesity or an increase in the levels of steroid hormones. Obese women with endometrial cancer also have significantly higher death rates from other obesity-related health problems, such as type II diabetes and heart disease, when compared to healthy women [26, 27]. In this project, I investigate the effects of four hormones that may be working in parallel with oestrogen to exert their effects in cancer cells and which are
dysregulated in obesity. These hormones are leptin, insulin, insulin-like growth factor-I (IGF-I) and adiponectin.

1.1.2. The white adipose tissue

White adipose tissue (WAT) mass, which is one of the two types of adipose tissue found in mammals (the other kind is brown adipose tissue), is characteristically increased in obese individuals [22, 23]. Indeed, WAT has many key roles in mammalian physiology such as glucose homeostasis, energy expenditure, source of many hormones, thermal insulator etc. [28]. Previous research indicates that WAT acts as a long-term fuel reserve, and this reserve can provide other organs with fatty acids for oxidation in cases of food deprivation.

A change in our understanding of WAT function came with the discovery of the cytokine-like factor, leptin [29], which revealed an active, endocrine role for WAT [19]. Leptin hormone is secreted principally from adipocytes (fat cells) within the WAT, and it acts in central organs, particularly the hypothalamus, and in peripheral organs. Neurons in the brain such as neuropeptide Y (NPY) and melanocyte-stimulating hormone (MSH) neurons release peptides in response to leptin secretion from adipose tissue and increased food intake by the humans [28]. However, leptin is not the only adipokine secreted by WAT, with adiponectin being another example [28, 30-32].

Obesity is associated with both systemic and local inflammation of WAT. Similarly, WAT from obese patients is infiltrated by leukocytes, including macrophages and T lymphocytes [12, 33], which may create a microenvironment that favours tumour growth and metastasis [12, 34]. Thus, it has been observed that WAT influences tumour progression including endometrial, oesophageal, colorectal and renal cancers [22, 23].

1.1.3. Obesity and the level of hormones

The levels of peripheral hormones, including leptin, adiponectin, insulin, and insulin-like growth factor-1 (IGF-I) are different between obese and lean individuals [16, 35]. Leptin concentrations are markedly lower in women with low BMI and higher in women with obesity [36, 37]. In contrast, adiponectin concentrations in plasma are significantly lower in
obese women than in normal weight subjects [38]. In the obese state, there is an increase in the size and number of adipocytes. This change in the composition of the adipose tissue is part of the reason for the increased secretion of leptin and a decrease in the secretion of adiponectin [16]. Obesity is also associated with an elevation of the insulin concentration [39, 40]. In contrast, IGF-I levels are markedly lower in obese subjects compared to lean, age-matched, controls [41]. It seems likely that over nutrition and excessive secretion of insulin (hyperinsulinaemia) in obese subjects modulates regulated growth response through the insulin stimulated production of IGF-1, and through the suppression of the secretion of hepatic insulin-like growth factor binding proteins (IGFBP-1 and IGFBP-2) [42]. Thus, as a consequence of their disrupted regulation, these hormones may have a crucial role in the carcinogenesis of endometrial cancer [16, 43] and possibly other cancers [12, 44].

1.2. **Hormones of obesity and cancer**

A number of hormones have been studied in relation to the development and progression of cancer. These include insulin, glucocorticoids, tumour necrosis factor α (TNF α), and reproductive hormones such as progesterone, testosterone and estradiol [45]. The most well-studied of these, and a key player in the development of various cancers, is oestrogen [46].

Oestrogens exhibit a broad spectrum of physiological functions ranging from regulation of the menstrual cycle and reproduction to modulation of bone density, brain function, and cholesterol mobilization. Despite the beneficial actions of endogenous oestrogen, sustained exposure to exogenous oestrogen is a well-established risk factor for various cancers [46-50]. However, other obesity-related hormones have rarely been investigated in a systematic way. Of the obesity-related hormones selected for study in this project, leptin, insulin and IGF-I have been reported to promote cancer proliferation [51-61]. Furthermore, adiponectin is secreted from the same tissue as leptin and is believed to inhibit the proliferation of cancer cells [62-64]. The effects of these four hormones on cell proliferation, vascular endothelial growth factor (VEGF) secretion, and apoptosis will be investigated in this project, and these hormones are discussed in the next sections of this introduction.
1.2.1. Leptin

Leptin (derived from Greek λεπτός, leptos, meaning "thin") is a protein hormone that plays a key role in regulating energy intake and expenditure, including appetite/hunger, metabolism, and the behaviour of cancer cells [31, 65-67]. Leptin’s sequence is highly conserved among mammalian species, and the disruption of leptin regulation can lead to obesity [68]. Thus, leptin was discovered in 1994 [29, 65] through the identification of a mutated gene which was responsible for the development of obesity in obese (ob/ob) mice, and so leptin is sometimes also called Ob protein. Very low leptin concentrations are found in the plasma of ob/ob mice, and these animals eat excessively [69]. Similar studies in another mutant, the obese (fa/fa) Zucker rat that exhibits hyperleptinaemia, hyperinsulinemia, and hyperlipidemia, led to the identification of the leptin receptor [70]. In humans, the leptin gene (Lep(ob)) is located on chromosome 7 [71] and encodes a protein with a molecular weight of 18 kDa. This protein contains a signal sequence which is cleaved to produce the mature leptin hormone of molecular weight 16 kDa [29], and leptin’s sequence is highly conserved among mammalian species [68]. The human leptin receptor gene is found within the 5.1 cM interval [46]. Ob-Rb (full-length and functional isoform, 48 kDa) and Ob-Ra (a short isoform, 42 kDa) are the main isoforms [72, 73].

Most recently, leptin receptors were found to have an association with human puberty [74]. The association between fat mass and pubertal timing is partly facilitated via the activities of the metabolic hormone leptin, which is considered as the key player in weight control [75]. It is well established that humans and mice lacking leptin (ob/ob) or the leptin receptor (db/db) are unable to reach puberty they are also likely to be infertile [76]. Genome-wide association studies (GWAS) of pubertal timing revealed that leptin cell signalling has a cross-talk with many genes that are involved with BMI, such as FTO, SEC16B, TMEM18 and NEGR1 [77]. On the other hand, a medical condition self-limited delayed puberty (DP), which is a highly heritable trait that often segregates in an autosomal dominant pattern, was found to be correlated with low leptin concentrations (hypoleptinaemia) in boys [78]. Disruption of neither leptin nor the leptin receptor did not exhibit a clear association with polymorphisms with DP [79]. Ghrelin, another appetite regulator, and other gut-derived peptides may also be
partly involved in the mechanism by which energy homeostasis regulates reproductive progression [79].

The level of circulating leptin is directly correlated with the total amount of fat in the body [12, 80-82], and leptin levels in obese individuals are higher than in lean individuals (26.9 ± 3.9 and 5.9 ± 0.7 µg/L) respectively [83]. Previous studies suggested that leptin was only synthesised in WAT, but it has now been established that the hormone is produced in a broad range of tissues and organs including the brown adipose tissue, stomach, placenta, mammary gland, ovarian follicles, certain fetal organs such as the heart and bone or cartilage, and perhaps even the brain [28, 66]. Primarily, leptin functions as an appetite reduction signal [84]. Fasting leads to a rapid inhibition of ob gene expression in WAT leading to a fall of leptin levels circulating in the blood. However, a reduction in the level of circulating leptin follows re-feeding [85]. These observations were supported by treating mice with leptin. Mice were either fed or injected with leptin in order to reduce food intake, with these treatments causing significant weight reductions [86-89]. Several central neuroendocrine systems also interact with leptin, and this interaction may further control food intake [90, 91]. For example, in case of fullness, leptin activates the NPY in the hypothalamus which leads to the inhibition of NPY-induced food intake or stimulation of MSH that leads to reduced food intake [85, 92-94]. In humans, the normal fasting leptin concentration in plasma ranges widely between 1.2 and 97.9 ng/ml, and it is about three times higher in women than in men [82].

Leptin is thought to function through the insertion of fatty acid channels into adipose tissue, limiting triacylglycerol deposition in other tissues [95]. Furthermore, leptin affects energy consumption, and acts as a major signal to the reproductive system particularly in relation to sexual maturation in females. Leptin may also be a key factor in angiogenesis and for the immune system [89, 96-98]. It influences a wide range of metabolic processes ranging from inhibition of insulin secretion to sugar transport stimulation [99-101].

1.2.1.1. The role of leptin in the tumour microenvironment

Leptin, the adipocytokine, is upregulated in overweight people, and many malignancy types are correlated with the presence of raised leptin levels; epidemiological studies have
confidently established this association [102]. Leptin is a key player in immunoregulation and may exhibit a connection whereby macronutrient intake and differences in the energy balance may have an impact on immune mechanism activation [103]. Leptin gene expression by adipose stromal cells (ASCs) may be enhanced by oestrogen [104]. Moreover, leptin is known to play a key role in oestrogen signalling in breast cancer cells via the activation of aromatase gene expression [104]. Cancer cell progression can be activated via leptin binding to oestrogen receptor (ERα) in ovarian, endometrial, and breast cancer cells [105]. Leptin is believed to increase cancer growth by a pathway which is via human telomerase reverse transcriptase (hTERT) expression. This was inhibited by treatment with activated vitamin D₃ via induction of miR-498 in cell line models [20, 106].

1.2.2. Insulin

Insulin is a peptide hormone composed of 51 amino acids and having a molecular weight of 5808 Da. It is produced in the islets of Langerhans in the pancreas: insulin’s name comes from the Latin insula for "island". The structure and sequence of insulin varies only slightly among species of mammals [107]. Insulin’s function is studied predominantly in regard to the physiological control of glucose metabolism. When glucose levels in the blood increase, the pancreatic β-cells will respond by releasing insulin. The effect of insulin secretion subsequently reduces glucose concentrations by suppressing gluconeogenesis, which takes place in the liver and other intermediate pathways. Insulin levels in obese persons were two-fold higher than in lean individuals (11.7 ± 1.3 and 5.6 ± 0.6 mU/L) respectively [83]. In addition, glycogenolysis taking place in muscle and fat tissue is blocked by the presence of insulin and that will reduce bioavailability of glucose in blood. Thus, insulin function is important to energy balance and adipocyte regulation [108]. In humans, insulin plays a crucial role in the regulation of glucose levels, and reduced insulin levels will lead to the development of hyperglycaemia [109]. In 1939, Himsworth and Kerr observed that obese and elderly patients had a low insulin response to increased glucose levels in the blood circulation even though the pancreas had increased the secretion of insulin. Their description was in line with what is now referred to as "insulin resistant syndrome" [110, 111]. However, insulin functions are not solely related to glucose metabolism. Insulin also increases sex
hormone synthesis and inhibits sex hormone binding protein which leads to increased oestrogenic and growth factor activities [40].

Insulin elicits its effects on cells via a tyrosine kinase receptor, the insulin receptor (InsR), and a hybrid insulin/IGF-I receptor. These receptors are highly expressed in cancer cells [41, 112-114]. Insulin binding to its receptor activates insulin receptor substrate-1 (IRS-1) and a series of chemical reactions and pathways inside cancer cells [115]. The two main pathways which are activated by insulin binding to insulin receptors are the mitogen-activated protein kinase (MAPK) and phosphoinositol-3 kinase/Akt (PI-3K/Akt) pathway [115].

It has been reported that insulin resistance, hyperinsulinemia, and type II diabetes, three pathologies which are related to obesity, may also correlate with endometrial cancer [116]. Once insulin binds to its receptor, it induces proliferative activities on several cell types especially cancer cells [58]. Other non-receptor mechanisms may occur such as changing expression of IGF-I and its binding proteins, or blocking of anti-proliferative activity of progesterone [117]. The mitogenic activity of insulin has been shown to be due to the action of insulin on suppressing the expression of IGFBP-I. Therefore, hyperinsulinemia may result in downregulation of IGFBP-I which may stimulate proliferation even in the presence of progesterone [116, 117].

1.2.3. Insulin-like growth factor-1 (IGF-I)

Insulin-like growth factor-1 (IGF-I), also known as somatomedin C, is a protein encoded by the IGF-I gene in humans [118, 119]. IGF-I is a hormone with a structure closely related to that of insulin. There are two types of insulin-like growth factors, IGF-I and -II, with amino acid sequences that are highly homologous. IGF-I is composed of 70 amino acid residues and IGF-II is composed of 73 [119]. IGF-I consists of a single chain with three intramolecular disulphide bridges. There are three protein receptors for IGF-I, which are insulin like growth factor-1 receptor (IGF-IR), insulin-like growth factor-II receptor (IGF-IIR) and insulin receptor (InsR) [87-89]. These different receptors may help IGFs to exert their function under specific circumstances [120-122]. Insulin shares some receptor-binding activity with IGF-I.
IGF-I is a growth factor that is central to a number of biological processes and its production is sensitive to nutritional state including glucose levels [123-125]. Accumulating evidence suggests dysfunction of IGF regulation underlies numerous pathologies, and the IGF system is involved in many pathophysiological activities in tumorigenic processes [122, 125]. IGF-I plays a critical role in growth in children and adults. An antagonistic peptide of IGF-I, mecasermin, is used for the treatment of abnormal growth during childhood development [126].

The correlation of endometrial cancer with obesity, type II diabetes and oestrogen led to an initiative to study the role of IGF-I in the biology of endometrial cancer. A significant number of random cases of type I endometrial cancer have been shown to develop upon stimulation with endogenous and exogenous oestrogens [127]. The effect of oestrogens, especially oestradiol (E2), as a cell proliferation stimulator is thought to be partly due to the induction of autocrine synthesis of IGF-I and upregulation of its receptor in the uterus [128]. However, expression of IGF-I encoding gene was detected mostly in the follicular and early luteal phase of menstrual cycle [127, 129]. As a consequence to the E2-dependent activation of IGF-I, mitogenic effects of IGF-I on the endometrium occur. Oestrogen also stimulates the synthesis of 17β-hydroxysteroid dehydrogenase and oestrogen sulfotransferase that contribute to the proliferation of endometrial cells [116, 127]. As a result of their expression, E2 is converted to a less active oestrone (E1) and oestrone sulfates, which are quickly expelled from the cells and the body. Progesterone on the other hand, counteracts these proliferative effects. In addition, the activity of progesterone contributes in the IGF-I binding protein (IGFBP-I) synthetises. IGFBP-I blocks the proliferative properties of IGF-I in the endometrium via controlling its availability for the cellular receptors [116]. Abnormal proliferation of endometrial cells occurs as a result of the excess production of oestrogen-induced IGF-I, and the absence of IGFBP-I synthesis which depends on the expression of progesterone [116, 127, 130].
1.2.4. Adiponectin

Adiponectin was identified in the mid-1990s. It is another protein hormone that is secreted from the adipose tissue [131], but it is also secreted from the placenta during pregnancy [132]. Human adiponectin is a 244 amino acid polypeptide [95] with molecular weight approximately 30 kDa [133], and it circulates in human plasma mainly as a 180 kDa, low molecular weight (LMW) hexamer and a high molecular weight (HMW) of 360 kDa [134]. However, while LMW adiponectin is the most important form in the circulation, HMW adiponectin is the more abundant form of intracellular adiponectin in cells such as adipose tissue [135]. AdipoR1 and AdipoR2 represent the two types of adiponectin receptor that have been identified. The two proteins are plasma membrane receptors with an internal N and external C terminals and so, interestingly, the location of N and C terminals are opposed to the topology of reported G protein-coupled receptors [136]. AdipoR1 and AdipoR2 occur on the cell membrane and in the cytoplasm of normal and malignant endometrial tissue [35].

Adiponectin plays a role in the suppression of the metabolic disruptions that may result in atherosclerosis [137], non-alcoholic fatty liver disease (NAFLD) and be an independent risk factor for metabolic syndrome [138]. Several studies observed that there is a strong negative association between adiponectin concentration and obesity [12], inflammation, insulin resistance, metabolic syndrome, cardiovascular disease, and cancer [139-141]. Adiponectin is found in the highest concentration of all hormones in human plasma [131] with a range from 3 to 30 µg/ml which accounts for up to 0.05% of total plasma proteins [142, 143]. In contrast to other hormones, adiponectin serum levels are found to be higher in lean women compared to obese (13.3 ± 1.8 and 8.6 ± 0.8 mg/L) respectively [83]. Adiponectin is considered to be an insulin-sensitising adipocytokine which means that adipocytes (low lipid storage, packed with fatty acids), suppress transcription of the adiponectin gene by secreting various pro-inflammatory, prothrombotic and angiogenic factors [144-146]. Additionally, oestrogen elevates adiponectin in the circulation of women in a manner that is independent of fat mass [135]. Also, in men, an inhibition of adiponectin HMW isoform in adipose tissue may occur due to the presence of testosterone [142, 147]. Reduction in adiponectin concentrations in the blood are closely related to the progression of type 2 diabetes and cardiovascular disease in human obesity [148, 149], and low adiponectin also has positive
association with cancer development [150]. Plasma adiponectin levels can be increased if obese individuals lose weight by following a diet regimen or through physical exercises [131, 151].

**1.2.5. Leptin, insulin, IGF-I and adiponectin and the growth of cancers**

Leptin, insulin, IGF-I and adiponectin have been observed to exhibit different effects on cancer cells. Measurements demonstrated that the proliferation of cancer cells was increased by leptin, insulin and IGF-I but not by the presence of adiponectin [139, 141, 143, 152-157]. The secretion of VEGF from cancer cells also increased in the presence of the leptin, insulin and IGF-I but not in the presence of adiponectin [158]. On the other hand, apoptosis has been shown to be suppressed by leptin, insulin and IGF-I but apoptosis was enhanced in the presence of adiponectin in different types of cancer [152-157].

Increasing epidemiological data and numerous *in vitro* studies have suggested a strong link between leptin and several types of cancer. An increase in the expression of leptin and its receptor (Ob-R) has been reported in many cancer types including gliomas, adenocarcinomas, and melanomas [159]. Increased proliferation as a result of leptin treatments have also been reported following *in vitro* experiments with gastric [160], breast [161-163], ovarian [164], prostate [165] and endometrial cancer cell lines [12, 166]. In contrast, leptin treatments show an inhibitory effect on the growth of pancreatic carcinoma cells *in vitro*, suggesting that leptin’s response is tissue-dependent [165].

Recent studies have reported that circulating adiponectin levels are inversely associated with the risk of malignancies associated with obesity [12] and insulin resistance [167]. In preclinical models, adiponectin inhibited the proliferation of several types of cancers [12, 168-170] including endometrial cancer [12, 137, 171], postmenopausal breast cancer [12, 169], leukaemia [172] and colon cancer [12, 173]. Moreover, low adiponectin levels have been associated with an increased incidence of gastric cancer [174], prostate cancer [175], and colon cancer [173]. Furthermore, a recent meta-analysis showed that low circulating adiponectin concentrations are correlated with an increased risk of endometrial cancer [43]. It is well established that adiponectin circulation levels are inversely correlated with BMI [12,
148, 176, 177], and lower adiponectin concentrations are found in obese individuals [148, 178]. Moreover, a study assessing the relationship between adiponectin concentration and BMI concluded that women with higher BMI and low adiponectin levels have a 6.5-times higher incidence of endometrial cancer than women with normal BMI and high adiponectin concentration [179]. Additionally, it has been suggested that tumours that grow in a low adiponectin environment are more likely to demonstrate a biological aggressive phenotype [180]. Adiponectin has also been shown to induce apoptosis in endometrial and hepatocellular cancer cells [12].

Several studies have shown that insulin signalling proteins, specifically the insulin receptor (InsR) and insulin receptor substrate-1 (IRS-1), may also be involved in the development of various tumours [181]. Insulin is not only implicated with an increase of colon cancer incidence [182, 183] but is also associated with colon cancer mortality [182-184] with the effects mediated by insulin resistance, hyperinsulinemia and high glucose diets [185]. Additionally, insulin was found to be associated with increases of pancreatic [186, 187] and breast [188, 189] cancer incidence and mortality [190, 191].

Obesity, diabetes, and insulin resistance are high-risk factors for the development of type 1 endometrial cancer [192]. Epidemiological studies have concluded that IGFs play an important role in tumour development. These studies have suggested that the high concentrations of IGFs and/or lower levels of IGF binding proteins present in cancer patients’ serum are involved in the increased risk of enhancement of several types of cancer including premenopausal breast [193, 194], prostate [195-197], lung [198-200], colorectal [201-203], endometrial [204], and bladder cancer [205]. IGF proteins and mediating signalling molecules are highly expressed in endometrial cancer [18, 206].

However, a recent in vivo study showed that there was no link between the risk for endometrial cancer and serum IGF-I levels. The study also found no statistically significant differences between serum IGF-I concentration in endometrial cancer as compared to healthy women, regardless of their menopausal status at the time of blood collection (most women were postmenopausal at the time of testing) [116].
The four hormones that will be studied in this thesis, leptin, insulin, IGF-I and adiponectin, have all been shown to be associated with oncogenic activity, although the mechanisms of action are yet to be clearly defined. For example there are conflicting conclusions on the effects that varied IGF-I levels have in connection to cancer incidence [204, 207, 208] with some studies reporting a positive correlation and others an inverse effect. Similarly, the parameters that may be involved in the regulation of the properties exhibited by adiponectin on cancer development are uncertain [12, 209, 210], and while insulin concentrations are raised in obese women with endometrial cancer [208, 211] it is unclear whether the connection between obesity to diabetes underlies this association [208]. In the case of leptin [212, 213] it is challenging to separate its association with increased cancer risk from its links with obesity since leptin is synthesised mainly by adipose tissue. However, leptin concentrations were found to have a strong positive association in women with endometrial cancer in both lean and obese patients [214]. The mechanisms that drive these endocrine-oncogenic associations are potentially complex [62] but the literature is without a definitive assessment of the plasma concentrations of these hormones in women with the various cancer and normal phenotypes.

1.3. Cancer progression and obesity

1.3.1. Cancer cell biology

In the process of cell division, all somatic cells undergo proliferation through a highly conserved cell cycle [215]. In order to produce new cells, the cell cycle involves five different phases: quiescence (G₀), Gap1 (G₁), DNA replication/synthesis (S), Gap2 (G₂) and mitosis (M). G₁ phase involves proteins and RNA synthesis in addition to DNA repair [216]. During these activities, cells may fall into quiescence entering the G₀ or resting phase [217]. Following quiescence, cells may re-enter the cycle when needed or differentiate into G₀ [216, 217].

DNA synthesis occurs during S phase and cells with newly-replicated DNA enter G₂ phase in preparation for nuclear division (mitosis) which occurs during M phase [218]. This
harmonised progression through the different phases of cell cycle, facilitated by cyclins and cyclin dependent kinases [219], is crucial to maintain genome stability [216]. Typically, cell proliferation is strictly monitored via different regulatory pathways where cell numbers are controlled via cell cycle checkpoints that, for example, pause the cell cycle and/or promote programmed cell death [215, 219]. Mutations in cancer cells may cause dysregulation of the cell cycle proteins and checkpoint genes leading to sustained cell proliferation that evades cell death signals. This is translated into continuous, uncontrolled cell division and proliferation and leads to the formation of tumours [216] (Figure 1.2). As a consequence to prolonged, active replication, dysfunctional apoptosis [216, 218], chromosome damage and, eventually, malignant transformation occurs [216, 218].

1.3.2. Cancer cells progression and proliferation

Cancer cells have the ability to maintain prolonged proliferation and escape growth suppression. These cancer cells exhibit a number of so-called hallmarks in their growth, including sustained proliferative signalling, the evasion of growth suppression, resistance to cell death enabling replicative immortality, the induction of angiogenesis, and the activation of invasion and metastasis [19, 220]. These cancer hallmarks and other characteristics have been proposed to be in association with cancers [18, 19]. The signals for these behaviours are carried by molecules with growth factor activity. In obesity, there is dysregulation of a number of endocrine factors that have been established to have growth-promoting properties. In addition, the hormones alter the regulation of other systems such as VEGF, which are involved in supporting tumour survival. The hormones trigger a cascade of signal transduction molecules which, in cells with oncogenic mutations, lead to cancer development [19]. Thus, tumour growth depends, to a large extent, on the increased and dysregulated proliferation of cancer cells. However, another component that plays a role in cell proliferation is the reduction of cell demise through the inhibition of the apoptosis processes. In obese women, the microenvironment is rich with growth factors e.g. leptin [20, 105], that are produced by fat cells [221].
Leptin is a proliferative hormone, and high leptin concentrations in the microenvironment will increase cell growth and proliferation [20, 105, 222]. Increased proliferation increases the opportunity for mistakes to be made during DNA replication [105, 222, 223]. As a result, the cells will become more prone to the accumulation of oncogenic mutations and more prone to become cancerous within the conducive microenvironment [20, 105, 223]. It has not yet been established as to which of the two steps, proliferation or mutation, occurs first. However, the dysregulation of hormones due to obesity may be contributing to cancer progression [20].

In summary, although cancer may develop along with obesity, it has not been reported that obesity can cause cancer. It should be noted though that obesity might initiate cancer if there are already oncogenic mutations or DNA replication mistakes [222, 223]. However, the work presented in this thesis investigates the effect of obesity-related hormones on already established cancer cells.

The tumorigenic process is a multistage process that involves a combination of gene mutations and changes in the microenvironment. One consequence is that cell proliferation takes place in an uncontrolled manner. Other phenotypic changes occur and tumours form [18]. Subsequently, the mutant cancer cells multiply and penetrate the wall of blood vessels as well as lymphatic tissue. Tumour cells metastasise to other tissue sites and regrow at new locations in the body [18] and lethality develops (Figure 1.2).
Figure 1.2 Tumorigenic processes of conversion of normal cells to tumour and carcinoma (The figure is based on a diagram in ‘Davidson’s Principle and Practice of Medicine’, 21st edition, ISBN Number 9780702050350, Edited by Brian R. Walker, Nicki R Colledge, Stuart H. Ralston, and Ian Penman, 2010) [193] and has been redrawn in a simplified form depicting proliferation and development.
1.3.3. Gene expression profiles and cancer risk in the obese

The molecular characterisation of tumours, in which tumour classification depends on the genetic profiling, currently promises improvement in clinical oncology studies because the process can subgroup patients in regards to prognosis and susceptibility to specific medications [224, 225]. It is thought that a similar signature might be recognised for the obese phenotype, along with various malignancy sites, as a step towards understanding of pathogenesis of obesity-related cancers and also for revealing possible novel treatment targets [20]. In this regard, gene microarray data showed different gene expression signatures for obese and lean patients with cancerous precursor cells for endometrial cancer [226]. Loss of the gene encoding tumour suppressor phosphatase and tensin homolog (PTEN), cancer suppressor, is correlated with developed progression-free survival. PTEN loss resulted in different protein alterations: canonical PI-3K pathway activation was exhibited only in the controls, while a reduction in the expression of the gene encoding b-Catenin (CTNNB1) and phosphorylated FOXO3A were obvious in obese patients [226]. The upregulation and activation of the PI-3K pathway in non-obese patients suggested that various targets may apply to different patient populations at the same disease site [227].

Breast tumours from obese patients have widespread differences in gene expression, including specific mutations, in comparison with overweight or lean patients, and an obesity-associated transcriptional signature of 662 genes was found using whole-genome analysis [20, 228]. In multiple, public gene expression data sets from breast cancers, this manifestation of the obesity signature patterns correlated with a gene expression signature for IGF signalling and, to a lesser extent, with lower levels of oestrogen receptors (ER) [20], and the presence of this obesity signature also correlated with a shorter time to metastases [228]. However, primary microarray data determined that mutations in 52 proteins from a smaller sample of tumours did not show any significant differences in mutations between obese and non-obese subjects, once modifications had been made for false discovery rates. This prevents application of these findings to produce a simple tumour protein biomarker panel [229].
1.3.4. Inflammation as a protumorigenic mechanism

The adjusted tumour microenvironment was central to research into the increased progression of tumours in obese subjects, and connected local and general inflammation with the developing hallmarks of cancer [19]. Chronic inflammation defines an abnormally extended or dysregulated defensive response that reflects the loss of tissue homeostasis [230], and which has been associated with the fundamental mechanisms of tumorigenesis [34, 231]. Intracellular signalling mechanism associated with inflammation include signal transducer and activator of transcription 3 (STAT3) and nuclear factor-kB (NF-kB), and these can be enhanced by somatic alteration or inflammatory incentives [231]. Cyclins are upregulated by STAT3 which in turns stimulates the cellular proliferation, and reduces cell death via activating anti-apoptotic proteins [232]. NF-kB stimulation might be caused by proliferative, inflammatory, and pro-survival gene expression, and may not be correlated with the regulation of cellular senescence [233]. Invasion and attaining metastatic potential and genomic instability of cancer cells due to the response to impaired DNA repair mechanisms and increased DNA damage [222, 223] were associated with epithelial-to-mesenchymal transition (EMT) [20]. Until now, it is uncertain which serum biomarkers for inflammation such as CRP, TNF-α, and IL-6 are linked with obesity-related cancer development [20, 234].

1.3.5. Cellular instability and changes in cellular energetics linked with obesity

One of the initial hallmarks of cancer is genomic instability [17]. This refers to the increased tendency of the genome to gain changes because of dysfunction during the processes of genome replication [235]. There are two mechanisms that can generate genomic instability, with these being the microsatellite instability (MIN) and chromosomal instability (CIN) pathways. Screening for the levels of MIN in tissues provides microsatellite-stable (MSS) or microsatellite-instable (MSI) profiles. Colorectal cancer in women has provided evidence of an association between obesity and an increased risk of microsatellite-instable high (MSI-H) [236]. In endometrial cancer, an MSI profile was associated with a high BMI [237]. A higher occurrence of chromosomal abnormalities has also been noticed in obese
compared with non-obese patients with endometrial cancer, providing further evidence linking genomic instability with the overweight state [238].

1.4. Cancer of the endometrium

In obese patients, there is a greatly increased risk for cancer in the uterus. For this reason, this project selected the endometrium as its focus and cancer cell lines from other tissues were studied for comparison.

1.4.1. Aetiology of endometrial cancer

1.4.1.1. Reproductive risk factors

The theory that infertility is a risk factor for endometrial cancer is in line with studies indicating higher risks for married, nulliparous women than for unmarried women. Thus, some researchers have discovered that infertile women encounter a three-to eightfold increase in risk [239, 240]. Mechanisms that may mediate this risk might variously be related to infertility-involved anovulatory menstrual cycles (i.e., prolonged exposure to oestrogens without adequate progesterone), high serum levels of androstenedione (i.e., an abundance of androstenedione is available for change to oestrone), and the absence of monthly sloughing of the endometrial lining (i.e., residual tissue may develop toward becoming hyperplastic). Moreover, nulliparity has been correlated with low levels of serum sex-hormone-binding globulin (SHBG), and this may lead to increase bioavailable oestrogen [241].

It has been known for a long time that the risk of endometrial malignancy decreases with growing parity, particularly among premenopausal women [242, 243]. Recently, consideration of this topic has concentrated on the ages at which these births happened. Research has identified a decreased risk with either older ages at last birth or shorter times since the last birth, and it has been suggested that this may reveal a defensive impact of mechanical clearance of initiated cells [244, 245]. An understanding of the actions of infertility on cancer risk must consider connections regarding specific birth control strategies such as oral contraceptives. However, it is noteworthy that a number of studies have found a
decrease in cancer risk among subjects using intrauterine devices, as revealed by a later meta-
analysis [246]. The nature of this clear, protective effect has not been explained despite the
fact that it is conceivable that the devices may influence risk by causing structural or
biochemical changes to the endometrium or circulating hormones. Another area of interest is
the impact of using fertility drugs, given that many studies have suggested that women using
ovulation-inducing drugs have a higher chance of endometrial cancers [247, 248]. Further, the
relationship of risk to breast feeding remains dubious, and while some studies propose that
lactation may offer protection [249], this has not been noted in all research [47, 245].

1.4.1.2. Menstrual risk factors

Most research has shown that age at menopause is specifically linked with the risk of
uterine disease. Around 70% of all women determined to have endometrial cancer are
postmenopausal. Most research indicates that there is an approximate two-fold increase in risk
for women with natural menopause after the age of 52 years compared with menopause
before the age of 49 years [250]. It has been suggested that the effect of increased age at
menopause on the risk of cancer developing may reflect longer exposure of the uterus to
oestrogen that influence the action in the absence of ovulation and progesterone. Overall,
however, the interplay between menstrual factors, age, and weight are complicated, as are the
biologic systems of these factors working in the pathogenesis of uterine malignancy [47].

1.4.1.3. Exogenous hormones

Exogenous hormones term refer to oral contraceptive, menopausal hormones and
tamoxifen. Studies have demonstrated that the impacts of hormonal treatment, both
unopposed oestrogens (that is, acting alone) and in combination treatments, may differ
depending on the characteristics of the user, most notably with a woman's BMI. Various
studies have suggested that the antagonistic impacts of unopposed oestrogens were most
noteworthy in non-obese subjects and that the clinically useful impacts of combined treatment
were most prominent in obese women [48-50]. Most information with respect to effects of
hormones are derived from research on consumers of pills. It is uncertain, however, as to
whether the utilisation of oestrogen patches, creams, or injections can influence risk. Given
associations of risk with even low-dose oestrogens, it is conceivable that these regimens may present some increase in the chances of risk [47].

1.4.1.4. Anthropometry and physical activity

Obesity is a well-recognised risk factor promoting uterine tumours and may represent up to 25% of cases [251]. Very overweight women seem to have extraordinarily high risks compared to normal weight women [50]. Obesity seems to influence both premenopausal and postmenopausal endometrial cancer [47].

Much research has focused on the role of physical activity in the aetiology of endometrial tumours. A potential relationship is naturally engaging, given that physical activity can lead to changes in the menstrual cycle, body fat distribution, and levels of endogenous hormones. Evidence shows a defensive effect of physical activity on uterine cancer risk, and no associations with body weight [47]. A current meta-analysis of prospective studies exhibited a reduced risk of endometrial cancer with medium to heavy physical exercise, and no association with known potential confounders such as obesity, menopausal hormone treatment utilize, and parity [252]. For the most part, studies that have assessed occupational and recreational physical activity have revealed decreased risks [47].

1.4.1.5. Smoking

A reduction in endometrial cancer risk among smokers has been reported, with current smokers having about half the risk of non-smokers [253-255]. Cigarette smoking has been connected to an early age of menopause in some communities and to reduced levels of endogenous oestrogens. Decreased risks might be more notable in overweight or obese patients [47, 256]. Currently, biological mechanisms underlying the negative association of smoking and endometrial cancer risk remain unclear. Adjustments in endogenous hormones or metabolic pathways are likely involved. One report [257] revealed that the negative association of smoking with endometrial cancer risk became strongly associated to higher serum androstenedione levels compared to lower serum oestrogen levels among overweight women [47, 257].
1.4.1.6. Medical Conditions

Multiple clinical studies have connected polycystic ovary syndrome (PCOS) to the elevated risk of endometrial cancer incidence, especially among younger females. It is unclear, however, whether this increased risk is related to obesity or not. A recent systematic review and meta-analysis suggested that women with PCOS had a three-fold higher risk of endometrial cancer [47, 258]. Various investigations have also noticed a high risk of endometrial malignancy among diabetic patients. A question that needs to be addressed, however, is whether this association is related to weight. Several research groups [259, 260] have proposed that the relationship holds when comparisons are confined to non-obese women or are adjusted for weight, but a strong association between diabetes and endometrial cancer risk among obese women in several studies has provoked interest for the aetiologic part of specific metabolic abnormalities such as hyperinsulinemia [47, 261, 262].

Other diseases, including hypertension, arthritis, thyroid conditions, anemia, and cholecystectomy, have all been proposed to influence the incidence of endometrial cancer, in spite of the fact that little reliable evidences has been presented [263, 264]. More recent research has also focused on the impacts of non-hormonal drugs on the risk of endometrial cancer development. Given speculation that the inflammatory process may be an essential part in the development of endometrial cancer [265], a number of papers have evaluated the associations of nonsteroidal anti-inflammatory drugs with endometrial cancer development but with conflicting outcomes [47, 263, 266, 267].

1.4.1.7. Familial factors

It is well established that 5% of the endometrial cancer incidence occurs in young subjects [268] as a result of family history of uterine cancer [269]. Moreover, subjects with a family history of colon growth are at increased risk [268]. Various investigations have concentrated on relationship of uterine cancer risk with mutual single nucleotide polymorphisms (SNPs), including those with obesity [270], hormone biosynthesis, metabolism or receptors [271, 272], and DNA repair [273]. More robust genome-wide
association studies (GWAS) were used in a study conducted in 2011, to identify the most important genes involved in increased risk of endometrial cancer [274]. This study revealed that a susceptibility locus close to HNF1B (rs4430796), that had a positive association with prostate cancer risk and inversely with T2D, was a significant predictor of endometrial cancer risk [274]. GWAS also identified that the SNP rs1202524, near the CAPN9 gene on chromosome 1q42.2, may host an endometrial cancer susceptibility locus [47, 275].

1.4.1.8. Environmental and occupational risk factors

Geographic variations in rates of endometrial cancer, with higher rates in certain industrial areas, has prompted the suggestion that specific environmental agents may influence risk. Given the well-recognised effect of hormones on the disease, there has been specific concerns about the potential effects of certain endocrine disruptors, including dichlorodiphenyltrichloroethane (DDT). Several studies have addressed this issue by looking at dichlorodiphenyldichloroethylene (DDE) levels, the active form of DDT, in the sera of cancer patients and controls, but have found no significant differences [276-278]. By contrast, few studies have focused on impacts of electromagnetic radiation as a risk factor for endometrial cancer but observations in regards to electric blanket or mattress covers have delivered mixed outcomes [47, 279, 280].

1.4.1.9. Hormones underlying risk factor associations

Few studies have reported on whether different endogenous hormones are associated with endometrial cancer risk. Key and Pike [281] suggested that endometrial carcinogenesis is reliant on uterine mitosis, which is increased by oestrogens and decreased by progesterone. A few studies have also suggested a positive correlation between endometrial cancer risk with serum androstenedione and testosterone levels [47, 127, 282, 283].

Obesity, which is suggested to cause elevated oestrogen levels, appears to represent a key risk factor for both uterine carcinoma and endometrial hyperplasia, yet the underlying mechanisms are not clear. A case-control analysis of serum oestrogen levels [283] revealed that the risk associated with obesity was not totally mediated by oestrogens in premenopausal women. However, a cohort study of postmenopausal women demonstrated that raised serum
oestrogen levels seemed to represent the main risk factor related to obesity [284]. A potential role for insulin and insulin-like growth factors (IGFs) in cancer risk has been suggested, although studies have not generally provided clear evidence of the role of either C-peptide, is a short 31-amino-acid polypeptide that connects insulin's A-chain to its B-chain in the proinsulin molecule, [285] or IGF levels [47, 211].

It is already known that oestrogen has a strong influence and is the key driver of the development of several cancers, including endometrial cancer but the possibility that some other factors may also be operating alongside oestrogen remains poorly researched [47-50]. In this project, therefore, I have investigated the potential of four hormones, leptin, insulin, IGF-I and adiponectin that are dysregulated in obesity for their ability to alter cancer cell behaviour.

1.4.2. Anatomy and physiology of endometrial cancer

The uterus is composed of myometrium (uterine muscle) and epithelial endometrium (the mucous membrane lining its cavity). The endometrium consists of groups of glands and stroma. Under the influence of ovarian oestrogen in the first half of the menstrual cycle, the endometrium proliferates and expands. Following this proliferative phase, ovulation occurs in a fertile cycle. In the second half of the cycle, the endometrium changes and develops secretory characteristics, including increasing levels of ovarian progesterone production. Later, the reduction of progesterone levels, which were maintaining the functional layer, results in the sloughing off of the tissue leading to menstruation [286, 287].

Under conditions of prolonged stimulation with E2 in the absence of progesterone, hyperplastic changes will take place in the endometrium and in time these may lead to the development of endometrial cancer. In some cases, these changes may be reversed by progesterone. Obese women are known to have higher levels of oestrogenic hormones and relatively lower progesterone levels than lean women. Indeed, the association of endometrial cancer and obesity is so strong that it is plausible that the changes in levels of reproductive steroids alone would be insufficient to generate the increased level of risk, and that effects produced by other obesity-related hormones are also required [288].
1.4.3. Epidemiology of endometrial cancer

Evidence provided by epidemiological studies and from both case control and cohort studies show a strong association between BMI of over 30 and endometrial cancer incidence [15, 289, 290], and a linear increase in the risk of endometrial cancers with increasing weight or BMI has been observed in most groups, [291]. In addition, an increased relative risk (6.3-fold) in the development of endometrial cancer in obese compared to lean women may occur due, in part, to the conversion of androgens to oestrogen [22].

The incidence rates of endometrial cancer have increased in a number of countries between the 1980s and 2010 [3], and in 2012 there were an estimated 320,000 new cases and 76,000 deaths from the disease worldwide [3], the annual incidence being 10-20 per 100,000 women [290, 292]. Incidence rates vary 20–30-fold between countries with close to two thirds of the registered new cases occurring in wealthy countries in Northern and Eastern Europe and North America, and with rates tending to be low in Africa and West Asia [3, 290]. Endometrial cancer is the sixth most common cancer in women around the world (almost 5% of all cancers in women). Increases in the elderly and obese in a population appear to be major contributing factors to this trend [3].

In the USA, endometrial cancer is the fourth most common type of cancer [293-296]. In the European Union, endometrial cancer ranks second in incidence among gynaecological cancers at 13 cases per 100,000 people per year [297] and is the third most common cause of death from gynaecological cancers after ovarian and cervical cancer [298, 299].

In New Zealand, endometrial cancer is the fifth most common cancer in women [298, 300]. There are four main ethnic groups in New Zealand; Maori, those of European origin, those of Pacific origin, and an Asian ethnic group who largely migrated to New Zealand in the last three decades [298]. Endometrial cancer rates are significantly higher among women of Polynesian descent than in Maori or European women [298, 300-303].

Most recently, a Ministry of Health of New Zealand report published in 2017 revealed that New Zealand was ranked third in obesity incidence in OECD, with obesity rates having increased notably over the past 15 years (the MOHNZ report). Moreover, the report highlighted that rates of endometrial cancer incidence also increased and were higher in
Pasifika women than in Maori or European women. It is tempting to relate these trends of increased endometrial cancer to the trend for increase obesity in these ethnicities.

In 2012, a similar study revealed that "Pacific and Maori women have higher incidence and mortality rates of uterine cancer. Women in the most deprived areas are more likely to present with an advanced stage of uterine cancer. Maori and Pacific women are less likely to present with well-differentiated tumours" [304].

The study concluded that Maori and Pacific women, and those from lower socioeconomic groups, are more likely to present with advanced cancer. However, the causes of variation in the epidemiology of endometrial cancer in New Zealand ethnic groups are not yet fully explained [304].

However, in 2016, a study was published that aimed to determine rates of which cancers follow changing ethnic inequalities over time in New Zealand. This study concluded that actions to further address the inequalities in the burden of cancer need to be developed [305]. These might include enhancing control of tobacco, decreasing rates of obesity and carcinogenic infectious agents, and focusing on increased access to effective cancer screening and improve health care services [305].

1.4.4. Characteristic of endometrial cancer

The normal endometrium may develop malignant changes that start as a confined tumour. In an advanced stage this tumour invades the adjacent myometrium and penetrates the uterine muscle to the serosal surface of the uterus. It may extend to surrounding organs such as the fallopian tubes, ovary, bladder, rectum, and cervical canal [306]. Local peritoneal spread of the disease occurs through lymphatic drainage to pelvic and para-aortic lymph nodes. However, less common is haematogenous (originating in or carried by the blood) metastasis [306] (Figure 1.3).
Figure 1.3 Images show the location and the progression of endometrial cancer. The anatomy of uterus. Abnormal growth of epithelial cell lining of endometrium started in a discrete area and changes into a malignancy later. (Image used with permission from Bausan Medical communications, Inc.- via OTRS. http://www.medicalook.com/Cancer/Endometrial_carcinoma.html).
1.4.4.1. Grades of endometrial cancer

The majority of endometrial tumours (80-90 %) arise within the epithelium of the uterine lining [290, 307, 308] and are categorised as endometrial carcinomas with the majority consisting of typical endometrioid adenocarcinomas [290, 309]. These typical endometrial carcinomas are further subdivided according to the International Federation of Gynaecology and Obstetrics (FIGO) classification (1988) into three architectural grades based on the percentage of solid tumour growth: Grade I tumours contain less than 5% solid architecture, grade II are those tumours with 6-50% solid architecture, while grade III tumours consist of more than 50% solid architecture [290, 307].

The remaining cases (10-20%) are rare cell types consisting of papillary serous carcinoma, clear cell carcinoma, papillary endometrioid carcinoma and mucinous carcinoma [308, 309]. Adenosquamous carcinomas are now categorized as typical endometrial carcinomas with squamous differentiation. The rare cell types are correlated with a later age of onset than grade I adenocarcinomas, have greater risk for extrauterine metastasis, and poorer prognosis [290, 308, 310-313].

1.4.4.2. Cell types

Cancer cell subtype is a good predictor of the biological response of endometrial cancer. Bokhman's clinical model has been the basis for categorising the pathogenesis of endometrial cancer [314], in which these tumours are classified into two types. Type I tumours (about 80%) are endometrioid carcinomas that usually present as a typical hyperplasia, first as a result of oestrogen stimulation, then followed by changes to malignancy. Women with type I tumours are mainly young [308], premenopausal or perimenopausal women, and are associated with high BMI, hyperlipidemia, nonovulatory cycles, infertility and late menopause [307]. This type of tumour is a low grade [308]. The risk of cancer incidence in overweight women increased by 50% compared to women of normal weight [315] and approximately 70–90% if type I (oestrogen-dependent) endometrial cancer patients are overweight [316].
It has been found that tumour progression may occur as a result of the development of non-endometrioid carcinomas type II tumours from pre-existing endometrioid carcinomas type I tumours [307, 317]. Type II tumours (20%) are non endometrioid carcinomas, or high grade predominantly serous and clear cell, which may arise from endometrial polyps or from precancerous lesions (endometrial intraepithelial carcinoma) [318, 319]. Type II tumours are not associated with oestrogen stimulation or hyperplasia. These tumours invade the myometrium and vascular spaces, and carry a high mortality rate [307]. These tumours are associated with high grade disease as well as more aggressive histologic subtype like papillary serous or clear cell carcinoma. Type II tumours are more common in older patients or those of African and American descent [308]. Histopathological images are provided (Figure 1.4).

1.4.4.3. Endometrial tumour genotype

Molecular regulation of the development of endometrioid carcinoma (type I) is different from those of serous carcinomas (type II) [307, 320, 321] and often exhibited microsatellite instability (MSI). Endometrial cells transform into endometrioid carcinoma through replication errors [290, 322, 323] and PTEN mutations [324, 325] and rarely exhibit p53 mutations [326]. However, the vast majority of endometrioid serous carcinomas (type II) exhibit p53 mutations and loss of heterozygosity on several chromosomes, and only occasionally show MSI [307, 326, 327].

Histological and morphological characteristics of type I and type II are shown in Figure 1.4. Type I endometrial cancer is usually confined to the surface of endometrium and grows slowly. On the other hand, type II seems to spread and form larger tumours, and grows aggressively. The morphology of these two types is also different. Type I endometrial tumour cells have the appearance of glandular structures, which is akin to normal endometrial glands. Type II tumour cells have serous papillary-like appearance, similar to serous ovarian cancer cells [3, 328].
Figure 1.4 Adenocarcinoma of the endometrium.
(A) Endometrioid carcinoma, type I.
(B) Nonendometrioid carcinoma, type II,
(C) Well-differentiated (grade 1) endometrioid adenocarcinoma, type I.
(D) Endometrioid serous carcinoma, type II.
Image used with permission from Dr. Jamie Prat [3, 328].

However, a wide range of mutations is responsible for tumour heterogeneity. Inactivation of the tumour suppressor gene \textit{PTEN} may result from MSI (45%), promoter hypermethylation (16%), or loss of heterozygosity (24%). \textit{PTEN} inactivation releases the PI-3K/AKT pathway, inhibiting apoptosis and resulting in tumour growth advantage. The protein, mammalian target of rapamycin (mTOR) is a kinase that regulates cell growth and apoptosis [329]. mTOR inhibitors have recently been developed as potential anticancer agents, and \textit{PTEN}-mutated tumours are particularly susceptible [3, 330]. Recent genomic and related analyses have indicated that most endometrioid tumours have few copy number alterations or \textit{TP53} mutations but frequent mutations in \textit{PTEN, CTNNB1, PIK3CA, ARID1A,
and KRAS, and novel mutations in the SWI/SNF chromatin remodeling complex gene ARID5B [331]. β-Catenin gene mutations occur in 20% of endometrioid carcinomas, correlate with MMP-7 and cyclin D1 overexpression, and are associated with good prognosis. Mutation of the ARID1A gene and loss of the corresponding protein BAF250a have been found in 29–40% of endometrioid carcinomas, 18% of serous carcinomas, and 26% of clear cell carcinomas [332, 333]. In contrast, nonendometrioid carcinomas often show TP53 and PPP2R1A mutations, inactivation of p16 and E-cadherin, c-ERBB2 amplification, STK15 alterations, and loss of heterozygosity at multiple loci [292, 334].

The mutations suggested in The Cancer Genome Atlas (TCGA), 2013 paper are; POLE ultra-mutated (Polymerase E enzyme 7-10% endometrial cancer are mutated), micro satellite instability hyper-mutated (DNA repetition gene), Copy-Number Low (LCN) short tandem repeat typing for samples less than 200 pg of template DNA within a cell) and Copy-Number High (HCN) (Figure 1.5).

In this study, Ishikawa cells are used. These are a type I endometrial cancer cell line which is obesity-E2-sensitive and because it has PTEN and PIK3CA mutations, it fits into Copy-Number-Low endometrial cancer molecular subtype. HEC-1A (also used in this study) is again type I endometrial cancer cell line and obesity-E2-sensitive. However, HEC-1A has p53 and PIK3R1 mutations and fits into Copy-Number-High endometrial cancer molecular subtype.

RNAseq and microarray methods are often used to measure gene expression level. While similar in purpose, there are a lot of differences between the two technologies [335]. It is noteworthy, that these cell lines indeed had responded to the obesity hormones that have been used in this study.
Figure 1.5 Non-silent mutation matrix for 13 SMGs with significantly different frequencies across the mutation spectra cohorts. A green bar indicates that at least 1 non-silent SNV or indel was identified in the tumor. *PTEN* and *TP53* distinguished the CN-high serous-like tumors from the remaining tumors. *PIK3CA* and *PIK3R1* mutations were mutually exclusive and *PIK3R1* was mutated significantly less in the CN-high group. *ARID1A* and *ARID5B* mutations also appear to be mutually exclusive and both have low mutation frequency in CN-high group. *KRAS* and *CTCF* mutations are rarely seen in the CN-high group. *CTNNB1* mutations appear more frequently in MSS tumors with lower mutation rates, than in endometrioid tumors with MSI. *RPL22* mutations were almost exclusive to cases with MSI. *FBXW7* and *PPP2R1A* mutations appear mutually exclusive, and are more common in CN-high serous-like tumors. *ARID1A* had a similar non-silent mutation frequency in MSI (36.9%) and MSS endometrioid tumors (42.2%), but a low frequency in high SCNA serous tumors (5%) [335a].
1.4.4.4. Management of endometrial cancer

Management depends on staging of the disease [336-341]. In general, treatment is mainly surgery and includes total hysterectomy, bilateral salpingo-oophorectomy, in case of I and II, with absence of visible metastasis [47]. More advanced disease required pelvic and para-aortic lymphadenectomy and omentectomy removing as much as possible of the tumour. This type of disease stage needs further postoperative adjuvant therapy such as chemotherapy, hormonal therapy and radiotherapy [341, 342]. Current clinical chemotherapy may include the use of Paclitaxel, Carboplatin, Doxorubicin and Cisplatin [342a]. Proper surgical staging offers better prognostic and therapeutic benefits for such women [336, 341] and may enhance the progress of targeted therapy and lead to better survival and less morbidity of overtreatment (radiation injury) and the effects of under treatment (recurrent disease, increased mortality) [341]. Especially in patients with type II endometrial cancer, systematic surgical staging requires additional omentectomy and biopsy of any suspected lesion [343]. Other reports also highlighted steps in clinical management of endometrial cancer [342, 344].

1.5. Vascular endothelial growth factor (VEGF)

VEGF, a heparin-binding glycoprotein, is a key regulator of physiological angiogenesis, which is the increased vascularisation that is necessary to transport oxygen and nutrients to tissues, including during embryogenesis, skeletal growth and reproductive functions [345]. Also, angiogenesis is essential to the development of solid tumours. VEGF-driven pathways have been demonstrated to play a major role in tumour angiogenesis by stimulating endothelial cell proliferation and inducing the budding of new blood vessels promoting the growth of tumour masses [346, 347]. Angiogenesis is the result of a cascade of physiological events that are linked to tumour neovascularisation; this process is very important for large tumours to obtain adequate oxygen and nutrients to maintain growth activity, and remove wastes. VEGF may be secreted from adipocytes and tumour cells, and overexpression is associated with a poor prognosis in endometrial cancer [3, 345]. Increased production of VEGF, frequently observed in hypoxia, nutrition depletion or inflammation, results in
increased endothelial cell proliferation and decreased apoptosis [345]. VEGF is also a potent permeability factor that promotes cell migration during invasion. The neovascularisation process for rapidly growing tumours may facilitate tumour metastasis and help in tumour spread [348]. The potential in cancer treatment of drugs that inhibit VEGF secretion and angiogenesis, such as bevacizumab and tyrosine kinase inhibitors, is being studied clinically in several cancer types [3, 349-355].

The VEGF gene is complex with several alternatively spliced isoforms, and the regulation of expression could differ between normal and tumour tissues [356]. The effects of VEGF are mediated by two high-affinity transmembrane tyrosine kinase receptors, VEGFR-1 and VEGFR-2 [107, 109, 110], which induce dissimilar cell signalling cascades. Moreover, the presence of the receptors also correlates with the pathological angiogenesis that is associated with tumours and other disorders.

Obesity elevates circulating VEGF secretion compared to lean individuals and high tumour expression of VEGF is associated with poor prognosis of obesity-related cancers [357, 358]. Overexpression of VEGF and angiogenesis are necessary for solid tumour growth and metastasis [111, 359-362]. In this project regulation by obesity-related hormones [363] of this angiogenic factor in endometrial, breast and ovarian cancer cell lines is investigated.
1.6. This Project

Choice of Cell lines: The model at the centre of this study is the endometrial cancer cell line Ishikawa. This cell line was originally isolated from a woman who had low grade type I endometrial cancer [308]. This is the most common subtype of endometrial cancers (comprising approximately 65% of presenting endometrial cancers) and is hence a relevant model. The Ishikawa cell line expresses active oestrogen [364], progesterone [365] and androgen receptors [366]. Evidence of hormonal responsiveness of Ishikawa cells in vitro by steroidal modification of proliferation, cellular function or gene expression is well-established [70]. Additionally, the activities that are exhibited by Ishikawa cells, which have a PTEN mutation, wild type p53 and represent type 1 subtype [367]. One additional endometrial cancer cell line, HEC-1A was also investigated in this study. HEC-1A has p53 mutation, a PTEN wildtype, K-RAS mutation, and this cell line is also representative of low grade cancer [331]. In this study, the responses to the individual hormones were also compared among cancer cells from different origins. A MCF-7 is representative of human ductal cell adenocarcinoma breast cancer [368]. This subtype of breast cancer has a substantially increased risk of developing cancer in obese women [369]. The fourth cell line, from an ovarian cancer, SKOV-3, is derived from metastasis ascites. Ovarian cancer onset is much less sensitive to obesity than either endometrium or breast tissues [370-372].

Hormones: The study considers the effects of four hormones, leptin, insulin, IGF-I and adiponectin. The first three are known as tumour-promoting hormones whereas adiponectin is a putatively anti-cancer peptide. Leptin is strongly associated with obesity and is the focus of much research investigating the effects of high BMI on metabolism and tissue function. Insulin also is associated with obesity and is the prominent link between diabetes and a number of tissue pathologies, including cancers. IGF-I has several properties similar to insulin and is also believed to exhibit growth-enhancing activities in the progression of many cancers. Thus these three peptides are known to have regulatory mechanisms that are intimately connected to BMI and have also been implicated in oncogenic processes. The activities of adiponectin are also modulated by factors associated with diabetes and is dysregulated in obese individuals. Again, although there have been reports that it has anti-tumorigenic effects,
the role(s) of adiponectin in cancers have not been well-defined. Thus the interactions of this hormone with the other three may provide insight into interactions that potentially occur in vivo.

**Signalling Pathways:** Also, this study attempts to document the important roles of selected proteins associated with PI-3K/Akt/mTOR, JAK/STAT3 pathways and AMPK protein. To understand these pathways in regulating cell behaviour, an inhibitor of each pathway is assessed. Using inhibitors will be useful to examine the cell signalling pathways that are involved in proliferation and VEGF secretion from cancer cells. A broad range of cell biological techniques will be employed in order to explore the role of the hormones in cancer cells.

It has been reported that two important signalling pathways, PI-3K/Akt and JAK/STAT3, exhibited cross-talk in three types of cell lines (hypothalamus, myeloproliferative neoplasms and ovarian cancer) via insulin receptor substrate (IRS) [53, 164, 373-375]. From these reports JAK was suggested to be the upstream molecule of these two pathways which provides a new target for therapeutic treatment of these diseases. Thus, I first hypothesised that the cross-talk of these pathways may also occur in Ishikawa endometrial cancer cell line. Therefore it was an initial aim to employ an in vitro model to investigate this proposition.

Previous studies identified the activation of adenosine monophosphate-activated protein kinase (AMPK) [376, 377] as a pathway mediating adiponectin activity. Indeed adiponectin is recognised as a stimulator of AMPK in many cancer cell types [136, 377-379], and exposing cancer cells to treatment with adiponectin enhances AMPK activation in endometrium [30] as well as colon [365-367], breast [380-383], prostate [143, 367], and liver [349]. The conditions of the study will be applied to investigate this pathway.

I acknowledge that the results found in experiments using in vitro cell lines may not always translate directly to the in vivo models. However, experiments designed under the right conditions, and with appropriate controls using properly authenticated cancer cell lines retain the properties of the cancers of origin [384] and certain relevant questions can be investigated and answered. An effect on cell behaviour by the hormones, albeit using an in vitro culture
model, will suggest that cancers are directly sensitive to dysregulated endocrinological processes that may alter growth patterns and VEGF secretion. In particular, signalling pathways associated with leptin, insulin, IGF-I and adiponectin in Ishikawa cells will be examined. The results may thereby suggest that an endocrinological approach could provide partial treatment options. Further, if the peptides have effects that vary between tissues, there may be advantage, in line with many contemporary views, in considering the target tissue or even the individual in understanding mechanisms of cancer progression in obese patients.

High leptin, insulin, and IGF-I concentration in the microenvironment will increase cell growth and proliferation [20, 105, 222] henceforth increase the opportunity for mistakes during DNA replication to occur and be repeated [105, 222, 223]. Thus, new mutations may appear due to the abnormal repair of DNA. As a result the cells will be more prone to accumulate oncogenic mutation and become cancerous within the conducive microenvironment [20, 105, 223].

Although cancer may develop along with obesity, however, it has not been robustly established that obesity can cause cancer. However, it is possible to suggest that obesity might trigger cancer if there is already an oncogenic mutation or DNA replication mistake [222, 223]. It is not determined yet which of the two steps, mutation or proliferation, occurs first. However, the dysregulation of hormones due to obesity is possibly contributing to cancer progression [20].

**The hypothesis:** In summary, the hypothesis of this study is that the obesity-related hormones, leptin, insulin, IGF-I and adiponectin can induce hormone-specific effects on behaviour of well-characterised cancer cell lines. In addition, these obesity-related hormones will elicit biological activities that will be associated with modulation of signalling pathways of cancer cell lines. This may provide new insight into details linking obesity with cancer progression and whether the hormonal effects are similar across all tissues. Also it is hypothesised that cross-talk between the PI-3K/Akt/mTOR and JAK/STAT3 pathways may also occur in Ishikawa endometrial cancer cell line. Opportunities for novel endocrinological treatments may emerge. Again, it must also be acknowledged that results found in *in vitro* cell lines do not always translate directly to *in vivo* models, where the milieu will include various
concentrations of many other cellular factors and hormones that may influence the response of the cancer cells.

**Aims of this project are**: (i) to document the proliferative and other effects of hormones on cancer cell lines, (ii) to study the effect of hormones on secretion of VEGF from cancer cells, (iii) to investigate signaling pathways that are associated with hormones modifying behaviours of cancer cells.
Chapter Two

Materials and Methods
2 MATERIALS AND METHODS

2.1 Cell Culture Media

2.1.1 M199 medium

2.2 g of sodium bicarbonate (Sigma Aldrich, Cat. No. S 5761-500 g, St Louis, MO, USA) and 12 g of M199 packaged medium (Gibco, Invitrogen, Cat. No. 31100-035, Auckland, NZ) were dissolved in 1000 ml of autoclaved Milli-Q water; then the medium was filtered through 0.2 μm filter membrane and stored at 4°C.

To 196 ml prepared basal media were added 2 ml of 1% (v/v) penicillin/ streptomycin, (Gibco Invitrogen, Cat. No. 15140-122-100 ml), 2 ml of 1% (v/v) Glutamax-ICTS (Gibco Invitrogen, Cat. No. A12860), 0.4% (v/v) fungizone (Gibco Invitrogen, Cat. No. 15290-018) (0.8 ml in 200 ml).

2.1.2 M199 medium with FBS

To prepare 200 ml of M199 medium supplemented with 10% (v/v) fetal bovine serum (FBS) medium, (Gibco Invitrogen, Cat. No. 10091-148-500 ml), 20 ml FBS 10% (v/v) were added to 180 ml prepared basal medium.

2.2 Cell Culture

2.2.1 Maintenance of cell culture

For experiments, cells were maintained in the M199 medium with FBS in culture flasks, (Corning flask 3065 (25 cm³ cell culture flask, angled neck)). To propagate cells, the medium was aspirated and discarded from the flask. Cells that attached to the base of the flask were washed with 3 ml of Phosphate Buffer Saline (PBS) (Invitrogen, Cat. No. 003002, Carlsbad, CA, USA). Then, 5 ml of diluted trypsin-EDTA (Gibco/Life, Cat. No. 15400-054-100 ml)
was added to the flask and incubated for 20 minutes at 37°C. This allowed cells to detach from the bottom of the flask. The resulting cell suspension was aspirated and transferred to a 15 ml tube. The flask was then rinsed with 3 ml of PBS. The cell suspension was centrifuged for 5 minutes at 400g. After centrifugation, the supernatant was aspirated and discarded. Then, 2 ml of the medium (M199 with FBS) was added to the cell pellet and triturated with a 1 ml pipette to disperse clumps of cells and obtain single cell suspension. An aliquot (100 μl) of the cell suspension was diluted with 100 μl of PBS in an Eppendorf tube prior to performing cell counting with a haemocytometer. Approximately 50,000 cells/well were seeded in a 24-well plate in M199 medium with FBS. Cells were at approximately 70% confluence in 37°C and 5%/95% CO₂/air incubator prior to addition of treatments and collection.

2.2.2. Treatment protocols

2.2.2.1. Hormone addition

Cells (5 x 10⁴ cells/well) were seeded and grown in 24 well plates (Corning 3524 - 24 well cell culture plate, flat bottom with a lid) in M199 medium with 10% FBS (1 ml). Cells were incubated for 48 hours to allow cells to attach and then medium discarded and replaced with M199 without FBS medium (1 ml). Cells were then incubated in FBS-free media for 24 hours to minimise the effects of remnant hormones and growth factors which may be derived from FBS. After 24 hours, media was removed from cell culture wells and medium containing selected concentrations of hormones in M199 without FBS was placed in wells to a total volume of 1 ml. The working concentrations of leptin (50 ng/ml), adiponectin (20 and 100 ng/ml), insulin (580 ng/ml), and IGF-I (100 ng/ml) were calculated from stock concentrations.

All hormones were purchased from GenWay Biotech Inc. (San Diego, CA, USA): Leptin (recombinant human leptin, Cat. No. 10-783-313250), adiponectin (adiponectin recombinant human, Cat. No. 10-002-38001), insulin (insulin human, Cat. No. 10-663-45749), and IGF-I (recombinant human IGF-I, Cat. No. CR1500C).
2.2.2.2. Signalling pathway modulation

Pathway inhibitors were used to investigate the signalling mechanisms of the hormones. Phosphatidylinositol 3-kinase (PI-3K) inhibitor LY294002 (LC Laboratories, Cat. No. L-7962, Woburn, MA, USA) was used at 20 μM. The analogue of rapamycin (mTOR) inhibitor everolimus (LC laboratories, Cat. No. E-4040) was used at 2 μM, and the JAK inhibitor, tyrphostin AG490 (Sigma-Aldrich, Cat. No. T3434-5 mg) was used at 100 μM. These inhibitors were included in the incubations as appropriate, concurrently with aforementioned growth factors. After 48 hours, the effects of experimental conditions were examined. Cells were collected for determination of growth activity, VEGF secretion and protein expression using a number of techniques including Western Blotting, flow cytometry, ELISAs, immunohistochemistry, and fluorescence microscopy (see following sections).

2.3. Measurements

2.3.1. Cell counting

Cell suspensions were diluted so that the cells did not overlap each other on the haemocytometer grid, and were uniformly distributed as it assumed that the total volume in the chamber represents a random sample. The cell suspension was mixed thoroughly before taking a sample to count. The cells were counted in selected squares so that the total count was approximately 100 cells (the number of cells needed for a statistically robust count). Selecting a specific counting pattern avoided bias, and the Bio-Rad Company protocol for cell counting was used. In brief, the haemocytometer and cover slip surfaces were cleaned with lens paper and ethanol. The cover slip was placed over the counting surface prior to adding the cell suspension. The cell suspension was introduced so that the surface was just covered by the cover slip. The counting chamber was viewed at 40x (Olympus CK40, Tokyo, Japan) and the counts were appropriately multiplied by the dilution factors used. In a preliminary study, leptin was used to determine whether there was glucose-dependent response to obesity-related peptides. This provided information for experimental design and also interpretation of some data in the literature.
To avoid the interference of diabetic status and oestrogen effect on cell proliferation. I chose M199 medium that has 5.5 mM glucose which is considered to be a normal physiological serum level, and compared it with DMEM-F12 that has a higher glucose concentration of 17.1 mM, a level associated with diabetes (see Appendices, Figure 10.1).

In addition, M199 medium has phenol red. Phenol red is known to be a weak oestrogen. However, oestrogen in M199 is unlikely to have an effect on cancer cell proliferation compared to other media [385]. Moreover, the effect is negligible due to the fact the same medium is used for the control cells and in worse scenario, the oestrogenic effects of M199 will have only a minor effect on cancer cell proliferation compared to the hormones investigated in the thesis.

On the basis of these results, M199 was chosen as the more relevant medium and to avoid a compounding factor associated with diabetes.

2.3.2. VEGF ELISA assay

Conditioned media from control and treated cells were collected for determining the amount of secreted VEGF. The cell medium was aspirated from wells with caution so as not to include cells, and 75 μl was left in the well while the pipette tip did not touch the cell layer. The cell-free conditioned media was transferred to Eppendorf tubes and centrifuged at 6700g at room temperature to remove debris and non-adherent cellular material. ELISA analysis was performed according to the manufacturer’s instructions (VEGF ELISA kit; DuoSet ELISA Development R&D System human VEGF, Minneapolis, MN, USA). The kit components consisted of Capture Antibody, Detection Antibody, Standard, Stop solution, Streptavidin-HRP, and Substrate solution. The cell numbers for corresponding cultures were counted and the level of VEGF that was secreted was determined per 10⁶ cells. The values of absorbance were measured at wavelength of 450 nm. The standard curve was plotted by Prism statistical software (GraphPad Software, La Jolla, CA USA). The working range was 50 – 2000 pg/ml and minimum sensitivity of the method is less than 50 pg/ml. Samples fell within this range without dilution. Results were then normalised to 1 million cells. The experiments were
performed with six replicates (three times in duplicate) and the experiment was performed at least three times on independent occasions.

2.3.3. Western blotting

Supernatant medium was removed and cells were washed with 0.5 ml PBS and lysed with 100 μL RIPA lysis buffer which was freshly prepared from constituents-(50 mM Tris base (2-Amino-2-(hydroxymethyl)-1,3-propanediol, Roche, Cat. No, 708976, USA), 150 mM NaCl (MERCK, Cat. No, K 33591704506, Germany), 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (SDS) (sodium dodecyl sulfate, BDH, Cat. No, 44244, UK), 1% NP-40 (acetyl phenol ethylene oxide, SIGMA Aldrich, Cat. No, N-6507, USA), 0.5% sodium deoxycholate, 1 mM NaVO₄, 10% glycerol (SIGMA Aldrich, Cat. No, G 6279), and 1 protease inhibitor cocktail tablet (Complete Mini, Roche, New Zealand), pH 7.4). The cell lysate was transferred to an Eppendorf tube and the total protein was measured by Bio-Rad DC protein assay kit (Cat. 500-0113, 500-0114 and 500-0115, CA, USA). The cell lysates were left on ice for a further 30 minutes. Sample buffer [0.2% (v/v) bromophenol blue, 25% (v/v) glycerol, 10% SDS in Tris-HCl, and pH 6.8] was added and protein lysates were boiled for 10 minutes. Prior to loading, the cell lysates were mixed and centrifuged at 9,700 rpm for 5 minutes. A total of 10 μg protein lysate was loaded and separated by SDS-PAGE using a 7% stacking gel and a 10% separating gel.

The SDS-PAGE was run at 120 V using Tris-glycine running buffer. The SDS-PAGE markers used were MagicMark™ XP Western Standard (Thermo Fisher Scientific, New Zealand) and Precision Plus Protein standard (Bio-Rad, Hercules, USA). Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (BioRad, Cat. No, 162-0177, USA) in ice cold transfer buffer (25 mM Tris base, 200 mM glycerin) at 100 V for 60 minutes. Specific immuno-detection was carried out by incubation with relevant primary antibodies. Antigens were revealed using the ECL developing solution (Amersham, ECL Prime Western Blotting Detection Reagent, ge-9466588; GE healthcare, UK). The protein bands were visualized and a densitometry analysis was performed using Alliance 4.7, Unitec (Cambridge, UK). Cell lysates were collected from at least 3 separated cell culture experiments.
Antibodies used in this study were all provided by Santa Cruz Biotechnology (Dallas, TX, USA). The primary antibodies used in this study were anti-AdipoR1 (sc-99183), anti-AdipoR2 (sc-99184), anti-Ob-R (sc-8325), anti-InsR (sc-711) and anti-IGFR (sc-7952) (Table 2.1). The two secondary antibodies used in this study were bovine anti-rabbit IgG-HRP (sc-2385) and bovine anti-mouse IgG-HRP (sc-2380).

The dilution of antibodies according to the suppliers was followed. In general, antibodies from Santa Cruz, the optimal dilution of antibody for Western blot was around 1/500-1/1000. Each time before I used each antibody, I have to optimise the dilution of the antibodies in order to use the appropriate dilution factor of the antibody as well as the appropriate blocker's concentration and type by doing several experiments with different concentrations of primary and secondary antibodies and blocker reagents.
## Table 2.1 Western Blotting antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>kDa</th>
<th>Ser. No.</th>
<th>Company</th>
<th>Blocking</th>
<th>Primary antibody dilution (10 µl)</th>
<th>Secondary antibody dilution (13 µl): R, rabbit; M, mouse</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. AdipoR (H-40)</td>
<td>42-44</td>
<td>SC-99183</td>
<td>Santa Cruz</td>
<td>5% Skim milk</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>2. Akt</td>
<td>56-63</td>
<td>SC-8312</td>
<td>Cell Signaling</td>
<td>1% BSA</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>3. AMPK</td>
<td>63</td>
<td>SC-25792</td>
<td>Santa Cruz</td>
<td>1% BSA</td>
<td>1/250 40 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>4. GAPDH</td>
<td>37</td>
<td>SC-25778</td>
<td>Santa Cruz</td>
<td>5% Skim milk</td>
<td>1/1000 10 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>5. IGFR</td>
<td>130</td>
<td>Sc-7952</td>
<td>Santa Cruz</td>
<td>4% BSA</td>
<td>1/200 50 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>6. InsR</td>
<td>95</td>
<td>Sc-711</td>
<td>Santa Cruz</td>
<td>4% BSA</td>
<td>1/200 50 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>7. Ob-R (H-300)</td>
<td>100-125</td>
<td>SC-8325</td>
<td>Thermo-Fisher</td>
<td>5% Skim milk</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>8. p-Akt</td>
<td>56-63</td>
<td>SC-101629</td>
<td>Santa Cruz</td>
<td>4% BSA</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>9. p-AMPK</td>
<td>63</td>
<td>SC-33524</td>
<td>Santa Cruz</td>
<td>4% BSA</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>10. p-Stat3(B-7)</td>
<td>86-91</td>
<td>Sc-8059</td>
<td>Santa Cruz</td>
<td>5% BSA</td>
<td>1/1000 10 µl 1/5000 2.6 µl</td>
<td>M Tyr705 epitope</td>
<td></td>
</tr>
<tr>
<td>11. p-Stat3(ser727)</td>
<td>86-91</td>
<td>9134</td>
<td>Santa Cruz</td>
<td>5% BSA</td>
<td>1/1000 10 µl 1/5000 2.6 µl</td>
<td>R Ser727 epitope</td>
<td></td>
</tr>
<tr>
<td>12. Stat3 (C-20)</td>
<td>86-91</td>
<td>Sc-482</td>
<td>Santa Cruz</td>
<td>3% BSA</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>13. Ob-R (C-20)</td>
<td>100-125</td>
<td>Sc-1832 P</td>
<td>Thermo-Fisher</td>
<td>5% Skim milk</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R Blocker</td>
<td></td>
</tr>
<tr>
<td>14. Insulin Rβ (C-19)</td>
<td>95</td>
<td>Sc-711 P</td>
<td>Santa Cruz</td>
<td>4% BSA</td>
<td>1/200 50 µl 1/10000 1.3 µl</td>
<td>R Blocker</td>
<td></td>
</tr>
<tr>
<td>15. AdipoR (C-14)</td>
<td>42-44</td>
<td>Sc-46748 P</td>
<td>Santa Cruz</td>
<td>5% Skim milk</td>
<td>1/500 20 µl 1/10000 1.3 µl</td>
<td>R Blocker</td>
<td></td>
</tr>
</tbody>
</table>

*All the primary antibodies were purchased from Santa Cruz Biotechnology Company (Santa Cruz Biotechnology, Dallas, TX, USA), except for #11 which was purchased from Cell Signaling Technology (Danvers, MA, USA).

*The same dilution of antibodies were used for Western blot and immunohistochemistry assays.
2.3.4. **Immunofluorescence microscope imaging**

To prepare cells for immunofluorescence microscopy, a coverslip was sterilised in 95% ethanol solution then left for 30 min in a sterile area inside a 12 well plate to dry. The coverslip was then washed in 500 µl medium. Then M199 with FBS medium up to 1 ml containing 50,000 cells of cell lines was added to the cover slip which was incubated for 48 hours to allow adherence and proliferation.

After 48 hours, the medium was removed and the cells on the cover slip were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 45 minutes at room temperature. The cells were washed with cold PBS (5 times x 5 minutes) at room temperature. To permeabilise the cells, cold methanol (95%) was added to cells for 15 min at 4°C, (to perform actin staining experiment, the fixation solution was free of methanol since methanol can disrupt the actin during the fixation process, thus I added 0.1% Triton X-100 in PBS onto fixed cells for 15 minutes in room temperature), and the cells were washed with cold PBS (4 times x 5 minutes) at room temperature. Cells were blocked with the blocker that was appropriate for each antibody (Table 2.1) for 60 minutes at room temperature. Again, cells were washed with cold PBS (4 times x 5 minutes) at room temperature.

Cells were incubated with primary antibody in 2.5% BSA in PBS at 4°C overnight. The same concentration of the antibodies used in Western blot was used for immunofluorescence. The cells were washed with cold PBS (4 times x 5 minutes) at room temperature. Incubation was performed in the dark at room temperature for 1 hour with secondary antibody conjugated with the fluorescent molecule Atto 594 (Sigma-Aldrich, Cat. No. 08717) to detect primary antibody staining and then Alexa 488 Phalloidin (CytoPainter Phalloidin iFluor 488 reagent, Cat. No. ab176753, Abcam, Cambridge, UK) was added to detect actin microfilaments and incubated for 15 minutes at room temperature. The cells were covered with foil to protect them from light. To stain DNA, cells were incubated with Hoescht (20 µg/ml) for 20 min at room temperature in the dark and cells were washed with a mixture of cold PBS and 0.05% Tween-20 (3 times x 10 minutes) at room temperature, followed by cells being washed with cold PBS (4 times x 5 minutes) at room temperature. Anti-fading solution
was added to the cells on cover slip. Controls for non-specific binding of primary antibody were performed using absorption blocking peptide where they were made available by the manufacturer. Controls for non-specific binding of the secondary antibody were also performed using just the second antibody without primary antibody.

Localisation of protein was visualised with fluorescence microscope (Carl Zeiss, Imager.Z1, Oberkochen, Germany). Optical sections (0.5µm thickness) were collected using the ApoTome module, and 3D images and z-stack data were generated using ZEN application software (Carl Zeiss). All images are computer-generated from optical sectioning.

### 2.3.5. Detection of apoptotic and necrotic cells using flow cytometry

The cells were seeded in 24-well culture plates at a density of $5 \times 10^4$ cells/well in M199 medium supplemented with 10 % FBS as a control medium or with treatments with the hormones for 48 hours. Cells were detached and collected in 1.5 ml tubes. Briefly, after centrifugation 10 minutes at 530g, the cell pellets were re-suspended in the Annexin assay buffer (200 µl). The buffer contained 10 mM PIPES, 140 mM NaCl, and 2.5 mM CaCl$_2$ (all from Sigma-Aldrich) and was adjusted to pH 7.4. Volume of 5 µl of Annexin-FITC was added to the Eppendorf tube to detect apoptotic cells. The re-suspended cell pellets were kept in the buffer for 20 mins at room temperature. Cells were then incubated with 1.3 µl of propidium iodide (PI) for 10 mins in the dark at room temperature (because PI is light sensitive) to detect necrotic cells. Then Annexin assay buffer (200 µl) was added again. 200 µl of cell suspension were added to 96-well plate in duplicate. Samples were then analyzed in a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Brea, CA, USA).

### 2.3.6. Cell lines

Four cell lines were studied in this project. Ishikawa cell line is a well-differentiated human endometrial adenocarcinoma cell. It is one of the most widely used human endometrial-derived cell culture models. Ishikawa cell line 3-H-12 No.138 was donated by the National Hospital Organization, Kasumigaura Medical Centre, Japan. To make a comparison between endometrial cancer cell lines, Ishikawa, other types of cancer cell lines
from other organs were investigated. These cell lines included an ovarian cancer cell line, SKOV-3, a breast cancer cell line, MCF-7, and a second endometrial cancer cell line, HEC-1A. SKOV-3 cell line was obtained from the American Tissue Culture Centre (ATCC), Number: HTB-77™; Homo sapiens, human, Ovary: Ascites, Adenocarcinoma, USA. MCF-7 cell line was obtained from ATCC® Number: HTB-22™; Homo sapiens, human, Epithelial, Mammary Gland, Breast; Derived from Metastatic Site: Pleural Effusion, Adenocarcinoma, USA. HEC-1A was obtained from ATCC® Number: HTB-112™; Homo sapiens, human, Uterus/Endometrium, Adenocarcinoma, USA. All cell lines were STR profiled and cells were found to be mycoplasma free.

2.3.7. Statistical analysis

Data were expressed as mean ± SEM from at least three independent experiments performed in triplicate. Statistical analyses were performed using GraphPad Prism 7. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) multiple range test or logarithmic analysis and Student's t-test or one-way analysis of variance (ANOVA) as appropriate. A value of $P < 0.05$ was considered to indicate a significant difference between parameters.
Chapter Three

The effects of leptin, insulin, IGF-I and adiponectin on the endometrial cancer cell line, Ishikawa
3 The effects of leptin, insulin, IGF-I and adiponectin on the endometrial cancer cell line, Ishikawa

3.1. Introduction

Obesity increases the risk of cancer incidence in overweight women by 50% compared to women of normal weight [315, 386], with endometrial cancer being particularly sensitive. The risk of endometrial cancer increases approximately 70–90% if type I patients are overweight [3, 15, 316]. Thus, obesity is estimated to account for 40% of endometrial cancer incidence [3], and as endometrial cancers are one of the most common causes of mortality from all types of cancer [294], obesity contributes directly to many cancer deaths.

3.1.1. Hormones

The manner in which obesity alters cancer risk is unclear. However, it is known that in obese individuals there is dysregulation of a number of factors including hormones, gene expression and feedback mechanisms. This project studies the effects of four obesity-related hormones, namely leptin, adiponectin, insulin, and IGF-I on endometrial cancer risk.

Leptin is a protein factor secreted primarily by white adipose tissue (WAT) but also by many other organs [28, 66]. There is a high correlation between plasma leptin and indices of body fatness including BMI [81, 82], and a correlation between elevated leptin concentrations and endometrial cancer has been observed [62]. This suggests that elevated amounts of leptin may be an important hormone in the development of endometrial cancer. Another adipokine hormone, adiponectin, is also primarily secreted from the adipose tissue [131]. It is believed to have opposite biological effects from leptin. The levels of adiponectin change in an obesity dependent manner with circulating plasma adiponectin levels inversely correlated with BMI [148, 176-178]. Adiponectin may also provide a link between obesity, insulin resistance, and diabetes [131, 139-141]. Recently, anti-tumour effects of adiponectin in several types of cancers, including endometrial cancer, have been reported [150, 168]. Insulin is a peptide hormone produced in the islets of Langerhans in the pancreas, and plays a crucial role in
glucose regulation [109]. Obesity, as defined by BMI, is associated with elevated insulin [39, 40], and higher insulin levels also are associated with increased risk of endometrial cancer [112, 114, 387]. IGF-I plays a critical role in growth in children and adults. Many studies have shown a significant correlation between the presence of IGF-I and development of types I and II endometrial cancer [388]. The growth of solid tumours depends on satisfactory nutritional support and the neovascularisation, that underpins the process, is dependent to a substantial extent on pathways activated by vascular endothelial growth factor (VEGF). VEGF is secreted from tumour cells themselves and has angiogenic, mitogenic, and vascular permeability-enhancing activities specific to endothelial cells [348]. Obesity elevates circulating VEGF secretion compared to that in lean individuals [389, 390] and high tumour expression of VEGF is associated with poor prognosis of obesity-related cancers [357].

This chapter will investigate effects of these four obesity-related hormones (leptin, adiponectin, insulin, and IGF-I) on an endometrial cancer cell line known as Ishikawa. In particular, the results of incubation of the cells with the hormones on cell number, level of apoptosis and VEGF secretion are measured.
3.2. Results

3.2.1. The presence of leptin receptors

For cancer cells to respond to leptin hormone, they should have detectable levels of expression of leptin receptors (Ob-Rs) to bind with the leptin ligand. Thus, the presence of leptin receptors (Ob-Rs) in Ishikawa cells was investigated. To ascertain this information the presence of Ob-Rs in endometrial adenocarcinoma Ishikawa cell lines was examined using immunofluorescent imaging with the collection of z-stacks on an optical sectioning microscope and with Western blot techniques. Immunostaining of Ob-R antigen in cell monolayers of Ishikawa was observed in both the plasma membrane, and cytoplasm of cells. This finding is in line with the localisation of substantial levels of leptin receptor in the cytoplasm of the cells from clinical endometrial cancer tissue [35].

Figure 3.1 Optical sectioning images in Ishikawa cells indicating the presence of leptin receptors. Red, Leptin receptors; Blue, Ishikawa cell nuclei; Green, actin. All images are computer-generated from optical sectioning.
Confirmation of leptin receptor protein expression was achieved using Western blot immunodetection. Figure 3.2 shows the expression of Ob-Ra short form (100 kDa), and Ob-Rb long form (125 kDa). The molecular weight was confirmed by position of the bands relative to the electrophoresis ladder.

Figure 3.2 Western blot detection of leptin receptors, Ob-Rs. Protein expressions of Ob-Ra, short form 100 kDa and Ob-Rb, long form 125 kDa. GAPDH represents the reference protein.

Whether the addition of leptin to cell culture of Ishikawa cells could alter the expression of leptin receptors was then investigated. As shown in Figure 3.3, the endometrial cancer cell line Ishikawa demonstrated a significantly increased level of leptin receptor long form, Ob-Rb, in the presence of leptin at 50 ng/ml compared to the control. This result may suggest that leptin hormone could alter its own receptor in order to provide cells with the process of growth and survival.
Figure 3.3 Densitometry of the long isoform of leptin receptor, Ob-Rb, expression. Densitometry of Ob-Rb expression of controls (Ctrl), and cells treated with leptin (50 ng/ml). n = 3. Data were analyzed using logarithmic analysis and Student's t-test (two-tailed). Data were denoted as statistically significant (**) when \( p < 0.01 \) compared to controls. Data are expressed as means ± S.E.M.

3.2.2. The effect of leptin on Ishikawa cell numbers

To study the effect of hormones on proliferation of Ishikawa cell numbers, Ishikawa cells were first exposed to selected concentrations of leptin for 48 hours. After treatment cells were counted using a haemocytometer to determine total cell number.
Figure 3.4 Cell numbers of controls (Ctrl) and cells incubated with selected concentrations of leptin. n = 3. Data were analysed using one-way ANOVA. ** $p < 0.01$, and *** $p < 0.001$ compared to control. Data are expressed as means ± S.E.M.

As shown in Figure 3.4, Ishikawa cell numbers increased significantly when treated with leptin at 20 ($p<0.01$), 50 ($p<0.001$), and 100 ($p<0.001$) ng/ml. In the experimental conditions of this study, 50 ng/ml leptin was associated with the highest cell numbers, compared to 20 and 100 ng/ml. As such, at 50 ng/ml (3 nM) leptin was used in following experiments.

3.2.3. **The effect of leptin on VEGF secretion**

VEGF is a potent protein molecule that plays a key role in the formation of new blood vessels around tumours and facilitates tumour progression. Understanding the biological activity and production of VEGF is of great interest. The relationship between leptin and the secretion of VEGF from Ishikawa cells was investigated. VEGF secretion was determined in the presence of the optimum concentration of leptin (50 ng/ml) which induced the greatest cellular proliferation.
Figure 3.5 The level of VEGF secretion from control (Ctrl) and cells incubated with 50 ng/ml leptin was determined and normalised to 1 million cells. n = 4. Data were analysed using Student's t-test (two-tailed). ** p < 0.01 compared to control. Data are expressed as means ± S.E.M.

As shown in figure 3.5, the secretion of VEGF was significantly reduced in the presence of 50 ng/ml leptin. This result suggests that leptin exerts a negative effect on VEGF secretion.

3.2.4. The presence of adiponectin receptors in Ishikawa cells

As previously mentioned, leptin and adiponectin show opposite roles in observations of biological activity. Therefore, the biological activity of adiponectin in a cell culture of Ishikawa is very crucial. To better understand its role, the presence of adiponectin receptor was investigated.

The presence of adiponectin receptors was confirmed. Immunohistochemistry was performed using antibodies against AdipoR1 and AdipoR2 (Figure 3.6). Antibody specificity was confirmed using an absorption peptide control. Localisation included substantial levels of adiponectin receptors in the cytoplasm of the cell line, similarly to reports of clinical endometrial cancer tissue [35]. Images of the receptors and the blockers were added to Appendices, antibody blocker section.
This result re-confirm the presence of adiponectin receptors in cell monolayers of Ishikawa, Western blotting was used. Ishikawa cells showed strong expression of adiponectin receptors at the molecular weights 42 and 49 kDa (Figure 3.7). The molecular weight was confirmed by position of the bands relative to the electrophoresis ladder. Bands at 49 and 42 kDa belonged to AdipoR1 and AdipoR2, respectively.

As leptin can elevate the level of Ob-Rb, is it possible that adiponectin may also alter the level of its receptor? To answer this question, cell monolayers of Ishikawa were exposed to two concentrations of adiponectin (20 and 100 ng/ml) for 48 h, and cell lysates screened for
the receptor using Western blotting. The results (Figure 3.8) revealed that adiponectin, unlike leptin, did not significantly increase the level of its own receptors. This results may suggest that leptin and adiponectin regulate cell responses in a distinctive fashion.

![Figure 3.7](image1.png)  
**Figure 3.7** Protein expression of adiponectin receptor 1, AdipoR1, and adiponectin receptor 2, AdipoR2 of Ishikawa cells. The band for AdipoR1 was observed at 49 kDa and AdipoR2 was at 42 kDa. GAPDH represents the reference protein.

![Figure 3.8](image2.png)  
**Figure 3.8** Densitometry of (A) AdipoR1 and (B) AdipoR2 protein expressions for Ctrl, cells treated with adiponectin (20 and 100 ng/ml) respectively. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. Data are expressed as means ± S.E.M.
3.2.5. The effect of adiponectin on Ishikawa cell numbers

Next, the effect on cell growth of selected concentrations of adiponectin was investigated. Recombinant human adiponectin containing the globular domain (20, 50, and 100 ng/ml) exhibited a concentration-dependent effect on cell growth (Figure 3.9).

![Figure 3.9](image)

**Figure 3.9** Cell numbers of Ishikawa cells were determined after treatment with selected concentrations of adiponectin or remaining untreated (Ctrl). n = 3. Data were analysed using one-way ANOVA. **p < 0.01 and ***p < 0.001 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.

Figure 3.9 shows that an n-shaped concentration effect was observed whereby at 20 ng/ml (0.66 nM), adiponectin was, surprisingly, stimulatory and significantly increased Ishikawa cell numbers and adiponectin at 50 ng/ml also increased endometrial cancer cell numbers. However, endometrial cancer cell numbers were reduced slightly but significantly compared to control when cells were exposed to adiponectin 100 ng/ml (3.3 nM).
3.2.6. The effect of adiponectin on VEGF secretion

Next, the relationship between VEGF secretions elicited by high adiponectin concentrations (100 ng/ml), and the adiponectin concentration that is close to the minimum physiological concentration in human (20 ng/ml), were examined in Ishikawa endometrial cancer cells (Figure 3.10).

![Bar graph showing VEGF secretion from Ishikawa cells treated with 20 and 100 ng/ml adiponectin (Adp20, Adp100) and control (Ctrl).](image)

**Figure 3.10** The level of VEGF secretion from Ishikawa cells treated with 20 and 100 ng/ml adiponectin (Adp20, Adp100), and control (Ctrl). Concentrations of VEGF were determined and normalised to 1 million cells. n = 3. Data were analysed using one-way ANOVA. *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.

Figure 3.10 shows that both 20 ng/ml and 100 ng/ml of adiponectin significantly increased the secretion of VEGF from Ishikawa cells. Thus, a concentration-related effect of adiponectin similar to that on proliferation was not observed for VEGF secretion where low adiponectin, 20 ng/ml, increased cell proliferation and 100 ng/ml adiponectin concentration reduced cell proliferation.
3.2.7. The effects of adiponectin and leptin on modulation of Ishikawa cells

It has been recognised that at any stage of cancer development, endometrial cancer cells may be exposed simultaneously to more than one growth factor. Thus, the effect of the putative inhibitory growth factor, adiponectin, in combination with the stimulatory factor, leptin, was investigated. Ishikawa cells were treated with combinations of adiponectin (20 or 100 ng/ml) with leptin. Cell numbers and VEGF secretion were determined.

![Figure 3.11](image)

**Figure 3.11** Cell numbers of Ishikawa cells treated with leptin and adiponectin individually and combined. The effect of leptin at 50 ng/ml, adiponectin at 20 ng/ml (Adp20), 100 ng/ml (Adp100) and combinations were compared to individual treatments and to untreated control cells (Ctrl). n = 3. Data were analysed using one-way ANOVA. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ compared to control. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.

Figure 3.11 shows that the combination of leptin plus adiponectin (Leptin+Adp20) increased cell numbers significantly, similar to the levels of individual treatments. However, the effect of the combination of both stimulatory hormones (Leptin+Adp20) was not an addition of the two stimulatory peptides alone. However, the addition of adiponectin at its
inhibitory concentration of 100 ng/ml to leptin (stimulatory) (Leptin+Adp100) was observed to counter the leptin activity. These data, together, may demonstrate that adiponectin has potential to influence leptin activity at its inhibitory concentrations but not at its stimulatory concentration.

**Figure 3.12** The level of VEGF secretion (ng/ml) when cells were exposed to Leptin, Adp20, Adp100 and a combination of Leptin+Adp20 and Leptin+Adp100 were determined and normalised to 1 million cells. n = 4. Data were analyzed using one-way ANOVA. **p < 0.01 and ***p < 0.001 compared to control. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.

Figure 3.12 shows that in incubations containing the combination of leptin (50 ng/ml) and adiponectin at 20 ng/ml (Leptin+Adp20) did not reduce VEGF compared to those cells that were stimulated with adiponectin 20 ng/ml alone. The stimulatory effect of adiponectin 20 ng/ml on VEGF secretion remained and the inhibitory effect of leptin was largely resisted.

On the other hand, although adiponectin at 100 ng/ml increased VEGF secretion significantly from Ishikawa cells, addition of leptin (50 ng/ml) to adiponectin at 100 ng/ml (Leptin+Adp100) led to VEGF secretion from Ishikawa cells being reduced to control level. This suggests that leptin may be involved in an anti-angiogenesis process via VEGF in contrast to its proliferative activity.
The results above raised a question: were the effects of adiponectin specific to leptin or did adiponectin also affect other hormones? Thus, two other obesity-related hormones, insulin and IGF-I were also investigated.

### 3.2.8. The effect of insulin on endometrial cancer cell line, Ishikawa

The presence of insulin receptors (InsR) in Ishikawa cells has been previously documented by a number of studies [391-395]. As shown in Figure 3.13, Ishikawa cells are positive for InsR. Also, insulin receptors were demonstrated to be present in the study conditions (Figure 3.13) using immunohistochemistry. Antibody specificity was confirmed by using an absorption peptide control.

![Image of Ishikawa cells showing insulin receptors, nuclei, and actin](image)

**Figure 3.13** Optical sectioning images of Ishikawa cells showing insulin receptors (Red), nucleus (Blue) and actin (Green). All images are computer-generated from optical sectioning.
To confirm the epifluorescence images that show that Ishikawa cells have a detectable level of insulin receptors (InsR), Western blotting was used. The molecular weight of 95 kDa was confirmed by position of the band relative to the electrophoresis ladder (Figure 3.14). The cytoplasmic and nucleus immunostaining of InsR in Ishikawa cells were in agreement with clinical samples [396].

![InsR and GAPDH](image)

**Figure 3.14** Protein expression of insulin receptor (InsR) in Ishikawa cells. GAPDH represents the reference protein.

The effects of insulin on the Ishikawa cell proliferation and VEGF secretion were examined by exposing Ishikawa cells to insulin concentrations previously used which ranged between 0.58-580000 ng/ml [392, 397-399]. These concentrations were close to the physiological, nanomolar concentrations (20, 100 and 500 nM). To keep unit usage consistent the units were changed to ng/ml. Cell numbers were counted and the amount of VEGF that cells secreted into the media was determined. The final amount of VEGF was normalised to total cell numbers in order to obtain a measure of VEGF secretion per cell.
Figure 3.15 Cell numbers of Ishikawa cells were determined after treatment with selected concentrations of insulin, 116, 580, and 2900 ng/ml or control (Ctrl). n = 3. Data were analysed using one-way ANOVA. *** $p < 0.001$ compared to control. Data are expressed as means ± S.E.M.

Figure 3.15 shows increased numbers for Ishikawa cells stimulated with selected concentrations of insulin. Insulin at 580 ng/ml (100 nM) demonstrated the maximal effect on increased cell number compared to control. As such, the secretion of VEGF was also determined at this concentration (Figure 3.16), and showed that insulin at 580 ng/ml significantly reduced VEGF secretion from Ishikawa cells.
Figure 3.16 The level of VEGF secretion from Ishikawa cells treated with insulin at 580 ng/ml (Insulin). Concentration of VEGF were determined and normalised to 1 million cells. n = 4. Data were analysed using Student’s t-test (two-tailed). *** p < 0.001 compared to control. Data are expressed as means ± S.E.M.

Next, the effect of adiponectin on the modulation of Ishikawa cells by insulin was examined.
Figure 3.17 Cell numbers of Ishikawa cells were determined after treatment with insulin and adiponectin individually and combined. The effect of insulin, Adp20, Adp100 and combination of insulin 580 ng/ml, and adiponectin 20 or 100 ng/ml (Insulin+Adp20 and Insulin+Adp100) were compared to individual treatment and to Ctrl. n = 3. Data were analysed using one-way ANOVA. ** p < 0.01 and *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.

The combination of insulin (580 ng/ml) and adiponectin at 20 ng/ml (Insulin+Adp20) increased cancer cell numbers significantly compared to control. The increase was not additive of the two peptides alone, despite both hormones having stimulatory effect on Ishikawa cell numbers. This result using insulin was similar to that of leptin combined with adiponectin at 20 ng/ml (Figure 3.11).

The addition of 100 ng/ml adiponectin to 580 ng/ml insulin (Insulin+Adp100) did not reduce the stimulatory effect of insulin on cell numbers compared to the control, although it did compared to insulin alone (Figure 3.17). This data is clearly different from Ishikawa cells stimulated with the combination of Leptin+Adp100 (Figure 3.11).
Figure 3.18 The level of VEGF secreted from Ishikawa cells treated with insulin and adiponectin individually and combined. The effect of Insulin, Adp20, Adp100 and combination of Insulin+Adp20, and Insulin+Adp100 were compared to individual treatment or untreated (Ctrl). n = 4. Data were analysed using one-way ANOVA. ** \( p < 0.01 \), and *** \( p < 0.001 \) compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.

The results in Figure 3.18 show that the combination of insulin and 20 ng/ml adiponectin (Insulin+Adp20) reduced VEGF secretion from Ishikawa cells significantly compared to 20 ng/ml adiponectin alone, and were close to the level obtained by insulin alone. The combination of insulin and 100 ng/ml adiponectin (Insulin+Adp100) reduced the VEGF secretion to control levels. It is interesting that this result is similar to the effects of adiponectin 100 ng/ml on leptin (Leptin+Adp100) (Figure 3.12).
3.2.9. The effect of insulin-like growth factor-1 (IGF-I) on endometrial cancer cell line, Ishikawa

It has been suggested that IGF-I shows biological activity in a manner similar to that of insulin. Therefore, it is important to investigate the effect of adiponectin and IGF-I, both alone and in combination. Ishikawa cells have detectable level of IGF-I receptors (Figure 3.19).

Figure 3.19 Optical sectioning images of Ishikawa cells showing IGF receptors (Red), nucleus (Blue) and actin staining (Green). All images are computer-generated from optical sectioning.

Localisation included levels of IGF receptor in the cytoplasm of the cell line, similarly to observations in ovarian cancer tissue [400]. The protein band at about 130 kDa of the IGF receptor was observed using Western blotting. The molecular weight was confirmed by position of the band relative to the electrophoresis ladder.
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Figure 3.20 Protein expression of IGF-I receptor (IGFR) for Ishikawa cells. The band for IGFR was observed at 130 kDa. GAPDH represents the reference protein.

Next, growth activity of Ishikawa cells in the presence of selected concentrations of IGF-I was determined in order to choose the most effective concentration to use in the next set of experiments of IGF-I.

Figure 3.21 Cell numbers of Ishikawa cells were determined after incubation with selected concentrations of IGF-I at 20, 100 and 500 ng/ml or remaining untreated cells (Ctrl). n = 3. Data were analysed using one-way ANOVA. *** $p < 0.001$ compared to control. Data are expressed as means ± S.E.M.

Figure 3.21 shows that 20, 100, 500 ng/ml of IGF-I increased Ishikawa cell numbers significantly in comparison with the control. The normal physiological range for IGF-I in the circulation is dependent on age, and varies from 90 - 360 ng/ml for age 40-54 years old, and
71–290 ng/ml for an age older than 55 years old. Consequently, 100 ng/ml of IGF-I was used in the following treatment because it is physiologically relevant and demonstrated the maximal increase of cell numbers in Ishikawa endometrial cancer cell line. As shown in Figure 3.22, there was a significant reduction in VEGF secretion from Ishikawa cells induced by the presence of IGF-I (100 ng/ml) (13 nM).

Figure 3.22 The level of VEGF secretion from Ishikawa cells treated with IGF-I at 100 ng/ml and Ctrl. n = 4. Data were analysed using Student’s t-test (two-tailed). ** p < 0.01 compared to control. Data are expressed as means ± S.E.M.
3.2.10. The effects of adiponectin on IGF-I modulation of Ishikawa cells.

Figure 3.23 Cell numbers of Ishikawa cells were determined after treatment with IGF-I and adiponectin individually and combined. The effect of IGF-I, Adp20, Adp100 and combination of IGF-I 100 ng/ml, and adiponectin 20 and 100 ng/ml (IGF-I+Adp20 and IGF-I+Adp100) were compared to individual treatment and to Ctrl. n = 3. Data were analysed using one-way ANOVA. ** p < 0.01, and *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.

The combination of 100 ng/ml IGF-I and 20 ng/ml adiponectin (IGF-I+Adp20) abolished the stimulatory effects of both on cell numbers and reduced it to control levels. This unexpected result indicates a further, previously unrecognised complexity in the activity of low adiponectin concentrations. However, addition of adiponectin at 100 ng/ml to IGF-I (100 ng/ml) (IGF-I+Adp100) led to a reduction in cancer cell numbers but to a level that was still higher than in the control incubations.
Figure 3.24 The level of VEGF secreted from Ishikawa cells treated with IGF-I and adiponectin individually and combined. The effect of IGF-I, adiponectin at 20 ng/ml (Adp20), adiponectin at 100 ng/ml (Adp100) and combinations, and untreated control cells (Ctrl) were compared. n = 4. Data were analysed using one-way ANOVA. ** \( p < 0.01 \), and *** \( p < 0.001 \) compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.

When combined, the IGF-I inhibition effect on VEGF secretion from Ishikawa cells had no effect on the stimulatory effect of adiponectin at 20 ng/ml. The combination increased VEGF secretion compared to control. This is a result similar to the observation observed for the effect of leptin (Figure 3.12) but different to that of insulin (Figure 3.16).

The presence of IGF-I in combination with adiponectin 100 ng/ml (IGF-I+Adp100) inhibited the stimulation effect of adiponectin on VEGF secretion from Ishikawa cells and the VEGF secretion level remained similar to the control (Ctrl) levels.
3.2.11. The effects of leptin, insulin, IGF-I and adiponectin on apoptosis levels in Ishikawa cells.

The hormones may have effects on survival of the cells via modulation of the apoptotic pathways. The effects of the hormones on apoptosis levels in Ishikawa cells were also investigated individually (Figure 3.25).

![Figure 3.25](image)

**Figure 3.25** The level of apoptosis in Ishikawa cells without treatment or treated with leptin, insulin, IGF-I and adiponectin at 20 ng/ml and 100 ng/ml. n = 3. Data were analysed using one-way ANOVA. *p < 0.05 compared to control. Data are expressed as means ± S.E.M.

It was observed that although there appeared to be a small trend, albeit not statistically significant, for increased levels of apoptosis in response to all of the four hormones, only the response to 100 ng/ml adiponectin was statistically significant. It appears that modulation of apoptosis is not a major process by which the hormones alter Ishikawa cell behaviour. Further, it was noted that the hormones had no statistically significant effect on control necrosis levels (6.6%).
3.3. Discussion

This chapter presented the effects of the presence of 20 and 100 ng/ml adiponectin in combinations with leptin, insulin and IGF-I, which are considered to be tumorigenic stimulators to endometrial cancer cells. When incubated with Ishikawa cells, leptin, insulin or IGF-I individually increased proliferation in agreement with previous studies [51, 159, 401]. In incubations with these hormones there was a decline in cell number at the highest leptin concentration. The reason for this is uncertain, but the onset of significant cell demise and detachment due to inadequate nutritional support are possible. Alternatively, a negative feedback process may have become prominent. Microscopic examination revealed some overgrowth associated with the highest concentration and such structures may have occurred as a result of rapid growth with resultant alteration of overall growth dynamics. Moreover, cells that accumulate will lose the 2D, monolayer, properties of the experiments.

The effects of adiponection acting alone showed concentration-dependent, differential effect on cell growth. Thus, 100 ng/ml adiponectin decreased cell growth, consistent with previous reports [402-404]. In contrast, a lower adiponectin concentration (20 ng/ml) increased cell number. This was an unexpected result in this study, and it is the first report of stimulatory activity of adiponectin in regard to proliferation of endometrial cancer cells. It is possible to speculate that this is due to cross-talk with other stimulatory pathways [137, 379]. To understand the underlying signalling pathway that led to increase cell proliferation it is important to find a reasonable explanation for such unexpected results. Thus, this novel observation notably extends properties of adiponectin in endometrial cancer to being associated with tumorigenic as well as the previously observed anti-tumorigenic characteristic [402].

The anti-tumorigenic activity of 100 ng/ml adiponectin, although not substantial, led to the investigation of the effects of this hormone on tumorigenc hormones. The effects of interactions of adiponectin, at both its lower (growth promoting) concentration and the higher (growth restricting) concentration, with the other growth promotors leptin, insulin and IGF-I were investigated.
3.3.1. Cell numbers when leptin, insulin or IGF-I are combined with adiponectin

Only a few papers have discussed the responsiveness of tumour cells to the presence of more than one growth factor. Adiponectin was observed to reverse proliferation stimulated by leptin in endometrial cancer cell line, SPEC-2 [376]. Also, adiponectin reduced the stimulatory effect of leptin in oesophageal cancer [35, 376, 405], hepatocellular carcinoma cells [406], colon [366] and liver cancer cell [359]. However, leptin was shown to block the antiproliferative effects of adiponectin on prostate cancer cells [407]. Habeeb et al. and Li et al. concluded that adiponectin reduced the phosphorylation of Akt and PI-3K in colorectal cancer and breast cancer, respectively, leading to reduced cell proliferation [408, 409]. Moreover, a previous report suggested that AMPK activation by adiponectin blocks STAT3 phosphorylation that is important for the action of the leptin stimulation effect [410]. The effects of leptin and adiponectin on self-receptor expression were investigated. It was noted, for example, that GnRH upregulates its own receptor and there is a myriad of examples of desensitisation and downregulation of receptors. In this study, combinations of 50 ng/ml leptin and 20 ng/ml adiponectin, and 50 ng/ml leptin and 100 ng/ml adiponectin were added to endometrial Ishikawa cancer cells. The results that showed that adiponectin at 100 ng/ml suppressed the effect of leptin (Figure 3.11) were in line with other researchers' findings [359, 366, 376, 405, 408, 409].

In contrast to effects of addition of adiponectin to leptin, the combination of 100 ng/ml adiponectin with insulin (Figure 3.17) had no detectable effect on the cell numbers produced compared to either factor added individually. This might suggest that the cell number attained was the maximum possible for the conditions. This seems unlikely because further proliferation with overgrowth has been observed on occasions in other conditions. Alternatively, the two factors may act via the same pathway or two pathways that converge to a common intermediary point where capacity of the pathway is reached. With IGF-I, a partial suppression was achieved by adiponectin (Figure 3.23). In this case, it is possibly a matter of balance of concentrations that underlies the incomplete suppression.
However, when adiponectin (20 ng/ml) (in contrast to 100 ng/ml) was added to leptin (50 ng/ml), the effect of leptin was absent and the level of proliferation was similar to that associated with by adiponectin (20 ng/ml) alone (Figure 3.11). Indeed, the effect of adiponectin is strongly dependent on its concentration; whereas 100 ng/ml had an inhibitory effect on cell number, 20 ng/ml was stimulatory. In combination with leptin, the cancer cell numbers were little different from those attained by the individual hormones. This is a notable finding because previously it had been hypothesised that low levels of adiponectin and high levels of leptin, as might be present in an obese woman [209] with endometrial cancer [411, 412], would have a doubled proliferation rate. This result, from investigating the effect of a combination, suggests that might not be so.

Similarly, 20 ng/ml adiponectin added to insulin resulted in a smaller number of cells to that attained by either peptide separately. Adding adiponectin (20 ng/ml) to insulin reduced cell number compared to each hormone individually. This suggests that there may be complex cross-talk between adiponectin and insulin stimulation pathways in the endometrial cancer cell line, Ishikawa.

However, the combination of 20 ng/ml adiponectin and IGF-I reduced Ishikawa cell numbers (Figure 3.23). This may suggest that IGF-I uses a different pathway to interact with adiponectin that is different from the pathways that are used by leptin and insulin. As such this raises the intriguing question on how two stimulatory proteins can, together, have no effect on cell number. However, there are methods to formally test for additively, synergism, or antagonism (Chou-Talalay Equation), but they require more detailed measurements of responses than were made in this thesis.

3.3.2. VEGF secretion following combination of leptin, insulin or IGF-I with adiponectin

To understand how angiogenic support maintains growth of solid tumours, it is vital to determine the effects of obesity-related hormones on VEGF secretion. Thus, the three hormones (leptin, insulin and IGF-I) caused similar reductions in VEGF secretion, in spite of selective effects on cell number growth noted above. The results therefore indicate that the
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factors do not have a uniform mechanism of action on the processes (such as proliferation and VEGF-induced angiogenesis) that are believed to lead to cancer development. However, caution must be exercised in suggesting the parameters measured here are directly related to the corresponding processes in vivo.

It has been suggested that VEGF is released by cancer cells under metabolic stress as a survival mechanism for the tumour [413]. Thus, the environment in which proliferation was being suppressed by adiponectin at 100 ng/ml (whereby a cell recognises effects deleterious to its survival) elicited increased VEGF secretion. Similarly, the increase in proliferation (indicating a low stress, favourable environment) induced by the tumorigenic hormones resulted in less VEGF secretion by those cells. Nevertheless, incubation with 20 ng/ml adiponectin which induces increased proliferation was also accompanied by increased VEGF secretion. This observation may suggest that the signalling pathways for proliferation and VEGF secretion that are activated by the adiponectin ligand (at a low physiological level) diverge within the cell, such that proliferation processes have a signalling switch that turns from positive to negative with adiponectin concentration but such a concentration-related switch is not present on the VEGF secretion pathway.

In this project, an interaction between the hormones was also observed on the level of VEGF secretion. Whereas adiponectin (100 ng/ml) elicited increased VEGF secretion, the addition of leptin in combination negated the effect (Figure 3.12). Figure 3.12 shows that leptin caused a reduction in VEGF secretion. On the other hand, 20 ng/ml adiponectin increased VEGF secretion significantly. VEGF secretion from the combination showed a similar action to 20 ng/ml adiponectin and the effect of leptin was abolished and looks like it had no inhibitory effect at all. Thus, the inhibitory effect of leptin was largely resisted by the stimulatory effect of adiponectin. Thus, the interaction was the opposite effect to that seen on proliferation where increased proliferation by leptin was inhibited by adiponectin. Nevertheless, the results are consistent with the view that the two hormones have opposite influences on tumour development. To my knowledge, this is the first demonstration that 100 ng/ml adiponectin stimulates VEGF secretion, and this is reduced by combination with 50 ng/ml leptin.
It appears, also, that combination of insulin or IGF-I with adiponectin has not hitherto been investigated. Here, it is reported that insulin with adiponectin at 100 ng/ml reduced the stimulatory effect of 100 ng/ml adiponectin on VEGF secretion to control levels (Figure 3.17). Similarly, IGF-I reduced adiponectin stimulation of VEGF secretion (Figure 3.24).

VEGF secretion from Ishikawa cells in the presence of 20 ng/ml adiponectin was also determined. VEGF secretion from cells exposed to 20 ng/ml adiponectin and leptin together was increased compared to that produced by adiponectin alone (Figure 3.12). On the other hand, insulin had an inhibitory effect on adiponectin’s stimulating activity on VEGF secretion (Figure 3.18). The response pattern for IGF-I was similar to that for leptin.

When interaction of tumorigenic hormones with adiponectin was investigated, cells in most of the incubations produced the same pattern of VEGF secretion response, namely that the response to tumorigenic hormone was increased in the presence of adiponectin and, conversely, the response to adiponectin was decreased in the presence of the tumorigenic hormone. The exception was displayed by IGF-I when mixed with adiponectin at 20 ng/ml where the mixture resulted in a level of VEGF secretion that was similar to that induced by adiponectin alone, different from the response when adiponectin at the higher concentration was used. In this context it may be of oncogenic relevance that IGF-I has been observed to be at lower levels in obese patients than in lean individuals [41]. BMI reduction was independently associated with IGF-I increase. For each point of BMI reduction, the mean increase of serum IGF-I was 4.39 ng/ml.

3.3.3. Summary

Leptin, insulin and IGF-I had similar effects on cell proliferation individually, however, these similarities were disrupted when combined with adiponectin. This may suggest that leptin, insulin and IGF-I use different pathways to elicit proliferation and VEGF secretion. For example, in the first experiment, although 50 ng/ml leptin caused robust increases in cell numbers, the addition of 100 ng/ml adiponectin to leptin removed the stimulatory activity. This may have implications for drug treatments that will need to target several pathways: some of these may be modified in obese women and interact differently with adiponectin. The
combination of factors described above provided evidence of potential interactions between obesity-related hormones.

Overall, the results indicate that the factors do not have a uniform mechanism of action on the processes that are believed to lead to cancer development and progression. The effects of individual compounds were not additive when mixed, emphasising that \textit{in vitro} data must be interpreted with caution as they relate to physiology and pathology, where extra regulating factors will also be involved. The use of combinations in this experimental \textit{in vitro} study also confirmed that different signalling molecules were used by different hormones with respect to the effects on cell number and VEGF secretion. It has been suggested that in regard to the inhibitory activity of adiponectin on leptin the ratio might be more important than absolute concentrations [12, 209, 210].

The results indicate that a number of aspects (such as interactions, pathways and concentrations) must be considered when extrapolating these \textit{in vitro} results to possible \textit{in vivo} circumstances in future studies.
Chapter Four

The effect of hormones on endometrial (HEC-1A), breast (MCF-7) and ovarian (SKOV-3) cancer cell lines.
Chapter Four

4 The effect of hormones on endometrial (HEC-1A), breast (MCF-7) and ovarian (SKOV-3) cancer cell lines

4.1. Introduction

It was shown in the previous chapter that Ishikawa epithelial cells responded to four obesity-related hormones, namely leptin, insulin, IGF-I and adiponectin. These results suggest that because these hormones are dysregulated in obesity, they may contribute to the increased risk of obesity-related endometrial cancer. However, it is uncertain whether these responses are specific to Ishikawa cells. Consequently, these hormones will be tested on a range of cancer cell lines including HEC-1A, an endometrial cancer cell line with different genotype from Ishikawa, MCF-7 a breast tissue cancer cell line that is believed to be sensitive to obesity-related factors [369] and an ovarian cell line, SKOV-3, which was chosen because ovarian cancer is suggested to be, at most, weakly associated or perhaps not associated with obesity [370-372].

High leptin levels are strongly associated with breast cancer progression, and leptin caused growth potentiation in breast cell lines (ZR-1 and MCF-7) [414, 415]. Also, despite a weak association between obesity and ovarian cancer cells [370-372], treatment of ovarian cancer cell lines with leptin resulted in growth stimulation of ovarian cancer cell lines by up-regulating genes and proteins responsible for enhancing cell proliferation [44, 416]. Further, raising insulin levels may be associated with the progression and development of breast [417] and ovarian cancers [370]. Consistent with insulin activity, IGF-I was also found to promote breast [414] and ovarian cancer cells [371, 418, 419]. Additionally, there was an inverse association between circulating adiponectin levels in vivo and the incidence of breast cancer linked to obesity [380, 381, 420-422] and in postmenopausal women [403]. Further, adiponectin has been suggested to be associated with ovarian cancer [370, 423] and adiponectin levels were found to be lower in capillaries of cancerous ovaries compared to normal ovaries [210, 424].

In addition, results in Chapter 3 indicated that the Ishikawa cells showed decreased proliferation in the presence of 100 ng/ml adiponectin (Adp100). This is in line with the
consensus that adiponectin at high concentration is considered to be anti-tumorigenic. In contrast, low concentration of adiponectin (20 ng/ml) (Adp20) stimulated proliferation of Ishikawa cells. Here, the three additional cell lines mentioned above were also investigated to determine whether these cells would follow a pattern of responses similar to that which was noted for Ishikawa cells. These observations may help to explain the link between obesity and progression of different types of cancer [127, 179, 425]. Thus, the cells were exposed to the four selected hormones. The concentrations used were the same as determined for Ishikawa cells, and cell proliferation, apoptosis and VEGF secretion were determined.
4.2. Results

4.2.1. Receptors

Following the findings in Ishikawa cells, it was established by immunohistochemistry with optical sectioning microscopy that the three cell lines possessed receptors for the four hormones being studied (Figure 4.1). The information was also confirmed by Western blot (Figure 4.2).

![Images of (A) HEC-1A endometrial, (B) MCF-7 breast and (C) SKOV-3 ovarian cancer cell lines. Images show receptors for leptin (Ob-R), insulin (InsR), IGF-I (IGFR) and adiponectin (AdipoR). Red, receptors; Blue, cell nuclei and Green, actin. All images are computer-generated from optical sectioning.](image-url)
Figure 4.2 Immunodetection bands for leptin receptor (Ob-R), insulin receptor (InsR), IGF-I receptor (IGFR) and adiponectin receptors (AdipoR) for HEC-1A, MCF-7 and SKOV-3 cancer cell lines. GAPDH is the reference protein.

Figure 4.2 shows that the molecular sizes observed were consistent with what was seen before.

4.2.2. Proliferation and VEGF secretion

The same protocol that was used before with Ishikawa cells was used to study the effects on cell numbers of these cell lines. Results for HEC-1A endometrial cell line (Figure 4.3) showed that both proliferation (A) and VEGF secretion (B) generally followed the pattern exhibited previously by the Ishikawa endometrial cells except that a low concentration of 20 ng/ml adiponectin did not increase cell number as was observed in Ishikawa cells. Leptin, insulin and IGF-I all increased cell numbers of HEC-1A line but none of the hormones modified the apoptosis levels in HEC-1A endometrial cells (Figure 4.3B).
Likewise, necrosis in the cells was not affected by culture with any of the hormones and was similar to that in control (3.7%).

VEGF secretion by HEC-1A cells following exposure to the four hormones was similar to the previously observed pattern on the Ishikawa cells (Figure 3.5, Figure 3.10, and Figure 3.22) whereby the growth-promoting hormones leptin, insulin and IGF-I caused less VEGF secretion and adiponectin increased secretion.

**HEC-1A**

![Figure 4.3](image)

Figure 4.3 (A) cell numbers, (B) apoptosis and (C) VEGF secretion from endometrial cancer cell line (HEC-1A) cells treated with selected hormones. Leptin 50 ng/ml (Leptin), insulin 580 ng/ml (Insulin), IGF-I 100 ng/ml (IGF-I), adiponectin at 20 ng/ml (Adp20) and 100 ng/ml (Adp100). n = 9. Data were analysed using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.
Leptin and IGF-I induced higher cell numbers for MCF-7 cells compared to controls. However, insulin had no effect on MCF-7 cell proliferation. It can be noted that the cell line, nevertheless, possessed insulin receptors (Figure 4.1B and Figure 4.2). Adiponectin had no

Figure 4.4 (A) cell numbers, (B) apoptosis and (C) VEGF secretion from breast cancer cell line (MCF-7) cells treated with selected hormones. Leptin, insulin IGF-I, Adp20 and Adp100. n = 9. Data were analysed using one-way ANOVA. ** p < 0.01 and *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.
notable effect on this cell line. It is interesting to note that apoptotic MCF-7 cells were increased after exposure to all four hormones (Figure 4.4B). Additionally, there were no changes in necrosis in cells cultured with leptin or insulin compared to control (11.75%). However, an increase in necrosis (17.55%) was observed with IGF-I, while necrosis decreased with adiponectin at both 20 and 100 ng/ml (6.0%, 7.0%).

The VEGF secretion response in MCF-7 cells was hormone dependent (Figure 4.4C). Again, leptin decreased VEGF secretion whereas both insulin and IGF-I caused increased levels of VEGF secretion. Despite MCF-7 cells having measurable level of adiponectin receptors, adiponectin, at either 20 ng/ml or 100 ng/ml had no effect on the VEGF secretion response (Figure 4.4C).

SKOV-3 exhibited a pattern markedly different from those of HEC-1A, MCF-7 or Ishikawa (Figure 4.3, Figure 4.4, Figure 4.5, Figure 3.4 and Figure 3.5). In spite of all four hormone receptors being present, there was no enhanced proliferative response to the four hormones (Figure 4.5C). Adiponectin, the hormone that has been associated with anti-tumorigenic activity, increased proliferation at 100 ng/ml although adiponectin at 20 ng/ml was without effect. None of the hormones incubated with SKOV-3 cells significantly modified apoptotic levels (Figure 4.5B) and nor were there changes in necrosis from control (5.5%). Insulin and IGF-I significantly increased secretion of VEGF. Adiponectin at both concentrations did not elevate secretion of VEGF.
SKOV-3

Figure 4.5 (A) cell numbers, (B) apoptosis and (C) VEGF secretion from ovarian cancer cell line (SKOV-3) cells treated with selected hormones. Leptin, Insulin, IGF-I, Adp20 and Adp100. n = 9. Data were analysed using one-way ANOVA. * \( p < 0.05 \), and *** \( p < 0.001 \) compared to control. Data are expressed as means ±S.E.M. The data are identical to previous figure.
4.3. **Discussion**

4.3.1. **HEC-1A - Endometrial cancer cell line**

The increase in HEC-1A cancer cell numbers in response to leptin was about half the number that was previously observed by Ishikawa cells. This suggests that HEC-1A cells are less sensitive to leptin stimulation than Ishikawa cell line. One possibility is that PTEN, a cancer suppressor, is a modulator of leptin activity and exhibits more suppressive activity in HEC-1A cells which is PTEN wild type than in Ishikawa cells which has a PTEN mutation [331]. Other factors, such as Ishikawa cells having half the apoptotic response compared to HEC-1A, however, clearly cannot be excluded from consideration. The semi-quantitative immunohistochemistry observations suggest the difference is not related to expression of leptin receptors.

The results are in agreement with other studies in which it was observed that insulin increased HEC-1A cell numbers [426-428], which is possibly related to over-expression of insulin receptor (InsR) in HEC-1A cells [426]. These results are also consistent with reports that insulin has been shown to exhibit an important impact on endometrial carcinoma cells [429] and insulin promoted endometrial carcinoma cell proliferation [430]. Also, it was noted that HEC-1A responded to IGF-I as did the other endometrial cell line, Ishikawa. The increase in HEC-1A cell numbers elicited by culture with IGF-I parallels that previously observed by Bermont et al. with IGF-I (13 nM) [431].

On the other hand, the HEC-1A endometrial cells behaved differently to those of Ishikawa cells in the presence of adiponectin. HEC-1A cell growth was suppressed by adiponectin at 20 ng/ml whereas that concentration of adiponectin provoked stimulation of Ishikawa cells (Figure 3.9). Similarly, growth was inhibited by adiponectin at 100 ng/ml (Figure 3.9). It is possible to speculate that mutational differences underlie the different response to adiponectin, compared to that of Ishikawa cells [150]. Recently, a study characterising Ishikawa and HEC-1A cells revealed that they differ greatly in gene expression and in their capacity for E2 formation during women’s reproductive years, and thus they represent different in vitro models [432]. A direct effect of adiponectin at a higher
concentration (10-40 µg/ml) on HEC-1A cell line via action on the cell cycle *in vitro* has been previously observed [150]. It is also possible that adiponectin at 100 ng/ml may inhibit proteins which are responsible for regulation of cell cycle phase in both Ishikawa and HEC-1A cell lines.

The results revealed that increased proliferation in HEC-1A cells was associated with reduced VEGF secretion and *vice versa* (Figure 4.3B). Interestingly, at the concentrations used, the decreased stimulation of proliferation rate by leptin and higher proliferation rate by insulin and IGF-I were associated with converse relative levels of VEGF secretion.

### 4.3.2. MCF-7 - Breast cancer cell line

The relationship between circulating leptin levels and breast cancer shows a positive association with cancer risk, and it has been suggested that leptin has an independent role in breast tumorigenesis [433]. Nevertheless, other researchers have concluded that it is difficult to explain the progression of breast cancer on the basis of serum leptin concentrations alone [434-436]. A recent study concluded that leptin may enhance breast cancer by enhancing the expression of several signalling pathways including JAK/STAT3, Erk1/2 and oestrogen that are responsible for increased progression in obese patients breast cancer [437].

Although no effect of insulin on MCF-7 growth was found, it has been suggested that insulin increases breast cancer growth through the modulation of the cell cycle, apoptotic factors and nutrient metabolism [438, 439]. However, those other studies used a concentration of insulin at 10 µM, which is 100 fold higher than the concentration used here. The observation of increased numbers of MCF-7 cells in the presence of IGF-I is in agreement with other investigators [440-443].

There was no effect on MCF-7 growth by adiponectin. However, Pfieler et al. suggested that there is a proliferative effect of adiponectin on breast cancer cells and that the effects of adiponectin on proliferation were oestrogen-dependent [444]. However, using oestrogen-deficient medium (as in this study) reduced this possibility of interactions.
Previously, an inhibitory activity of adiponectin on MCF-7 has been reported [382, 445]. It has been noted that circulating adiponectin levels are inversely correlated with the incidence of breast cancer [169, 180, 380, 381, 420, 421] and the development of breast cancer [169]. High adiponectin concentrations correspond with reduced proliferation of breast cancer cells in the \textit{in vivo} milieu [169, 180, 380, 420, 421] where indirect effects such as those seen, for example, on Ishikawa cells (Figure 3.9) can occur.

The patterns of VEGF secretion of MCF-7 cells did not always follow that of the other cell lines. The response to leptin was orthodox; insulin, while not promoting or suppressing proliferation, provoked increased VEGF secretion; and, IGF-I induced increased VEGF secretion that was associated with enhanced proliferation. Interestingly, there was no change in either proliferation or VEGF secretion in the presence of adiponectin.

4.3.3. \textbf{SKOV-3 - ovarian cancer cell line}

The results obtained with SKOV-3 cells are in line with others who observed that these cells are not affected by the presence of leptin [164] in adherent monolayer cell cultures [374, 446]. Some studies have reported that leptin and Ob-R are increased in ovarian cancer, and are associated with poor progression-free survival [374, 446]. On the other hand, in studies using a substantially different culture model, namely floating cell aggregates of SKOV-3 cells, leptin was observed to promote proliferation and growth [374, 447].

Although there was no response to insulin at the same concentration that promoted proliferation in the other cell lines, insulin induced an increase in SKOV-3 cell numbers when they were grown in a different medium (DMEM-F12) [419]. The discrepancy of these results may be due to the differences in cell culture media and possibly the different glucose concentration in the medium.

Recently, a positive role of IGF-I on progression of ovarian cancer in \textit{in vivo} nude mice and patient tissue samples was also documented [448, 449].

Adiponectin, the hormone in this group that has most commonly been associated with anti-tumorigenic activity, increased proliferation at 100 ng/ml although adiponectin at 20
ng/ml was without effect. The concentrations used here again reveal the potential for adiponectin to have tumour-promoting activity when there are conditions, perhaps such as poor vascular supply, that are associated with cell stress. The link between ovarian cancer and adiponectin remains uncertain. A case-study concluded that BMI is higher in ovarian cancer patients and that adiponectin is lower [450] and a link to ovarian cancer has been suggested [370], although it has also been suggested that the leptin/adiponectin ratio may be more pertinent [12, 209, 210] and it may be important in the hormone effects measured.

These responses to leptin, insulin and IGF-I that were observed are consistent with the observations in some studies [372] that ovarian cancer risk is not increased by obesity although a meta-analysis suggests that there is an associated risk [370, 423, 451] which may be weak [371]. The cell proliferation response by SKOV-3 cells to adiponectin at 20 ng/ml and 100 ng/ml suggests an opposite response to, say, Ishikawa endometrial cells, in that the hitherto unrecognised stimulatory activity of adiponectin was exhibited, but at a higher concentration of adiponectin on SKOV-3 than on Ishikawa cells.

There was an increased response to insulin in VEGF secretion (relative to proliferation) in SKOV-3 cells despite the fact that no change in proliferation was observed. This may be due to insulin’s role in facilitating the movement of energy molecules, leaving SKOV-3 cells in nutrition deficit. This might lead to increased VEGF secretion despite there being no effects on cell proliferation. VEGF secretion by cells cultured with adiponectin exhibited the usual reversal relative to proliferation that was commonly observed during this project.

4.3.4. Summary

In overweight patients, low adiponectin levels have been observed to be strongly associated with increased cancer cell numbers [452] and in adipose tissue VEGF production was increased and adiponectin apparently has a direct inhibitory effect on VEGF secretion and angiogenesis in endothelial cells [453]. In contrast, other studies have found that adiponectin has a stimulatory effect on human umbilical vein endothelium cell (HUVEC) growth and VEGF secretion [379]. Thus, from these findings, it may be suggested that adiponectin action is cell-type dependent.
Further emphasising the complexity of the hormone systems is the variation of responses when a variety of cell lines were studied. Thus, there was a cell-type dependent component to the effects of the hormones. It is uncertain whether this is a result of the genotype and/or whether there are other tissue-specific factors. The former seems more likely to at least partly explain details of the responses. This is because Ishikawa cells and HEC-1A cells, both endometrium-derived, exhibited differences in response to, for example, 20 ng/ml adiponectin. Extrapolation of these results may indicate that the regulation of tumour development in vivo will be a complex matter affected by concentrations of numerous growth factors as well as other oncogenic entities.

The roles of obesity in generating the levels of these compounds and modulating their activities are fundamental to understanding the increased risk of endometrial cancer, and other cancers, for overweight individuals.

This is the first study to use a series of cell lines with a group of hormones that are dysregulated in obesity. The study design revealed, for the first time, variations in overall response patterns to the hormone factors and differences between cell lines. It can be noted that the results parallel clinical observations. In particular, these obesity-related hormones did not affect the proliferation in the ovarian cell line (SKOV-3) but did so in the other two.

Whether some of the changes in cell number might have been a result of the hormones modifying the level of apoptosis was examined. Similarly to Ishikawa cells (Chapter 3), cells of the other endometrial-derived cell line, HEC-1A, also lacked a robust apoptosis response to the hormones. In regard to apoptosis levels, MCF-7 was the most responsive of the cells studied. All four hormones, including adiponectin and both 20 and 100 ng/ml, induced increased apoptosis in the breast cells compared to control cells. In concert with the cell number response, SKOV-3 cells did not significantly modify apoptotic behaviour in the presence of the four hormones, although mean values were arithmetically varied. Thus, in general, it may be suggested that the apoptosis responses of cancer cells are at least partly dependent on factors other than the obesity-related hormones in the panel studied here.

The patterns of VEGF secretion as they relate to proliferation in the same treatment incubation with one of the obesity-related hormones are noteworthy. In the 20 treatments (i.e.
Chapter Four

4 cell lines – Ishikawa, HEC-1A, MCF-7, SOKV-3, and 5 hormone treatments - leptin, insulin, IGF-I, adiponectin 20 ng/ml and adiponectin 100 ng/ml) a qualitative categorisation (i.e. increase or no change or decrease induced by a hormone treatment) was made for each response of a pair of proliferation and VEGF secretion responses. Approximately three-quarters (14/20, 70%) were inverse or absent (8 were [proliferation *INCREASED* & VEGF secretion *DECREASED*]; 2 were [proliferation *DECREASED* & VEGF secretion *INCREASED*]; 4 were [NO CHANGE & NO CHANGE]) (Table 4.1).

<table>
<thead>
<tr>
<th>VEGF secretion</th>
<th>Increase</th>
<th>2</th>
<th>3</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No change</td>
<td></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease</td>
<td>1</td>
<td></td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

| Cell Number    | Decrease | No change | Increase |

Table 4.1 The Table notes the number of pairs of responses regarding the the *direction of change* in cell number related to the concurrent effect on VEGF secretion. The numerals are numbers of pairs with the designated relationship as explained in the text. Note the more than 2-fold predominance of pairs (in bold) that have no change for both parameters or opposite direction of change ((2+4+8=14), compared to pairs with similar direction of change (3+2+1=6). Cochran-Mantel-Haenszel test: p=0.033

These responses were consistent with a previous observation that a sensed deficiency in the cell (metabolic reduction of proliferation by oxaliplatin) induced a compensatory response such as increased VEGF secretion [413]. Conversely, a low level of drug (oxaliplatin) that had low chemotoxicity resulted in less cellular secretion of the survival factor, VEGF [454]. The observations in this study were consistent with that notion and expand the concept to noting that cancer cells can also recognise a stressful condition associated with hormone ligands and modulate the survival response accordingly (Cochran-Mantel-Haenszel test: p=0.033). It is plausible that conservation of energy underlies this relationship [455]. However, exceptions were observed and there were six pairs that did not fit this pattern (3 were [proliferation *NO CHANGE* & VEGF secretion *INCREASED*], and 2 were [proliferation *INCREASED* & VEGF secretion *INCREASED*] and one was [proliferation *DECREASED* & VEGF...
secretion DECREASED] which indicates that predicting clinical outcomes will require complex modelling of VEGF control [456, 457].

This project reports observations that obesity-related hormones can act directly at the level of the cancer cell. At times, there is conflict between conclusions from in vivo monitoring and in vitro culture effects. Such observations may be partly due to paracrine pathways that function in cancer development [458]. In such cases, the circulating peripheral blood levels are of secondary relevance. Additionally, full conclusions cannot be drawn from the simple response to the hormone alone. The potential for interactions between hormones was documented in the previous chapter. Also, it has been observed that leptin can regulate breast cancer initiation, growth and metastatic progression via interaction with various growth factors including oestrogen, insulin, IGF-I, angiogenic pathways and inflammatory cytokines [459]. This implies caution should be shown in extrapolating unmodified interpretations to physiological conditions. Further, growth, apoptosis and VEGF secretion as targets for the hormones were determined, but other functions may also be affected, such as metastatic potential and the risk of recurrence [386, 460].

Thus, the results point to tissue type or cell line-dependent oncogenic potential of the studied hormones i.e. leptin, insulin, IGF-I and adiponectin, that may be related to genetic variability between the cell lines or pathophysiological organs. It must be acknowledged that the results found in the in vitro cell lines experiments may not always translate directly to the in vivo models. In this study, some preliminary data were obtained suggesting that hormones that are affected by obesity will influence cancer cells in definite conditions. It is noteworthy that these cell lines responded non-identically to the obesity-related hormones used in this study.
Chapter Five

Leptin signalling pathways that modulate proliferation in Ishikawa cells
Chapter Five

5 Leptin signalling pathways in modulating proliferation in Ishikawa cells

5.1 Introduction

Leptin exerts its biological actions through the activation of its receptors. Leptin receptors (Ob-Rs) belong to the class I cytokine receptor super-family and their signal transduction pathways are associated with the phosphatidylinositol 3-kinase (PI-3K) / protein kinase B (Akt) / mammalian target of rapamycin (mTOR) pathway [461] and the Janus kinase family and signal transducers and activators of transcription-3 (JAK/STAT3) pathway [462]. The regulation of these pathways [463, 464] by leptin with regard to proliferation in an endometrial cancer cell line (Ishikawa) was investigated in this chapter.

Signalling activity of JAK/STAT3 is triggered by the binding of leptin to its cell membrane receptors. This leads to receptor dimerisation, which appears mandatory to complete the signalling process [465, 466]. Cytoplasmic tyrosine kinases' expression of the JAK/STAT system are induced by the activation of class I cytokine receptors [72, 467].

Homodimerization of leptin receptors occurs after the ligand, leptin, binds to the extracellular domain of the receptor (Ob-Rb). Ob-Rb then activates the JAK/STAT3 pathway [45] with the binding of leptin to Ob-R leading to phosphorylation of tyrosine amino acid residues at the JAK domain inside the cell. The phosphorylated tyrosine sites act as docking locations for the SH2 and SH3 domains of the STATs [468, 469]. After binding to the phosphorylated intracellular domain site of JAKs STAT3 protein becomes phosphorylated forming p-STAT3. After activation, STAT3 proteins dimerise and move to the nucleus [470]. Intranuclear STAT3 recognises STAT3-specific DNA-binding elements [471] and binds to target gene(s) [338] which eventually leads to leptin-induced increase of cell proliferation in endometrial cancer [470, 472].

The PI-3K/Akt/mTOR pathway has been linked to the pathophysiology of several neoplastic diseases [461, 473]. Activation of this pathway can be a result of defects in PI-3K or Akt genes, inactivation of phosphatase and tensin homolog (PTEN), or basal activation of
upstream regulatory steps such as receptor tyrosine kinases [461, 473]. *PI-3K* gene mutations have been reported in type I endometrial cancer [473, 474] and data from genomic studies support activation of the PI-3K pathway in endometrial cancer [331]. *In vitro* pre-clinical studies have also reported that endometrial cancer cell proliferation inhibition can be interrupted by PI-3K pathway stimulation [475]. Clinical trials using target agents have produced results that support a role for PI-3K/Akt/mTOR mediated signalling in endometrial carcinoma [476, 477].

It is well established that leptin uses the PI-3K/Akt/mTOR pathway to increases cancer cell proliferation. Hence, PTEN can act as cancer suppressor by converting PIP3 to PIP2, and thus it acts as a modulator of leptin activity and exhibits more suppressive activity in HEC-1A cells where PTEN is wild type and active than in Ishikawa cells in which has PTEN is mutated and inactive. During leptin activation, PTEN acts as facilitator in phosphorylation of Akt and increases cell proliferation via the PI-3K/Akt/mTOR pathway [476, 477].

Although many articles treat these pathways as separate but parallel, some suggest that these two pathways, PI-3K/Akt/mTOR and JAK/STAT3, may overlap [53, 164, 373, 375]. Thus, there may be potential for cross-talk between these two pathways in endometrial cancer and such activity might then have implications for prevention or treatment of endometrial cancer.

In this project, it was previously demonstrated that leptin, an obesity-related hormone, modulated Ishikawa cell behaviour. This chapter, therefore, will investigate the hypothesis that leptin stimulates the activation of both PI-3K/Akt/mTOR and JAK/STAT3 pathways, and that both pathways are involved in leptin activation and modulation of cell growth in Ishikawa cell line.
5.2. Results

To study whether the effects of leptin on stimulating cell growth utilise PI-3K/Akt/mTOR signalling cascade, the PI-3K inhibitor LY294002 was used to probe the pathway. After performing preliminary studies to determine the optimum concentration for inhibitor, an LY294002 concentration of 20 μM was selected for these experiments.

![Graph](A) cell numbers and (B) VEGF secretion in Ishikawa cells of untreated cells (Ctrl), cells treated with PI-3K inhibitor, LY294002 (20 μM), leptin 50 ng/ml (Leptin), and a combination of leptin and LY294002 (Leptin+LY294002). n = 3. Data were analysed using one-way ANOVA. ** p < 0.01 and *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.

As shown in Figure 5.1, and consistent with results in chapter 3, leptin alone significantly increased cell numbers of the Ishikawa cell line. However, LY294002 significantly reduced cell numbers of leptin-treated Ishikawa cells compared to leptin alone. Although without leptin stimulation, LY294002 alone did not show a measurable effect compared to controls (Figure 5.1A). Secretion of VEGF from controls and LY294002 alone were not different. Leptin alone significantly reduced VEGF compared to the control.
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However, the combination of leptin and LY294002 did not show a further reduction of VEGF compared to cells stimulated by leptin alone (Figure 5.1B). These data suggest that leptin activates growth activity in the Ishikawa cell line via a PI-3K molecule, but not alteration of VEGF secretion.

The regulation of VEGF associated with the activity of adenosine mono phosphate kinase (AMPK) is well described [478, 479]. Thus, the activation of leptin on AMPK may indirectly regulate VEGF secretion. Thus, the expressions of AMPK and p-AMPK in the presence of leptin were also investigated with Western blots (Figure 5.2) that were quantified by densitometry (Figure 5.3).

![Figure 5.2](image.png) Protein expression of AMPK and p-AMPK for untreated cells (Ctrl) and cells treated with leptin (50 ng/ml). GAPDH is the reference protein.

![Figure 5.3](image.png) Densitometry of (A) AMPK and (B) p-AMPK expression for untreated cells (Ctrl), and cells treated with leptin (50 ng/ml). n = 3. Data were analysed using logarithmic analysis and Student's t-test (two-tailed). Data are expressed as means ± S.E.M. The data are identical to previous figure.
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As shown in Figure 5.3, leptin stimulation of Ishikawa cells did not change total expression of AMPK or phosphorylated AMPK (p-AMPK) protein. This result indicates that in these circumstances AMPK may not be associated with leptin-activated pathways.

Next, the expression and activities of the protein kinase B, Akt in the signalling pathways were investigated to determine whether the reduction in cell numbers induced by LY294002 was associated with the phosphorylation and activation of Akt. This possibility was investigated by assessing the total protein expression of Akt and the phosphorylated form (p-Akt) in the absence and presence of LY294002 using Western blots and densitometer measurement of the bands.

Figure 5.4 Akt and p-Akt protein expression for untreated cells (Ctrl), Ishikawa cells treated with LY294002 alone, cells treated with leptin (50 ng/ml) (Lep50), and cells treated with a combination of leptin and LY294002 (20 µM) (Lep50+LY20). GAPDH is the reference protein.

Figure 5.5 Densitometry of (A) Akt and (B) p-Akt protein expression for untreated Ishikawa cells (Ctrl), LY294002, Leptin, and a combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.
As shown in Figure 5.4 and Figure 5.5A, leptin increased the total expression of Akt, and this effect was inhibited by the presence of LY294002.

Similarly, the level of p-Akt was increased in the presence of leptin and significantly reduced by the PI-3K inhibitor LY294002 (Figure 5.4 and Figure 5.5B). The results suggest that PI-3K mediation of the leptin-stimulation pathway via Akt proteins is likely to be crucial.

Whether the effect of leptin on cell proliferation is also mediated through the mTOR, a downstream protein from the PI-3K/AKT mediated route, was then determined. To ascertain this role, an mTOR blocker, everolimus [480] was introduced at a concentration of 2 µM to leptin-stimulated Ishikawa cells. As shown in Figure 5.6, everolimus significantly reduced the cell number of leptin-treated cells but it did not demonstrate a similar effect on non-stimulated cells. Overall, data may suggest a classical PI-3K/Akt/mTOR pathway triggered by the stimulation of leptin is essential for cell proliferation of Ishikawa cell line.

**Figure 5.6** Cell numbers were determined after treatment with mTOR inhibitor, everolimus (Everolimus) (2 µM), leptin 50 ng/ml (Leptin), and a combination of leptin and everolimus (Leptin+Everolimus) or remained untreated cells (Ctrl). n = 3. Data were analysed using one-way ANOVA. **p < 0.01, and ***p < 0.001 compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.
To ascertain the effects of mTOR inhibitor on Akt protein activation and phosphorylation, the expression of Akt and p-Akt proteins in the absence and presence of mTOR inhibitor, everolimus, and leptin were investigated. As shown in Figure 5.7, Ishikawa cells did not exhibit any difference in Akt and p-Akt protein expression in the presence of everolimus compared to Akt and p-Akt protein expression for leptin alone (Figure 5.7). These data suggest that mTOR is a downstream protein of the PI-3K/Akt activated pathway acting after Akt.

![Figure 5.7](image)

**Figure 5.7** Densitometry of (A) Akt and (B) p-Akt protein expression for untreated Ishikawa cells (Ctrl), everolimus, leptin, and a combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. **p < 0.01 and ***p < 0.001 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.

It is well known that leptin can activate JAK/STAT pathway and elicit cell growth. To investigate the effect of leptin on Ishikawa cancer cell growth via the JAK/STAT3 pathway, the JAK inhibitor Tyrphostin AG490 [481] was employed.
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Figure 5.8 (A) cell numbers and (B) VEGF secretion in Ishikawa cells of untreated cells (Ctrl), cells treated with Tyrphostin AG490 (100 μM) and Leptin alone, Leptin+JAK inhibitor, Tyrphostin AG490, at 1 μM (Leptin+Tyrphostin1), 10 μM (Leptin+Tyrphostin10) and 100 μM (Leptin+Tyrphostin). n = 3. Data were analysed using one-way ANOVA. ** p < 0.01, and *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.

As shown in Figure 5.8A, leptin increased cell numbers compared to the control. Tyrphostin AG490 (100 μM) alone had no effect on growth of Ishikawa cells. Tyrphostin AG490 significantly reduced leptin-stimulated growth potential in a concentration-dependent manner with 100 μM Tyrphostin AG490 producing the largest reduction in Ishikawa cell number, an effect which was significant compared to cell numbers in the presence of leptin alone and control incubations. For this reason, 100 μM Tyrphostin AG490 was used in the following experiments.

These results showed that, along with the PI-3K/Akt/mTOR pathways, leptin is also able to trigger cell proliferation of Ishikawa via the JAK protein. It is also important to note that Tyrphostin AG490 alone, or acting in combination with leptin, does not impact secretion of VEGF from Ishikawa cells (Figure 5.8B). It is plausible to hypothesise that leptin
stimulates cell proliferation by using the JAK protein, which consequently activates Akt as well as STAT3 the downstream protein.

To investigate the possibility of a link between the JAK and PI-3K/Akt/mTOR pathways, the expression of PI-3K pathway intermediate molecules such as Akt and p-Akt were examined in the presence of JAK inhibitor Tyrphostin AG490.

Figure 5.9 Akt and p-Akt protein expression of untreated cells (Ctrl), Ishikawa cells treated with Tyrphostin AG490 (Tyrphostin) (100 µM), cells treated with leptin (50 ng/ml) (Leptin) alone, and cells treated with a combination. GAPDH is the reference protein.

Figure 5.10 Densitometry of (A) Akt and (B) p-Akt protein expression for untreated Ishikawa cells (Ctrl), Tyrphostin AG490 (JAK inhibitor), Leptin, and a combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.
Figure 5.9 and Figure 5.10A show significant increases in total Akt protein expression in the presence of leptin compared to the control while a significant reduction in Akt protein expression occurred when cells were exposed to a combination of leptin and Tyrphostin AG490 compared to incubation with leptin alone. Moreover, addition of inhibitor alone also led to the reduction of Akt protein expression.

Similarly, as shown in Figure 5.9 and Figure 5.10B, p-Akt increased significantly in the presence of leptin. Exposing Ishikawa cells to the JAK inhibitor Tyrphostin AG490 alone or in combination with leptin reduced p-Akt significantly compared to p-Akt protein expression of control and incubation with leptin, respectively. This data may suggest that Akt protein was also stimulated via the JAK protein, and is therefore a potential mediator that links PI-3K/Akt interaction with the JAK activation pathway.

It is well established that the STAT3 protein is a downstream regulatory molecule from JAK. In order to confirm the effect of the stimulation of JAK molecule on STAT3, the protein expression of STAT3 and p-STAT3 in the presence of Tyrphostin AG490 was also studied.

<table>
<thead>
<tr>
<th>STAT3</th>
<th>91 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-STAT3(Ser727)</td>
<td>91 kDa</td>
</tr>
<tr>
<td>GAPDH</td>
<td>37 kDa</td>
</tr>
<tr>
<td>Leptin 50 ng/ml</td>
<td>-</td>
</tr>
<tr>
<td>Tyrphostin 100 µM</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 5.11** STAT3 and p-STAT3(Ser727) protein expression in Ishikawa cells treated with Leptin (50 ng/ml) and Tyrphostin AG490 (100 µM) alone, and a combination. GAPDH is the reference protein.
Figure 5.12 Densitometry of (A) STAT3 and (B) p-STAT3(Ser727) protein expression for untreated Ishikawa cells (Ctrl), Tyrphostin AG490, Leptin, and a combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to controls. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.

Figure 5.11 and Figure 5.12A show significant reductions in total STAT3 protein expression in the presence of Tyrphostin AG490 compared to leptin-treated cells and the control. Additionally, as shown in Figure 5.11 and Figure 5.12B, a reduction in phosphorylated STAT3 protein occurred after Ishikawa cells were exposed to the combination of leptin and Tyrphostin AG490 compared to Ishikawa cells treated with leptin alone. Figure 5.12A also shows that Tyrphostin AG490 inhibits basal STAT3 expression and leptin-induced STAT3 to the same level. In addition, Figure 5.12B shows that there is a significant reduction of leptin-induced phosphorylation compared to leptin alone.

Next, it was determined whether the PI-3K pathway is inhibited by LY294002 when the JAK/STAT3 pathway was still active and STAT3 is expressed. Alternatively, if LY294002 inhibits total STAT3 expression, then PI-3K may have a direct stimulatory effect on STAT3 and p-STAT3 as different from stimulation by JAK.
Figure 5.13 STAT3 and p-STAT3 expression in Ishikawa cells treated with Leptin (50 ng/ml) and PI-3K inhibitor LY294002 (20 μM) alone, and a combination. GAPDH is the reference protein.

Figure 5.14 Densitometry of (A) STAT3 and (B) p-STAT3(Ser727) protein expression for untreated Ishikawa cells (Ctrl), LY294002, leptin, and a combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to controls. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.

Figure 5.13 and Figure 5.14A show an increased expression of STAT3 in leptin-treated cells compared to control. Similarly, Figure 5.13 and Figure 5.14B show an increase in STAT3 phosphorylated at Ser727 in the presence of leptin compared to control. Addition of LY294002 reduced the effect of leptin. Further, the p-STAT3 amount is reduced to less than
control. These data may suggest that, leptin-induced cell proliferation in Ishikawa cells activates a complex interaction of the PI-3K/Akt/mTOR with the JAK/STAT proteins.

5.3. Discussion

In these experiments, it was demonstrated that leptin-stimulated cell growth of the endometrial cancer cell line Ishikawa was inhibited by both a PI-3K inhibitor and also by a JAK inhibitor. This suggests that leptin induces cell growth in Ishikawa cells through two different pathways, PI-3K/Akt/mTOR and JAK/STAT3. The proposed pathways are shown in diagrammatical form in Figure 5.15, constructed on the basis of the observations in this chapter. The pathways show JAK as the upstream molecule for PI-3K/Akt/mTOR pathway as well as for STAT3 in Ishikawa cancer cells.
Figure 5.15 Proposed pathways involved in the action of leptin on regulation of proliferation of Ishikawa endometrial cancer cells. (A) before addition of inhibitors and (B) after the addition of the inhibitors. The proposed mechanism involves two pathways, the PI3K/Akt/mTOR pathway (in red) and the JAK/STAT3 pathway (in green). The points of activity of the pharmacological modulators used in this study are shown in black. The observations are consistent with a JAK/PI-3K/STAT3 signalling axis in endometrial cancer cells.
Leptin exerts its effect on cancer cells via activation of specific pathways inside the cell leading to increased DNA transcription in the nucleus and increased proliferation and tumour growth. PI-3K/Akt/mTOR and JAK/STAT3 are major downstream pathways which have been identified as being involved in leptin action during endometrial cancer cell proliferation [461, 462, 482].

The first pathway is PI-3K/Akt/mTOR which is well known for its hyperactivation in many cancers. In order to investigate the involvement of PI-3K/Akt/mTOR pathway in leptin's stimulatory effect [461, 482] on endometrial cancer cell growth, the PI-3K inhibitor LY294002 was used. Addition of LY294002 to leptin significantly reduced endometrial cancer cell numbers compared to leptin alone (Figure 5.1) which is consistent with the decline in phosphorylated form of Akt (p-Akt) compared to leptin alone (Figure 5.4 and Figure 5.5B). The results suggest that PI-3K and Akt are key proteins in leptin-induced growth stimulation which is in line with findings of others [373, 461, 473, 483].

To ascertain the role of mTOR activation that followed PI-3K and Akt activation, the mTOR inhibitor everolimus was used. Everolimus did not modulate cell numbers in non-treated control incubations (Figure 5.6). This suggests that Ishikawa cells may use JAK/STAT3 or another pathway to maintain basal proliferation. However, addition of everolimus to leptin led to a reduction in leptin’s stimulatory effect on cells and this suggests that mTOR protein is also a downstream step that is associated with activation of the PI-3K/Akt/mTOR pathway by leptin (Figure 5.6). However, the presence of mTOR inhibitor everolimus did not affect the leptin-induced activation and phosphorylation of Akt protein significantly (Figure 5.7) but did alter cell numbers (Figure 5.6). This confirmed that Akt is upstream from mTOR. Everolimus is known to inhibit the phosphorylation of mTOR, and so inhibit cell proliferation. These findings are similar to published data and together suggest that leptin initiates a cell signalling cascade in endometrial cancer cells that causes tumour progression via the stimulation of PI-3K/Akt/mTOR pathway [373, 374, 461, 473]. In addition, the JAK/STAT3 pathway can be activated by leptin. The binding of leptin to its receptor leads to recruitment of JAK protein that binds to the intracellular domain of the leptin
receptor. JAK becomes phosphorylated and that in turn phosphorylates STAT3 to p-STAT3 which dimerises with another STAT3 and moves to the nucleus to initiate a transcription process [45, 468-470, 472, 484].

To ascertain if this pathway is involved in the growth of endometrial cancer cells in the presence of leptin, a JAK inhibitor, Tyrphostin AG490, was used. Alone, Tyrphostin AG490 reduced cancer cell numbers compared to control cell number, and in combination with leptin it inhibited the leptin stimulation effect on Ishikawa cells (Figure 5.8). Activation of JAK/STAT3 pathway by leptin has been reported to stimulate ovarian cancer cell growth and Tyrphostin AG490 was reported to abolish the leptin effect on ovarian cancer cells [339]. In this study, Ishikawa cells exhibited a similar response to the presence of Tyrphostin AG490 (Figure 5.8). Blocking JAK activity with Tyrphostin AG490 led to significantly reduced expression of STAT3 and p-STAT3 (Figure 5.11 and Figure 5.12). Tyrphostin AG490 also inhibited the baseline expression of STAT3 and p-STAT3 suggesting that in Ishikawa cells the JAK/STAT3 pathway is active in maintaining basal levels of cell proliferation (Figure 5.11 and Figure 5.12).

In this study, the PI-3K inhibitor, LY294002, inhibited expression of both total STAT3 and phosphorylated STAT3 (Figure 5.13 and Figure 5.14), and, conversely, the JAK inhibitor also reduced activation of Akt (Figure 5.9 and Figure 5.10A). This raises the question as to whether PI-3K is a protein upstream from both STAT3 and Akt or whether activation of STAT3 is only by JAK. These results indicated at least a connection between these two pathways.

Here, the relationship between JAK pathway and PI-3K pathway in endometrial cancer cell line Ishikawa was investigated. The expression of Akt and p-Akt proteins as intermediates of PI-3K/Akt/mTOR pathway in the presence of JAK inhibitor, Tyrphostin AG490, was determined. Interestingly, Tyrphostin AG490 reduced expression of Akt and p-Akt (Figure 5.9, and Figure 5.10). This suggests that JAK influences the PI-3K/Akt/mTOR pathway and STAT3 phosphorylation at the same time. On the other hand, the PI-3K inhibitor LY294002 inhibited increases in STAT3 protein expression induced by leptin to levels similar to those present in control cells and cells treated with LY294002 alone (Figure 5.13 and
Chapter Five

Figure 5.14A). It has been observed that Ishikawa cells phosphorylate STAT3 specifically at Ser727 instead of Tyr705 which is the site phosphorylated in cervical cells in the presence of leptin [484]. It has also been established that PI-3K also activates STAT3 phosphorylation specifically at Ser727 location [485, 486]. Two antibodies were used to detect expression of p-STAT3 proteins. The first was against p-STAT3(Tyr705), which is specific for JAK-mediated phosphorylation. The second was against p-STAT3(Ser727), which is specific for PI-3K pathway stimulation [484].

The absence of p-STAT3 phosphorylation at Tyr705, an effect specific for the kinase activity of JAK, caused by the presence of leptin in Ishikawa cells was reported previously [484]. Sharma et al. and others showed the presence of leptin resulted in phosphorylation of STAT3 at Tyr705 residue within 3 minutes, with this being reduced after 15 minutes of exposure. This expression regulates gene expression and leads to cell division being abolished within 24 hours post-treatment [51, 487]. This effect may have also occurred in my experiments at early exposure times. However, the continued exposure of Ishikawa cells to leptin beyond 24 hours may result in the Ishikawa cells developing alternative pathways through which to continue the proliferation. Hence, exposure to the stimulatory agent leptin for 48 hours did not show any expression of p-STAT3(Tyr705) although expression of p-STAT3(Ser727) was very obvious after 48 hours post-treatment. This may suggest that Ishikawa cells recruit another p-STAT3 residue, Ser727, which is specific for PI-3K, to continue the proliferation process via another pathway which is specific to this protein and in this case is PI-3K/STAT3.

In other words, p-STAT3(Ser727) was detected and its expression was reduced significantly in cells treated with a combination of leptin and LY294002 compared to those cells treated with only leptin (Figure 5.13 and Figure 5.14B). The results are consistent with findings that reported that Ishikawa cells did not express p-STAT3(Tyr705) protein but revealed only p-STAT3(Ser727) protein in the presence of leptin [484]. Moreover, a PI-3K inhibitor, LY294002, partially reduced the leptin stimulation effect on total STAT3 to control levels (Figure 5.13 and Figure 5.14A) but reduced significantly the p-STAT3 expression (Figure 5.13 and Figure 5.14B). Thus, the results suggest that by blocking PI-3K a cancer cell may use JAK as an alternative or additional pathway. Thus phosphorylation of p-STAT3 at
Chapter Five

Tyr705 is not active in Ishikawa cells but p-STAT3(Ser727) is receptive to PI-3K action to stimulate STAT3 activity. In addition, Figure 5.11 and Figure 5.12A show that Tyrophostin AG490 inhibits basal STAT3 expression and leptin-induced expression to the same level. Moreover, Figure 5.7 and Figure 5.12B show a strong reduction of leptin-associated phosphorylation. These reductions were further than with PI-3K inhibitor, LY294002. Thus, there may be some LY294002-independent inhibition occurring, possibly via direct JAK to STAT3 interaction.

In summary, Sharma et al. suggested that in the presence of leptin, cells expressed p-STAT3(Tyr705) at very early times of exposure (3 minutes), and this effect vanished within 24 hours [51]. However, continuous exposure of Ishikawa cells to leptin for more than 24 hours may cause the cells to utilise an alternative mechanism to continue proliferation. This may explain the observation of the expression of Ser727 residue on p-STAT3 in order for cells to continue to proliferate. This may occur via other pathways, possibly PI-3K, which is specific to this protein. However, a comprehensive understanding of the underlying mechanisms is essential to unravel the overlap between the pathways.

The observations above suggest that the interaction between the pathways JAK/PI-3K/STAT3 is a time-dependent process. Carino and colleagues also investigated the effects of leptin on benign and cancerous endometrial epithelial cells [488]. They suggested that leptin is a key molecule for the activation of JAK and downstream signalling proteins (MAPK and PI-3K) in endometrial cancer cell lines (An3Ca, SK-UT2 and Ishikawa). They demonstrated that inhibition of JAK with a specific tyrosine kinase inhibitor (AG490) in the presence of leptin led to decreased phosphorylation of leptin-induced p-STAT3 and MAPK (p-ERK1/2). However, inhibition of JAK alone did not affect the basal levels of phosphorylated MAPK or Akt [488].

JAK is a protein upstream of both PI-3K and STAT3(Ser727) in endometrial cancer cells. Thus, inhibiting JAK should lead to inhibition of both pathways. Further, PI-3K may have a direct stimulatory effect on STAT3 activation and phosphorylation. The work presented in this chapter is the first to provide such evidence in Ishikawa cells of the novel cross-talk between the pathways. Additionally, a reduction in STAT3 and p-STAT3 proteins
occurred in the presence of JAK inhibitor, Tyrphostin AG490. Consequently, inhibition of JAK may lead to suppression of STAT3 and p-STAT3 expression by either interference with the effect of JAK on PI-3K and/or on a direct activity of JAK on STAT3.

A time course study to investigate the sequence of the activation of STAT3 and phosphorylation for both residues, Tyr705 and Ser727, would be useful for finding the appropriate time to inhibit the stimulatory action of leptin on Ishikawa cancer cell line.

Other studies suggested that following phosphorylation of JAK induced by leptin binding to its cellular receptor, other signalling pathways such as PI-3K pathway in ovarian cancer are triggered via MAP kinase activation [374] or via activation of MEK/ERK1/2 pathway [373]. However, it has been suggested that in myeloproliferative neoplasmas (MPN) [375] and in hypothalamus [53] JAK is the upstream protein of PI-3K and the activation of PI-3K by JAK is via insulin receptor substrate (IRS). IRS may be involved in the endometrial cancer pathway also. However, in this study the presence of IRS was not determined. This study nevertheless extends evidence of the involvement of a JAK/PI-3K pathway to endometrial cancer cells.

These results in endometrial cancer cells again support findings of others [53, 164, 373-375] in ovarian cancer cells, myeloproliferative neoplasms and hypothalamus in regard to the stimulatory effect of JAK on PI-3K in endometrial cancer cells. In this chapter, evidence was provided that the two pathways may be involved in leptin stimulatory effect on endometrial cancer growth and are not differently separated or parallel pathways. JAK/STAT3 and PI-3K/Akt/mTOR pathways apparently had cross-talk and JAK is the upstream protein in endometrial cancer cells.

The observations suggest that the effect of leptin on Ishikawa cells is time dependent and JAK blockade may be an interesting area for further research as a therapeutic target in endometrial cancer cell proliferation in obese endometrial cancer patients who are overexpressing leptin.
Chapter Six

Adiponectin signalling pathways in Ishikawa cells
6 Adiponectin signalling pathways in Ishikawa cells

6.1. Introduction

Adiponectin exerts its effect on the cancer cell via two protein receptors (AdipoR1 and AdipoR2). These AdipoRs are proteins that have an intracellular N terminal and extracellular C terminal region, characteristics which make the AdipoRs different from G protein-coupled receptors [1]. AdipoR1 and AdipoR2 were previously reported to be found in the cell membrane and in the cytoplasm of cells in normal and malignant endometrial tissue [2][35], and have different affinities to adiponectin isoforms [1]. AdipoR1 has high affinity for a globular adiponectin isoform (GAd) and low affinity for a full-length isoform (FAd), while AdipoR2 has moderate affinity for both GAd and FAd isoforms [1].

Binding of adiponectin to one or both of the receptors triggers a series of signalling events inside cells that include the activation of adenosine monophosphate (AMP) protein kinase (AMPK) [3]. In this project, biological activities of adiponectin associated with the activation of the PI-3K/Akt/mTOR, JAK/STAT3 pathways and AMPK protein were investigated in the endometrial cancer cell line, Ishikawa.
6.2. **Results**

6.2.1. **Modulation of adiponectin receptors**

The levels of AdipoR1, and AdipoR2 in the Ishikawa cell line were investigated in the presence of adiponectin at two different concentrations, 20 ng/ml (Adp20) and 100 ng/ml (Adp100). GAPDH is the reference protein in the Western blotting studies. As shown in Figure 6.1 and Figure 6.2 adiponectin at 20 ng/ml or at 100 ng/ml did not alter the protein level of AdipoR1 and AdipoR2 when compared to the control.

![Western blot images](image)

**Figure 6.1** The level of AdipoR1 and AdipoR2 in untreated Ishikawa cells, cells treated with Adp20, and Adp100. GAPDH is the reference protein.

![Densitometry graphs](image)

**Figure 6.2** Densitometry of (A) AdipoR1 and (B) AdipoR2 expression for Ishikawa cells untreated (Ctrl) and treated with Adp20 and Adp100. n = 4. Data were analysed using logarithmic analysis and one-way ANOVA. Data are expressed as means ± S.E.M. The data are identical to previous figure.
Adiponectin has been observed to exhibit biological activities opposite to the activity of leptin in that adiponectin can inhibit cancer cell proliferation [489]. However, the influence of adiponectin on expression of leptin receptors in cells remains unknown. For this reason, it may be worthwhile investigating whether adiponectin can change the amount of leptin receptor. Ishikawa cells were exposed to Adp20 and Adp100; proteins from each condition were analysed by Western blots.

**Figure 6.3** Expression of leptin receptor (Ob-Rb) in untreated cells, and cells treated with Adp20 and Adp100. GAPDH is the reference protein.

**Figure 6.4** Densitometry of Ob-R expression for Ishikawa cells untreated (Ctrl) and treated with Adp20 and Adp100. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. Data were denoted as statistically significant (*) when $p < 0.05$ compared to controls. Data are expressed as means ± S.E.M.

It is very interesting that the level of Ob-Rb was significantly increased in Adp20 stimulated cells. However, this increase was not observed in cells treated with Adp100 (Figure 6.3 and Figure 6.4).
6.2.2. Effects of adiponectin on adenosine monophosphate kinase (AMPK)

The binding of adiponectin to its receptors has been observed to induce the activation of AMPK, which in turn regulates cellular functions including VEGF secretion [478, 479]. As such, it would be profitable to know whether adiponectin at 20 ng/ml and 100 ng/ml can elicit similar effects on the total level of AMPK protein and its active form (phosphorylated AMPK, p-AMPK) in Ishikawa cells.

![Image of AMPK and p-AMPK expression](image)

Figure 6.5 Expression of AMPK and p-AMPK in untreated cells, and cells treated with Adp20 and Adp100. GAPDH is the reference protein.

![Image of densitometry](image)

Figure 6.6 Densitometry of (A) AMPK and (B) p-AMPK expression in Ishikawa cells untreated (Ctrl) and treated with Adp20 and Adp100. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05, and ** p < 0.01 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.
As shown in Figure 6.5 and Figure 6.6, the presence of 20 ng/ml adiponectin increased the levels of AMPK and p-AMPK significantly compared to controls. However, the protein levels of AMPK and p-AMPK were not changed in the presence of 100 ng/ml adiponectin.

In the previous chapter, the cellular activities of leptin associated with the PI-3K/Akt/mTOR and JAK/STAT3 pathways were described. Furthermore, two experimental inhibitors (LY294002 and Tyrphostin AG490) were used to examine these pathways that might be responsible for leptin’s growth promoting effect. Here, the same inhibitors were employed to investigate whether adiponectin could also activate PI-3K/Akt and JAK/STAT3 signalling cascade.

6.2.3. The effect of adiponectin on cell numbers and VEGF secretion

The targets of the pathways that were established above were compared to results of experiments that examined function. The effects on cell number and VEGF secretion of adiponectin at both its growth-promoting concentration (20 ng/ml) and its apparent anti-cancer concentration (100 ng/ml) for Ishikawa cells were examined.

First, the effect of Tyrphostin AG490 was determined and the effects of LY294002 on adiponectin modification of cell number and VEGF secretion were assessed. The results showed that growth promoting and VEGF secreting effects of Adp20 were lowered by the presence of Tyrphostin AG490 (Figure 6.7). This may suggest that JAK mediated pathway may regulate cell proliferation.
Figure 6.7 Ishikawa (A) cell numbers and (B) VEGF secretion of untreated cells (Ctrl), cells treated with Tyrphostin AG490, Adp20, and in combination. n = 3. Data were analysed using one-way ANOVA. ** p < 0.01, and *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. The data are identical to previous figure.
Figure 6.8 Ishikawa (A) cell numbers and (B) VEGF secretion of untreated cells (Ctrl), cells treated with LY294002, Adp20 and in combination. n = 3. Data were analysed using one-way ANOVA. * p < 0.05, and *** p < 0.001 compared to control. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.

To investigate this unique function of Adp20 activating Akt protein, the PI-3K inhibitor was used to treat Ishikawa cells and cell numbers were determined. The secretion of VEGF was also studied. As shown in Figure 6.8A, Adp20 significantly elevated cell numbers compared the control, and LY294002 compromised Adp20 growth promoting effect. A similar pattern is observed in the secretion of VEGF (Figure 6.8B). These findings suggest that Adp20 promotes cell proliferation and VEGF level via PI-3K protein. On the other hand the effects of Adp100 were not affected by either Tyrphostin AG490 or LY294002 (Figure 6.9 and Figure 6.10).
Figure 6.9 Ishikawa (A) cell numbers and (B) VEGF secretion of untreated cells (Ctrl), cells treated with Tyrphostin AG490, Adp100 and in combination. n = 3. Data were analysed using one-way ANOVA. ** $p < 0.01$, and *** $p < 0.001$ compared to control. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.
These results, overall, indicate that the signalling pathways activated by adiponectin are dose-dependent effects.

6.2.4. Effects of adiponectin on PI-3K/Akt pathway

The association of adiponectin with the PI-3K/Akt/mTOR pathway was investigated. Ishikawa cells were treated with the two different concentrations, Adp20 and Adp100, and protein levels of Akt and the phosphorylated Akt (p-Akt) were documented using Western blotting (Figure 6.11).
Figure 6.11 Protein levels of Akt and p-Akt in untreated cells, and cells treated with Adp20 and Adp100. GAPDH is the reference protein.

Figure 6.12 Densitometry of (A) Akt and (B) p-Akt expression for Ishikawa cells untreated (Ctrl) and treated with Adp20 and Adp100. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.

Figure 6.11 and Figure 6.12A show a significant increase of total Akt protein and p-Akt protein in the presence of Adp20 but there was no change in Adp100 stimulated cells.

It is interesting to note that, as shown in Figure 3.9, Adp20 significantly increased cell numbers in the Ishikawa cell culture. It is likely that Adp20 promotes growth activity via the activation of Akt protein as shown here by results in Figure 6.11 and Figure 6.12B.
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It is very important to understand whether Adp20-stimulated cell growth may be associated with the activity of Akt protein, which lies downstream from PI-3K in the signalling pathway. Therefore, to ascertain this, proteins from Ishikawa cells were again subjected to Western blotting.

![Western blot analysis of Akt, p-Akt, and GAPDH.

<table>
<thead>
<tr>
<th></th>
<th>Adp20</th>
<th>LY294002</th>
<th>Adp20+LY294002</th>
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<tbody>
<tr>
<td>Akt</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>p-Akt</td>
<td>-</td>
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<td>GAPDH</td>
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**Figure 6.13** Expression of Akt and p-Akt in Ishikawa cells in untreated cells, and cells treated with Adp20, LY294002 and a mixture of both inhibitors. GAPDH is the reference protein.

![Densitometry plots for Akt and p-Akt expression.

**Figure 6.14** Densitometry of (A) Akt and (B) p-Akt expression for Ishikawa cells untreated (Ctrl) and treated with Adp20, LY294002 and in combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. *p < 0.05 and ***p < 0.001 compared to controls. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.
As shown in Figure 6.13 and Figure 6.14, adiponectin at 20 ng/ml increased the expression of Akt and this was reduced back to the control level by LY294002. A similar effect was observed with the phosphorylated form of Akt, as shown in Figure 6.14B. These results suggest that the proliferation-promoting effect of adiponectin 20 ng/ml was mediated via the PI-3K/Akt pathway.

To examine if Adp100 could produce a similar Akt stimulating effect as shown by Adp20. It is very interesting to notice that Adp100 failed to increase the level of Akt and p-Akt, Figure 6.16. These findings suggest that the PI-3K/Akt/mTOR pathway may not be involved in the biological activity of 100 ng/ml adiponectin in Ishikawa cells.

**Figure 6.15** Expression of Akt in Ishikawa cells in untreated cells, and cells treated with adiponectin at 100 ng/ml (Adp100), PI-3K inhibitor, LY294002 (20 µM) and a combination. GAPDH is the reference protein.

**Figure 6.16** Densitometry of (A) Akt and (B) p-Akt expression for Ishikawa cells untreated (Ctrl) and treated with Adp20, LY294002, and in combination. n = 12. Data were analysed using logarithmic analysis and one-way ANOVA. Data are expressed as means ± S.E.M. The data are identical to previous figure.
6.2.5. Effects of adiponectin on an association of PI-3K with AMPK

The expression of AMPK protein was also examined in the presence of Adp20 alone and in combination with LY294002.

![Protein expression of AMPK and p-AMPK in Ishikawa cells in untreated cells, and cells treated with Adp20, LY294002 and in combination. GAPDH is the reference protein.](image1)

**Figure 6.17** Protein expression of AMPK and p-AMPK in Ishikawa cells in untreated cells, and cells treated with Adp20, LY294002 and in combination. GAPDH is the reference protein.

![Densitometry of (A) AMPK and (B) p-AMPK expression for Ishikawa cells untreated (Ctrl) and treated with Adp20, LY294002 and in combination. n = 4. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05 and ** p < 0.01 compared to controls. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.](image2)

**Figure 6.18** Densitometry of (A) AMPK and (B) p-AMPK expression for Ishikawa cells untreated (Ctrl) and treated with Adp20, LY294002 and in combination. n = 4. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05 and ** p < 0.01 compared to controls. Data are expressed as means ± S.E.M. Stars without connections indicate comparison to control incubation. The data are identical to previous figure.
Figure 6.14, Figure 6.17 and Figure 6.18, show that the presence of PI-3K inhibitor, LY294002, abolished the stimulatory effect on AMPK of Adp20, leading to a reduction of the expression of AMPK and phosphorylated AMPK. This may suggest that PI-3K acts on AMPK, and may regulate the activation and phosphorylation of AMPK.

6.2.6. Effects of adiponectin on an association of PI-3K with JAK/STAT3

In Chapter Five, it was suggested that PI-3K could act upstream from STAT3 following leptin binding. For that reason, it is worthwhile to investigate whether STAT3 is activated by adiponectin in the presence of LY294002. Here the expression and activation of STAT3 protein in response of Adp20 and LY294002 alone and combination of both were investigated. The results indicate that LY294002 reduced the total levels of STAT3 and p-STAT3 in a Adp20-stimulating condition (Figure 6.20). The effect of Adp100 on total STAT3 and p-STAT3 were also assessed. However, Adp100 had no effect on the levels of STAT3 or p-STAT3 in any tested conditions (Figure 6.21, Figure 6.22).

![Figure 6.19 Protein expression of STAT3 and p-STAT3 in Ishikawa cells in untreated cells, and cells treated with Adp20, LY294002 and in combination. GAPDH is the reference protein.](image-url)
Figure 6.20 Densitometry of (A) STAT3 and (B) p-STAT3 expression for Ishikawa cells untreated (Ctrl) and treated with Adp20, LY294002 and in combination. n = 4. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05 and ** p < 0.01 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.

Figure 6.21 Protein expression of STAT3 in Ishikawa cells in untreated cells, and cells treated with Adp100, LY294002 and in combination. GAPDH is the reference protein.
Figure 6.22 Densitometry for (A) STAT3 and (B) p-STAT3 expression for Ishikawa cells untreated (Ctrl) and cells treated with Adp100, LY294002 and in combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. ** p < 0.01 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.

Taken together, the results shown in Figures 6.13 to 6.20 suggest that Ishikawa cells stimulated with 20 ng/ml adiponectin can activate the PI-3K, Akt, AMPK, and STAT3 proteins. However, these activations are limited in Adp100-treated cells (Figure 6.22).

It is also important to understand the Adp20 stimulating effects in Ishikawa cells, possibly occurs via the activation of JAK protein. To examine this possibility the JAK blocker, Tyrphostin AG490, was included in cells that were stimulated with Adp20.

Ishikawa cells were exposed to Adp20, Tyrphostin AG490, and a combination of Adp20 and blocker and the expression of the intermediary molecules that are involved in the JAK/STAT3 pathway were determined including Akt (Figure 6.23, Figure 6.24), AMPK (Figure 6.25, Figure 6.26) and STAT3 (Figure 6.27, Figure 6.28).
Figure 6.23 Protein expression of Akt and p-Akt in Ishikawa cells in untreated cells, and cells treated with Adp20, Tyrphostin AG490 and in combination. GAPDH is the reference protein.

Figure 6.24 Densitometry of (A) Akt and (B) p-Akt expression for Ishikawa cells untreated (Ctrl) and cells treated with Tyrphostin AG490, Adp20 alone, and in combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.
Figure 6.25 Protein expression of AMPK and p-AMPK in Ishikawa cells in untreated cells, and cells treated with Adp20, TY AG490 and in combination. GAPDH is the reference protein.

<table>
<thead>
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<th>Treatment</th>
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<tr>
<td>Adp20 + TY AG490</td>
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63 kDa
63 kDa
37 kDa

Figure 6.26 Densitometry of (A) AMPK and (B) p-AMPK expression in Ishikawa cells untreated (Ctrl) and cells treated with Tyrphostin AG490, Adp20 alone, and in combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * p < 0.05 and ** p < 0.01 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.
Chapter Six

Figure 6.27 Protein expression of STAT3 and p-STAT3 in Ishikawa cells in untreated cells, and cells treated with Adp20, TY AG490 and in combination. GAPDH is the reference protein.

Figure 6.28 Densitometry of (A) STAT3 and (B) p-STAT3 expression for Ishikawa cells untreated (Ctrl) and cells treated with Tyrphostin, Adp20 alone and in combination. n = 3. Data were analysed using logarithmic analysis and one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.

The results confirmed that the presence of JAK inhibitor, Tyrphostin AG490, significantly reduced the Adp20-stimulated expression of Akt (Figure 6.23, and Figure 6.24), AMPK (Figure 6.25, and Figure 6.26), and STAT3 (Figure 6.27, and Figure 6.28) proteins. The expression of these proteins was limited to being similar to or less than the expression in
untreated cells. This suggests that Adp20 may also activate JAK/PI-3K/Akt pathway and possibly in a similar manner to the cellular stimulation of leptin.
6.3. **Discussion**

Adiponectin is secreted exclusively by white adipose tissue (WAT) [143, 177] and has been shown to have anti-tumorigenic effects on tumour growth [143, 490]. Also obesity, as defined by BMI, is negatively correlated with the circulating concentrations of this adipokine hormone, adiponectin. It has been suggested that the development of obesity-related malignancies, including endometrial cancer [491], may be linked to the lowered adiponectin concentrations encountered in that condition [169, 173, 174, 179, 411].

This project has revealed that adiponectin demonstrates an anticancer effect on Ishikawa cells at concentration of 100 ng/ml (Adp100). However, adiponectin has an opposite, tumorigenic effect at a concentration of 20 ng/ml (Adp20). This effect has not previously been reported for adiponectin. Experiments investigated how this unusual and varied response was transduced.

Untreated Ishikawa cells expressed AdipoR1 and AdipoR2 proteins (Figure 3.8). The presence of Adp100 had no effect on the expression of AdipoR1 and AdipoR2 protein expression at 48 hours post-treatment (Figure 6.2) compared to untreated Ishikawa cells. However, the expression of Ob-R leptin receptor protein was investigated in the presence of Adp20 and Adp100. As shown in Figure 6.4, the lower adiponectin concentration increased the expression of leptin receptors significantly compared to control. This suggests that low adiponectin levels may modulate Ob-R and hence the effect of leptin on endometrial cancer cells. However, there was no change in Ob-R protein expression (Figure 6.4) induced by high adiponectin.

In many aspects, adiponectin 20 ng/ml activity is closely similar to the action of leptin on endometrial cancer cells with regard to increasing endometrial cancer cell numbers (Figure 3.4 and Figure 3.9).

Previous studies identified the activation of adenosine monophosphate-activated protein kinase (AMPK) [376, 377] as a pathway mediating adiponectin activity. Indeed, adiponectin is recognised as a stimulator of AMPK in many cancer cell types [136, 377-379], and
exposing cancer cells to treatment with adiponectin enhances AMPK activation in endometrium [30] as well as colon [365-367], breast [380, 381, 409], prostate [143, 367], and liver [349]. However, in the current study, the presence of higher adiponectin concentrations for 48 hours incubation did not activate the phosphorylation of AMPK protein.

Moreover, this study demonstrates for the first time that cross-talk exists between AMPK and JAK/PI-3K/Akt/mTOR in the presence of low adiponectin. The results suggest that following adiponectin ligand binding, JAK activates PI-3K which in turn stimulates AMPK and Akt expression and phosphorylation. AMPK activated by PI-3K in the presence of adiponectin may explain the increase in VEGF secretion in endometrial cancer cells [478, 479]. It appears that VEGF secretion is controlled separately from cell proliferation because VEGF secretion by leptin is not mediated by PI-3K. Nevertheless PI-3K is involved in activity of adiponectin at 20 ng/ml.

The difference between the presence of leptin and adiponectin at 20 ng/ml concentration may be exemplified by the secretion of VEGF from Ishikawa cells that is associated with AMPK protein activation and phosphorylation. Leptin induced a reduction in VEGF secretion although Ishikawa cells did not exhibit a significant change in expression of AMPK or p-AMPK proteins in the presence of leptin compared to untreated cells (Figure 5.2, Figure 5.3), which in this case does not involve PI-3K/Akt/AMPK pathway. However, 20 ng/ml adiponectin significantly increased AMPK and p-AMPK expression (Figure 6.6), consistent with other observations [478, 479].

The results overall indicate there is a complex array of pathways that are involved in regulating VEGF secretion. According to these data, the following schema for the adiponectin signalling pathway is proposed (Figure 6.29). Here, it was demonstrated that the population of leptin receptors may be involved in endometrial cancer cell growth that is modulated by adiponectin. Also, PI-3K is upstream of AMPK and Akt and modifies the phosphorylation of AMPK and Akt, which in turn leads to increased cancer cell numbers.
Figure 6.29 Depicted is a proposed set of signalling pathways associated with adiponectin (20 ng/ml) in endometrium adenocarcinoma (Ishikawa) cells consistent with the observations. Adiponectin activated the PI-3K pathway, which in turn led to (i) increased expression of Akt and its phosphorylation; (ii) increased expression of AMPK and its phosphorylation; (iii) an increase in STAT3 synthesis and its phosphorylation. Additionally, involvement of JAK phosphorylation was observed.
Chapter Seven

Insulin and IGF-I: proliferation and VEGF secretion signalling pathways in Ishikawa cells
Chapter Seven

7 Insulin and IGF-I: proliferation and VEGF secretion signalling pathways in Ishikawa cells

7.1. Introduction

Insulin is one of several obesity-related growth factors that may be associated with cancer cell proliferation and survival [387, 428]. Insulin resistance occurs in a variety of conditions such as obesity, polycystic ovary syndrome (PCOS) [492], type 2 diabetes mellitus and hypertension, and may also be associated with increased endometrial cancer risk [429, 493]. Insulin exhibits an important impact on endometrial carcinoma cells [429] and serum insulin levels are correlated with progression of endometrial adenocarcinoma [494]. It is possible that hyperinsulinemia associated with obesity underlies this relationship in different types of cancer [494-496]. The binding of insulin to its receptors (InsR) generates a series of reactions commencing with the phosphorylation of the insulin receptor and its substrate (IRS) proteins, which then activate downstream molecules such as the PI-3K/Akt/mTOR pathway [391, 393-395].

The insulin-like growth factor system is also known to be associated with increased body weight, diabetes, and hyperinsulinemia [206, 497]. The receptor for IGF-I, IGF-IR, is a trans-membrane tyrosine kinase receptor that has several functional features in common with InsR [120, 121, 125]. IGF-I ligand binding induces auto-phosphorylation of IGF-IR [498]. This in turn results in the activation of signalling pathways, which, like insulin, include the PI-3K/Akt/mTOR signalling cascade [498]. The expression levels of IGF-IR are also significantly higher in endometrium adenocarcinoma than in the normal endometrium [499].

It was previously noted that insulin and IGF-I promoted proliferation of Ishikawa cells (Figure 3.15, Figure 3.21). Thus, this chapter studies the effects of insulin and IGF-I on the PI-3K/Akt/mTOR pathway to determine whether there was a potential association of InsR and IGF-I with proliferation in the Ishikawa endometrial cancer cell line [392]. Further, it has also been observed that both insulin and IGF-I reduced VEGF secretion from Ishikawa cells (Figure 3.16, Figure 3.22). And so in the absence of robust data on mechanisms of insulin-
and IGF-I-regulated VEGF secretion in these cells, the relationship of VEGF and AMPK activation was also investigated.
7.2. **Results**

7.2.1. **The effect of insulin on signalling proteins**

It has been suggested that insulin’s promotion of tumour cell proliferation and survival involves the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway [500-502]. Therefore, the responses of Akt and p-Akt to insulin were investigated. As shown in Figure 7.1 and Figure 7.2, insulin significantly increased the expression and phosphorylation of Akt. This increase in Akt and p-Akt confirms the involvement of PI-3K/Akt/mTOR pathway in cell signalling behaviour in the presence of insulin.

![Figure 7.1](image1.png) **Figure 7.1** Protein expression of Akt and p-Akt in untreated Ishikawa cells (-) and cells treated with 580 ng/ml insulin. GAPDH is the reference protein.

![Figure 7.2](image2.png) **Figure 7.2** Densitometry of the amount of (A) Akt and (B) p-Akt proteins for cells treated with 580 ng/ml insulin and untreated cells (Ctrl). n = 3. Data were analysed using logarithmic analysis and Student's t-test (two-tailed). * p < 0.05 and *** p < 0.001 compared to controls. Data are expressed as means ± S.E.M.
It is believed that AMPK is involved in the stimulatory pathway of VEGF secretion [379, 478, 479]. Therefore, the levels of both AMPK and p-AMPK were investigated in Ishikawa cells by Western blotting. The inhibition of AMPK expression and phosphorylation that occurred in the presence of insulin (Figure 7.3, Figure 7.4) may be a contributing factor to the reduction in VEGF secretion from Ishikawa cells.

**Figure 7.3** AMPK and p-AMPK proteins expression in untreated Ishikawa cells (-) and cells treated with 580 ng/ml insulin. GAPDH is the reference protein.

**Figure 7.4** Densitometry of (A) AMPK and (B) p-AMPK expression in untreated Ishikawa cells and cells treated with insulin. n = 3. Data were analysed using logarithmic analysis and Student's t-test (two-tailed). *p < 0.05 compared to controls. Data are expressed as means ± S.E.M.
7.2.2. Effect of insulin like growth factor-1 on signalling proteins

The effect of treatments with IGF-1 on signalling proteins were also investigated. Akt protein expression was investigated to ascertain whether signalling molecules that IGF-I activates include the PI-3K/Akt/mTOR pathway. Figure 7.5, and Figure 7.6 show that there was a significant increase of Akt protein expression and phosphorylation in the presence of IGF-I compared to the control.

![Image](image_url)

**Figure 7.5** Protein expression of Akt and p-Akt in untreated Ishikawa cells (-) and cells treated with 100 ng/ml IGF-I. GAPDH is reference protein.

![Image](image_url)

**Figure 7.6** Densitometry of (A) Akt and (B) p-Akt expression in untreated Ishikawa cells (Ctrl) and cells treated with 100 ng/ml IGF-I. n = 3. Data were analysed using logarithmic analysis and Student's t-test (two-tailed). * p < 0.05 and *** p < 0.001 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.
AMPK protein expression was also investigated in order to determine whether IGF-I activates this pathway, as this may also be associated with secretion of VEGF from Ishikawa cells. As shown in Figure 7.7 and Figure 7.8, the presence of IGF-I decreased expression of AMPK, and this reduction was significant for p-AMPK compared to the control.

![Figure 7.7](image)

**Figure 7.7** Protein expression of AMPK and p-AMPK in untreated Ishikawa cells (-) and Ishikawa cells treated with IGF-I (100 ng/ml). GAPDH is reference protein.

![Figure 7.8](image)

**Figure 7.8** Densitometry of (A) AMPK and (B) p-AMPK expression for untreated cells (Ctrl) and cells treated with IGF-100. n = 3. Data were analysed using logarithmic analysis and Student's t-test (two-tailed). * p < 0.05 compared to controls. Data are expressed as means ± S.E.M. The data are identical to previous figure.
7.3. **Discussion**

In this chapter, it was shown that both insulin and IGF-I activated the PI-3K/Akt pathway in Ishikawa cells, and down-regulated AMPK expression and phosphorylation. Unfortunately, because of time limitations, it was not possible to investigate the contribution of JAK/STAT3 pathway in the activation process of insulin and IGF-I in Ishikawa cells nor the effect of the inhibitors that were used in the previous chapters.

The PI-3K/Akt/mTOR pathway is widely associated with cell proliferation. Consequently, it is reasonable to suggest that the observed increase of Ishikawa cancer cell numbers induced by both insulin and IGF-I (Figure 3.15, Figure 3.21) may be related to the endocrine regulation of this pathway (Figure 7.1, Figure 7.2, Figure 7.5, Figure 7.6). These results are consistent with other studies [399, 496, 502] in tissue cell lines such as HEC-1A, colorectal and myoblast cell lines in which it has been proposed that insulin increases cancer cell proliferation via the PI-3K/Akt pathway.

Increases in Akt and p-Akt expression may have led to the inhibition of the activation of AMPK, thus disturbing the cellular energy balance that is partly regulated by AMPK [455, 503] and suppresses the breakdown of ATP to AMP which in turns activates the kinase protein. Thus, cancer cells may divert energy expenditure while the proliferation process occurs [455].

Because AMPK has a central role in upregulating VEGF secretion and hence the presence of insulin reduced the expression of AMPK and p-AMPK and reduced VEGF secretion from HEC-1A cells [397]. Bermont et al. treated HEC-1A cells with 10 nM insulin for up to 24 hours and observed a high VEGF level in the conditioned medium compared to untreated ones due to low insulin concentration. In this study, the insulin concentration used was ten-fold higher (100 nM) than in Bermont's study [397] which also tested hypoxia [345] which shares with insulin the ability to regulate the expression of some stress-induced genes by similar mechanisms in Ishikawa cells.
The effects of insulin and IGF-I on Akt activation and phosphorylation were similar to those of leptin (Chapter 5). However, neither insulin nor IGF-I replicated the action of leptin on AMPK activation. This suggests that the down-regulation of AMPK protein expression in endometrial cancer cells observed in this chapter might possibly occur via different intracellular complexes than those recruited by leptin. This aspect will require further investigation.

The other signalling protein investigated was AMPK. Treatment with insulin or IGF-I led to reduced levels of AMPK and p-AMPK. It might be speculated that the high concentrations of insulin and IGF-I for 48 hours and the non-hypoxic environment reduced the stress on the cells, which in turn reduced AMPK activation and led to inhibit VEGF secretion from the Ishikawa cells. Thus, the reduction of AMPK and p-AMPK protein expression might be associated with the reduced VEGF secretion from Ishikawa cells that was previously observed (Figure 3.16, Figure 3.22). To my knowledge, this is the first study that reveals the potential for a negative mechanistic association between either insulin or IGF-I and VEGF secretion from endometrial cancer cells.

It is suggested that there is an inverse association between Akt and AMPK activation and the presence of insulin and IGF-I. Cancer cell proliferation requires significant amounts of energy to maintain the processes of growth and division associated with proliferation. Thus, the increase in expression of Akt, a mediator of the cell proliferation process, partly silences AMPK phosphorylation and reduces VEGF secretion and saves energy.

There is a strong reason to conduct a study using a series of pharmacological inhibitors such as LY294002, everolimus and Tyrphostin AG490 to further investigate the signalling pathways that are involved in the responses of Ishikawa cells to the presence of insulin and IGF-I, just as were conducted with leptin and adiponectin. Unfortunately, however, because of the time limits, I was not able to conduct such a study, and I consider this for future study.

These observations show that insulin and IGF-I have parallel effects on both cell number and VEGF secretion in Ishikawa cells and, further, that the effects of the two hormones on the signalling molecules that were investigated are similar. However, in the light of the previous observation that IGF-I was shown (Chapter 4, Figure 4.3, Figure 4.4, and
Figure 4.5) to have differential growth effects on different types of cancer cell lines which did not parallel insulin, it would be informative in future to expand this study of mechanisms to further cell lines to investigate these intriguing observations.
Chapter Eight

Conclusion
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8 CONCLUSION

There is a strong interest in the connection between obesity and an increased risk of the onset of cancer. This thesis has been an investigation into aspects of this relationship, and possible links between cancer progression and obesity-related hormones are reported. These hormones were leptin, insulin, IGF-I and adiponectin.

In pre-clinical studies these hormones exhibit a spectrum of biological activities in regards to their actions on different types of cancers. There have, however, been only a limited number of studies in which these hormones have been systematically tested with gynaecological cancers. Interest in this study has focused on endometrial cancer and a well differentiated endometrial cancer cell line, Ishikawa, was chosen to be the centre of the study. This cell line is widely used in pre-clinical studies of endometrial cancer [504-506]. For comparison and controls alternative cell lines were also included. These lines were a second endometrial cancer cell line, HEC-1A, a breast cancer cell line, MCF-7, and an ovarian cancer cell line, SKOV-3.

The four obesity-related hormones included in this study are secreted by at least three different tissues, namely the white adipose tissue which secretes leptin and adiponectin, the pancreatic β-cells which secrete insulin and the liver which is responsible for the secretion of IGF-I. The effects of these hormones on the Ishikawa cell line were determined through different approaches. Cell numbers, which were determined as an indication of cell proliferation, the level of apoptosis, the secretion of VEGF as a putative tumorigenic marker, and protein expression of selected signalling molecules were all measured. Also, immunohistochemistry and Western blotting were utilised to complement the other methodologies. The effects of these hormones were individually studied, and combinations of adiponectin with either leptin, insulin or IGF-I were also investigated.
Chapter Eight

The working hypothesis in this study was that leptin, insulin and IGF-I would promote cancer growth, and in particular the growth of endometrial cancer, along with the secretion of VEGF, while adiponectin would inhibit these tumorigenic processes.

As hypothesised, it was observed that leptin, insulin and IGF-I increased Ishikawa endometrial cancer cell proliferation in a manner that was influenced by the hormone concentration. Similar increases in proliferation were also found in HEC-1A cells and MCF-7 cells, with the exception of insulin which failed to promote cell proliferation in the latter cell line. Notably, the ovarian cell line, SKOV-3, exhibited different behaviour. These results all agree with extensive reports in the literature that these hormones all induce cell proliferation, with leptin known to cause proliferation in Ishikawa, HEC-1A, and MCF-7 [157, 507-509], insulin to generate increased cell numbers in Ishikawa, HEC-1A and MCF-7 [510-513], and IGF-1 also known to induce increased cell proliferation in HEC-1A, MCF-7 [514, 515]. On the other hand, the hormone adiponectin exhibited effects that varied with concentration such that both anti-cancer activity was observed with a reduction in cell number caused by 100 ng/ml adiponectin while pro-tumorigenic activity was observed with an increase in cell number when only 20 ng/ml was used. The details of the effects of adiponectin were also cell line-dependent. Adiponectin at both 20 and 100 ng/ml significantly reduced the proliferation of HEC-1A cells but adiponectin did not have these effects on MCF-7 cells. In SKOV-3 cells, 100 ng/ml adiponectin increased cell proliferation while 20 ng/ml adiponectin had not effect on proliferation.

In this study, the effects of adiponectin acting alone showed concentration-dependent, differential effect on cell growth. The observations of a tumorigenic effect of 20 ng/ml adiponectin, a concentration which has the ability to increase proliferation of Ishikawa cells, is novel and runs counter to published studies on the effects of adiponectin which reduces endometrial and MCF-7 cancer risk [402-404, 516]. It is possible to speculate that this unusual effect might be due to stimulation of multiple stimulatory pathways, as has been suggested in other systems [137, 379]. Thus, this novel observation extends the properties of adiponectin in endometrial cancer to an association with tumorigenic as well as the previously observed anti-tumorigenic characteristics [402]. Further, that 20 ng/ml adiponectin has the potential to increase cell proliferation in Ishikawa cells suggests that it may impact on in vivo
oncogenic responses that result from obesity because concentration of adiponectin in this range are biologically relevant. Adiponectin is found in the highest concentration of all hormones in human plasma [131] with a range from 3 to 30 µg/ml which accounts for up to 0.05% of total plasma proteins [142, 143]. In contrast to other hormones, adiponectin serum levels are found to be higher in lean women compared to obese (13.3 ± 1.8 and 8.6 ± 0.8 mg/L) respectively [83].

There is still discussion regarding the place of obesity in risk of ovarian cancer. Clinical studies have concluded that for ovarian cancer patients BMI is higher and adiponectin concentrations are lower [450], thus suggesting the link to ovarian cancer [370]. *In vitro*, this study showed that the SKOV-3 cells had no proliferative response to leptin, insulin and IGF-I which is in a manner consistent with literature observation [372]. These observations are also consistent with the fact that ovarian cancer risk is not increased by obesity, although meta-analyses suggest that there is an associated risk [370, 423, 451] which may be weak [371]. The results obtained in this study which show limited effects of the proliferative hormones on the SKOV-3 ovarian cancer cell line are consistent with, at most, a low increased risk due to obesity. Alternatively, if there is an increased risk it is not mediated via the endocrinological factors investigated in this study. Thus, the initial hypothesis was only partly confirmed in that SKOV-3 cells did not respond to the presence of the stimulatory obesity-related hormones, leptin, insulin and IGF-I. However, the cell proliferation response by SKOV-3 cells to adiponectin at 20 ng/ml and 100 ng/ml suggests an opposite response to Ishikawa endometrial cells, in that the hitherto unrecognised stimulatory activity of adiponectin was exhibited, but at a higher concentration of adiponectin on SKOV-3 than on Ishikawa cells.

Interpreting the results presented here is complex. The results suggest, for example, that the low adiponectin concentrations and high leptin concentrations that are found in obese women with endometrial cancer [209, 411, 412] could have an additive effect because, as shown in Chapter 3, both have stimulatory effects on proliferation. However, later experiments in which combinations of leptin and low concentrations of adiponectin were tested together indicated that the proliferative effects of these hormones were not additive. Further, although individual treatments with insulin and IGF-I also increased endometrial cancer cell numbers at all selected concentrations, the interactions of leptin, insulin or IGF-I
when in combination with adiponectin gave different results. These observations are a warning about making simple speculations concerning the activities of hormones \textit{in vivo}. The implication is that in a pathophysiological environment, manipulating the concentration of a single hormone will not necessarily reduce tumorigenicity because the overall effect \textit{in vivo} is not a simple mathematical equation, as found \textit{in vitro}, but rather a complex network controlled by multiple, redundant pathways. In the case of obesity, a reduction in BMI will modulate the endocrine environment in a complex way, but this may have the overall effect of reducing the risk of endometrial cancer, although results may be expected to vary between individual women.

Also unexpected were the effects of the different hormones on the secretion of VEGF, a protein that stimulates the formation of new blood vessels and is critical for tumorigenesis \cite{517, 518}. This project revealed that the presence of the tumorigenic hormones leptin, insulin, and IGF-I often reduced VEGF secretion from the cells. Thus, the results partially refuted the original hypothesis. In contrast, both 20 and 100 ng/ml adiponectin significantly increased VEGF secretion from Ishikawa cells. Again, the effects of adiponectin on VEGF were also the reverse of the original hypothesis. Typically, therefore, the effects of a hormone on cell proliferation and on VEGF secretion were most often in opposite directions \textit{in vitro}.

It is possible to speculate on why this inverse relationship between proliferation and VEGF secretion occurs in culture cells. It is possible that cell signalling events occur that indicate that the local environment contains satisfactory nutrition. In that case, a subsequent signal might reduce VEGF secretion because increased vascularisation is unnecessary: reducing VEGF production would mean that the cell could conserve its energy expenditure. A sensor based on energy balance could regulate this effect, perhaps through detecting the AMP/ATP ratio \cite{519, 520}. Thus, it is possible that because of the increased ratio of ATP to AMP, the expression of AMP kinase is reduced, and that this leads to lower secretion of VEGF by the cells.

Thus, AMPK is believed to play a crucial role in regulating VEGF secretion. Indeed, AMPK expression was elevated in this study in the presence of adiponectin along with increased VEGF secretion. However, leptin did not have an apparent effect on AMPK expression, although leptin reduced VEGF secretion. Moreover, insulin and IGF-I decreased
p-AMPK activity in association with lowered VEGF secretion. The variation of the association between AMPK expression and VEGF secretion indicates that the hormonal effects on VEGF secretion might be mediated via more than just AMPK protein regulation, possibly incorporating the energy-regulating capacity of AMPK [519].

Although numerous studies have reported increases in VEGF secretion following treatments with the hormones leptin [521-523], insulin [524-526] and IGF-I [527, 528] this study clearly did not observe increases in all cases. It is possible that the experimental design and M199 culture conditions used, which involved FBS-free conditions, a low glucose concentration (normal level) and the absence of oestrogen supplementation, have an effect on cell proliferation and might have enabled exposure of the proposed, underlying energy-related process. Thus, with reduced glucose in the media, the energy supply was lower and energy conservation becomes important, and so in most cases in this study, energy expenditure was prioritised to proliferation over VEGF secretion. This is a situation that may correspond to some in vivo circumstances when, for example, vascular supply is poor, and in vivo other, non-endocrinological VEGF-stimulating pathways may be activated.

Cell signalling pathways that are involved in the activities of leptin, adiponectin, insulin and IGF-I were studied in this study, with those of leptin and adiponectin investigated in the most detail. The PI-3K/Akt pathway is involved in cell proliferation [511, 529]. These experiments demonstrated that leptin stimulation of Ishikawa cell growth was inhibited by a PI-3K inhibitor, LY294002. A JAK inhibitor, Tyrphostin AG490, also suppressed the leptin stimulation of endometrial cancer cell growth. For these reasons, JAK may be a regulatory protein for the PI-3K/Akt/mTOR pathway [53, 375, 447] in endometrial cancer cells. Akt and p-Akt expression were also enhanced by treatments with 20 ng/ml adiponectin which stimulated cell growth, and 100 ng/ml adiponectin which did not enhance Akt expression nor promote cell division. STAT3 protein also appears to be an intermediate molecule in the pathways of JAK and PI-3K. There was, as a result, evidence to suggest there is cross-talk between PI-3K/Akt/mTOR and JAK/STAT3 pathways which are associated with cell growth in endometrial cancer. No other papers have reported such interactions in endometrial cancer, as different from observations of the paths acting in parallel. Interactions have been reported in only three tissues and none was endometrial cancer, thus this study complements the sparse
A previous study [35] indicated that endocrine ligand receptors are located on cell membrane, and perhaps in cytoplasm, but failed to provide evidence for this such as images. Using the optical sectioning imaging capacity of the ApoTome system, the collection of Z-stacks confirmed for the first time that the receptors for the endocrine ligands are present in these cancer cell lines, and that they are located in the cytoplasm and nucleus. The separation or convergence of signalling following receptor occupancy by the ligand is potentially a point of regulation in cancer responses to a hormone, and might be relevant in the variations in proliferative responses to the hormones studied here.

In the present study, treatment with insulin and IGF-I increased expression of Akt and phosphorylated Akt, suggesting that Ishikawa cells use the PI-3K/Akt as a pathway for hormones to increase cell number. Further study is required to dissect the point(s) at which the different signalling pathways PI-3K/Akt/mTOR and JAK/STAT3 interact, and which molecules are involved when hormones, which did not act identically when combined with adiponectin, are acting together. When extrapolating these results to the in vivo situation it is important to note that leptin receptor expression was modulated by adiponectin. This observation indicates the potential for interactions to occur between hormones through modulating the responsiveness of the second hormone by affecting its receptor expression. Thus, a full understanding of the in vivo milieu is dependent on defining the interactions between the different components of hormonal signalling and reception. The literature is generally deficient of these sorts of investigations, although several studies have investigated the two WAT-derived hormones, leptin and adiponectin, because they have putatively opposite effects, leptin increased cancer cell proliferation while adiponectin reduced cancer cell proliferation [530-532]. Other hormones will also interact and such studies are a logical extension of the current project.

Other pathway studies that included, for example, the Erk pathway [52, 533] would be an expansion to this project that might yield valuable data because it acts as a mediator between stimulatory pathways such as MEK and MAPK pathways in endometrial cancer. Investigating the multiple opportunities for cross-talk might yield insights into the
fundamental signals involved in tumour progression, and the signalling redundancies that are present that provide survival paths for the growth and development of a cancer. A deeper characterisation of the signalling processes that are modified by the presence of a second or even a third ligand would begin to provide the basis for a model on which to build hypotheses.

VEGF secretion is believed to centrally require AMPK [478]. PI-3K is located upstream of AMPK and activates the phosphorylation of AMPK. However, AMPK is possibly not the only regulator of VEGF secretion in the experimental conditions used in this study because there was no detected effect of leptin on AMPK expression in spite of leptin’s modulation of VEGF secretion. Additionally, because the reduction in VEGF induced by leptin is relatively small, the relative insensitivity of the Western blot densitometry measurements might mean that changes in AMPK activation and phosphorylation could not be detected in this instance. Also, the higher adiponectin concentration (100 ng/ml) did not enhance AMPK and p-AMPK expression despite this higher adiponectin concentration resulting in increased VEGF secretion from Ishikawa cells. These results suggest that VEGF is signalled via multiple, different pathways and that the concentration of adiponectin plays a role in choosing the relative activation of these pathways.

Adiponectin may exert its effects on endometrial cancer cells via cross-talk with leptin pathways [534]. Also, the identity of this proposed signalling link between increased proliferation and decreased VEGF secretion remains to be determined, although other data that suggest a positive correlation between cell proliferation and VEGF secretion suggests that there may be a vital survival response component based on an energy sensor [489, 535]. Therefore, there may be value in undertaking experiments in different culture media containing a range of constituents or with a range of concentrations of FBS.

Emphasising the further complexity of the hormone systems was the variation in responses when the other cell lines were studied. In other words, there was a cell-type dependent component to the effects of the hormones. It is uncertain whether this is a result of the genotype and/or whether there are tissue-specific factors. The former seems likely to explain, at least partially, the differences in the responses of Ishikawa cells and HEC-1A cells, both of which are endometrium-derived cancer lines. These lines exhibited differences in
response to, for example, 20 ng/ml adiponectin which increased Ishikawa cells proliferation, while with HEC-1A cell proliferation was reduced. These results indicated that the regulation of tumour progression *in vivo* will be a complex matter affected by concentrations of numerous growth factors as well as other oncogenic entities.

The differences in this study between the effects of adiponectin at 20 ng/ml and 100 ng/ml provide a ready example of the importance of details, with the role of concentration revealed in these new and intriguing results in multiple ways:

(a) In some case the results of the two concentrations were similar. They both did not alter proliferation of MCF-7 but increased proliferation of SKOV-3 cells at high concentration, and both decreased proliferation of HEC-1A cells. The two concentrations tended to be always equally active on VEGF secretion - they increased VEGF secretion from both Ishikawa cells and HEC-1A and both had no effect on MCF-7 or SKOV-3 cells. Also adiponectin at neither 20 ng/ml nor 100 ng/ml concentration altered adiponectin receptor expression.

(b) In other cases adiponectin at 20 ng/ml was active but at 100 ng/ml was not - adiponectin at 20 ng/ml had a positive effect on signalling molecules AMPK, Akt and STAT3 whereas adiponectin at 100 ng/ml had no effect. Similarly adiponectin at 20 ng/ml increased leptin receptor expression in Ishikawa cells, adiponectin at 100 ng/ml did not.

(c) Conversely, in Ishikawa cells on leptin-associated increase of proliferation, adiponectin at 100 ng/ml had a decreasing effect but adiponectin at 20 ng/ml had none and in the otherwise unresponsive SKOV-3 cells adiponectin at 100 ng/ml increased proliferation.

(d) Opposite effects were observed in Ishikawa proliferation (adiponectin at 20 ng/ml increased proliferation, 100 ng/ml was associated with a small decrease in proliferation). So can I say that adiponectin is actually acting as an anti-cancer hormone, as described by some researchers? Further studies are required.

Identifying the roles of obesity in generating these compounds and in modulating their concentrations and activities is fundamental to understanding the increased risk of endometrial, breast and ovarian cancer in overweight individuals. The systematic approach taken to examining the effects in the selected four cell lines provides new findings and offers new understanding in this field. The observations are consistent with each of the four
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hormones having a role in cancers that occur and proliferate in the dysregulated environment that is a result of obesity. In particular, it was interesting to note the lower sensitivity to obesity-related hormones by the ovarian SKOV-3 cell line compared to endometrial and breast lines.

However, an expanded series of ovarian cancer lines should be investigated to determine whether these finding can be replicated across a wider range of mutations and cancer subtypes. Reviewing the results here – that the hormones have more effect on three of the cell lines but not so much on the ovarian cell line – raises the intriguing possibility that there are tissue-related properties. There is a wide range of ovarian cell lines available, and undertaking a project on only ovarian lines would be a useful study and would seem to offer a most interesting and efficient approach. Nevertheless, it would be profitable to also investigate other endometrial and breast cell lines.

Further insights would also be gleaned by studying the RNA expression of relevant genes after being exposed to the hormones. Thus, the endpoint in this project has provided excellent entry points for further experiment, but digging deeper will provide a different sort of information on points of regulation at translation or transcription control.

The experiments undertaken in this project indicate that the dysregulated endocrine environment associated with obesity has the potential to support the progression of tumours. The results revealed parts of the network that makes up the mechanism processes recruited by the hormones studied. On the other hand, the observations also pointed to the necessary involvement of other entities, which were not studied here, for the full engagement of the hallmarks of cancers.
8.1. Limitations of the study

It is acknowledged that this study used only a small number of endometrial cancer cell lines which might not represent the complete diversity of endometrial cancer genotypes. In addition the conclusions that can be drawn from the experiments with both breast cancer and ovarian cancer cell lines remain preliminary, although the results obtained in this study do open the door to future studies. Furthermore, it must also be acknowledged that results found in *in vitro* cell lines do not always translate directly to *in vivo* models, where the milieu will include various concentrations of many other cellular factors and hormones that may influence the response of the cancer cells. Such variations and combination cannot really be replicated in the experimental conditions in the laboratory.

I used 2D as representative of the actions of these hormones on cancer cell lines. Using 3D cell model, explant models and genetically modified mice could add more value to this study but because of time constraints, I could not conduct all these experiments. Results from 2D cell monolayer is an early indication of very important knowledge that could lead to further investigation of the roles of obesity-related hormones in the stimulation of PI-3K and JAKs pathways in animal models. Moreover, 2D models provide useful information – most of our knowledge of e.g. pathways, receptor activation, etc has come from 2D in vitro incubations.

Even with these limitations, however, the results from this study remain exciting and can direct further research that might take the initial steps in the move towards an *in vivo* study.
8.2. **Future studies**

*i* Of highest priority will be the replication of these experiments in other endometrial cancer cell lines as well as in non-cancerous normal cells in order to confirm the general attributes of the observations and for comparison.

*ii* The details of the proposed energy sensor, linking between increased proliferation and decreased VEGF secretion, remain to be determined. Thus, there may be value in undertaking experiments in culture media that provide a range of metabolic stress (e.g. hypoxia) and energy supply (e.g. glucose concentrations) in order to justify the relationships between low and high adiponectin concentrations, and increased VEGF secretion from cancer cell lines. Moreover, signalling pathway experiments covering the action of 20 ng/ml adiponectin and PI-3K activation and the expression of AMPK should be completed. These findings open the door for further studies that will provide information on translation or transcription control. Further, details of the intriguing oncogenic and inhibitory pathways activated by 20 ng/ml adiponectin would also be gleaned by studying RNA expression.

*iii* Expansion of the project into *in vivo* conditions would also provide additional information enabling a fuller description of oncogenic processes as well as providing a comparison between *in vitro* and *in vivo* microenvironments. Alternatively, clinical studies might be undertaken in which measurements of hormones in obese women with cancer are compared to other phenotypes including diabetes.

*iv* Treating the endometrial cancer cell line, Ishikawa, with normal chemotherapy treatment such as Paclitaxel, Carboplatin, Doxorubicin and Cisplatin drugs that used on patients to investigate the effects of these drugs on cell proliferation and expression of receptors.

*v* There are several models in animals with defined genotypes, perhaps including the obese Zucker rat that can be used to explore the roles of the hormones in cancer development where the absence of a hormone, or its functional receptor, might be
coupled to orthotopic implantation of tumour cells, with these experiments leading to further insights.

\textit{vi}) It is noteworthy that all four cancer cell lines have oestrogen receptors [432, 536, 537]. To investigate whether these receptors are involved in positive or negative action on cancer cell proliferation, experiments with an oestrogen receptor negative cell line, or blocking the oestrogen receptors with tamoxifen are very important.
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Appendices
Appendices

Figures

Figure 10.1. Comparison of Ishikawa cell growth in the presence of leptin at selected concentrations in two media, M199 and DMEM-F12 which have different glucose concentrations. M199 has 5.5 mM and DMEM-F12 has 17.1 mM glucose concentrations.

Figure 10.2. Random samples for Western blot full molecular weight membrane. The samples in first membrane were Ctrl, Leptin, Adp20, Adp100, Insulin and IGF-I. In second membrane Ctrl, Leptin, Adp100, Insulin, IGF-I, Leptin+Adp100, Insulin+Adp100 and IGF-I+Adp100.
The optical section thickness was 0.5 µm for each image. Red = the identified receptor, Blue = nuclei, and Green = cell membrane (actin). All images are computer-generated from optical sectioning.
Leptin receptor (Ob-R) in Ishikawa
Appendices

Insulin receptor (InsR) in Ishikawa
Appendices

IGFR in Ishikawa
Adiponectin receptors (AdipoR) in Ishikawa
Ob-R in HEC-1A
Appendices

InsR in HEC-1A
Appendices

IGFR in HEC-1A
Appendices

AdipoR in HEC-1A
Appendices

Ob-R in SKOV-3
Appendices

InsR in SKOV-3
Appendices

IGFR in SKOV-3
Appendices

AdipoR in SKOV-3
Appendices

Ob-R in MCF-7
Appendices

InsR in MCF-7
Appendices

IGFR in MCF-7
Appendices

AdipoR in MCF-7
Peptides positive controls
Peptides used as blocker for receptors

Figure 10.3. Immunohistochemical images for receptors of leptin, adiponectin and insulin before and after addition of blockers. For Ob-R and InsR, the primary antibodies concentration were 1/500, and for IGFR was 1/200. Secondary antibodies were used at a concentration of 1/10000 for the three blockers.
Material and Methods protocols

Western blot

Sample preparation

Supernatant medium was removed and cells were washed with 0.5 ml PBS and lysed with 100 μl RIPA lysis buffer prepared from constituents - (50 mmol/ Tris base (2-Amino-2-(hydroxymethyl)-1,3-propanediol, Roche, Cat. No. 708976, Indianapolis, IN, USA), 150 mmol/l NaCl (Sodium chloride, Merck, Cat. No. K 33591704506, Darmstadt, Germany), 5 mmol/l ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate (SDS) (BDH, Cat. No. 44244, UK), 1% NP-40 (acetyl phenol ethylene oxide, Sigma-Aldrich, Cat. No. N-6507), 0.5% sodium deoxycholate, 1 mM NaVO₄, 10% glycerol (Sigma-Aldrich, Cat. No. G 6279-1 litre), and 1 protease inhibitor tablet, pH 7.4). Cell culture plates were left on ice for 20 minutes. Cells were scraped off from the bottom of the wells using a pipette tip and the cell lysate was transferred to an Eppendorf tube and the total protein concentration in a cell lysate was measured by Bio-Rad DC protein assay kit (Cat. No. 500-0113, 500-0114 and 500-0115, Hercules, CA, USA).

SDS gel preparation

Working solutions for SDS-GEL

2M Tris-HCl (pH 8.8), 100 ml.

Tris base (24.2 g) was added to 50 mL Milli-Q water, then concentrated HCl (Sigma-Aldrich) was added slowly to reach pH 8.8 and distilled water was added to a total volume of 100 ml and stored at room temperature.

1M Tris-HCl (pH 6.8), 100 ml.

Tris base (12.1 g) was added to 50 mL Milli-Q water. Concentrated HCl was added slowly to reach pH 6.8 and water was added to a total volume of 100 ml and stored at room temperature.
2M Tris-HCl (pH 7.4), 100 ml.

Tris base (24.2 g) was added to 50 ml Milli-Q water then pH adjusted by adding concentrated HCl slowly to reach pH 7.4 and water was added to a total volume of 100 ml. The solution was stored at room temperature.

10% SDS (w/v), 100 ml.

SDS (10 g) was added to 100 ml Milli-Q water and stored at room temperature.

50% glycerol (v/v), 100 ml.

100% glycerol (50 ml) was added to 100 ml Milli-Q water. Solution was stored at room temperature.

10x sample buffer.

Tris-HCl pH 6.8 (600 µl), 5 ml of 50% Glycerol, 2 ml of 10% SDS, and 2 ml of 1% bromophenol was added to 5.4 ml Milli-Q water to a total volume of 10 ml. The solution was stored at -20°C.

Stock solutions for SDS-GEL

Solution B, 100 ml.

75 ml of 2 M Tris-HCl (pH 8.8) (1.5 M) was added to 4 ml 10% SDS (0.4%) and Milli-Q water was added to a total volume of 100 ml and stored at 4°C.

Solution C, 100 ml.

50 ml of 1 M Tris-HCl (pH 6.8) (0.5 M) was added to 4 ml 10% SDS (0.4%) and Milli-Q water was added to a total volume of 100 ml and stored at 4°C.

10% ammonium persulphate, 5 ml.

Ammonium persulphate (Boehringer, Cat. No. 100 190, Phoenixville, PA, USA) (0.5 g) was dissolved in 5 ml Milli-Q water and stored at -20°C.
Appendices

Electrophoresis buffer, 1 litre.

Tris base 25 mM (3 g), glycine (Sigma-Aldrich, Cat. No. G 8898-1 Kg) 192 mM (14.4 g), and SDS (1 g) were dissolved in Milli-Q water to a total volume of 1 litre and stored at room temperature.

Transfer buffer, 1 litre.

Tris base (1.93 g) and glycine (9 g) were dissolved in Milli-Q water to a total volume of 1 litre and stored at 4°C.

Tris-Buffer-Saline plus Tween-20 (TBS-T).

NaCl 140 mM (8.18) g was dissolved in 10 ml of 2 M Tris-HCl pH (7.4) 20 mM. Then Milli-Q water was added to a total volume of 1 litre. The solution was autoclaved and cooled. Tween-20 (Sigma-Aldrich, Cat. No. P 5927-500 ml) (1 ml) was added and stored at room temperature.

SDS-PAGE Separating Gel (10%), preparing 2 gels.

4.17 ml of Milli-Q water were added to 2.5 ml of solution B (see paragraph above) then Acrylamide/Bis 40% (N,N’-methylene-bis-acrylamide, Bio-Rad, Cat. No. 161-0148) (3.33 ml) was added. Then ammonium persulphate 10% (50 µl) also was added. TEMED (5 µl) was added and mixed before pouring the solution in the apparatus. Water was added to the top of the glass and the apparatus left in a 37°C incubator for 1 hour to polymerise.

SDS-PAGE Separating Gel (12%), preparing 2 gels.

3.5 ml of Milli-Q water were added to 2.5 ml of solution B then 4 ml of acrylamide/bis 40%, 50 µl of ammonium persulphate 10% and TEMED 5 µl were added directly before pouring the solution into the apparatus. Addition of water continued to the top of the glass. The apparatus was placed in a 37°C incubator for 1 hour to polymerise.

SDS-PAGE stacking Gel (7%), prepare 2 gels.

2 ml of Milli-Q water were added to 1 ml of solution C then acrylamide/bis 40 % (1 ml), ammonium persulphate 10 % (40 µl), TEMED (5 µl) were added and mixed together directly before pouring the solution into the apparatus. The corners were maintained in a
proper position on the top of the glass and the apparatus was left at room temperature for 30
minutes to polymerise.

**Preparation of cell lysate for SDS-GEL**

Sample buffer (10 µl of 10x) was added to each 100 µl of cell lysate. Tubes were boiled
at 100°C for 10 minutes then cooled down and the tubes were placed on ice. When tubes
reached room temperature they were centrifuged at 8040g for 7 minutes. Samples were kept
on ice until loading. The samples were stored in a freezer for further experiments.

The samples containing 10 µg of the total proteins were loaded in each well of SDS-
GEL as shown below.

<table>
<thead>
<tr>
<th>MW ladder</th>
<th>Ctrl</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>Dye</th>
</tr>
</thead>
</table>

The marker contained 4 µl Precision Plus protein Dual color standard (Bio-Rad, Cat.
No. 161-0374) and 1 µl Magic Mark Western standard (Invitrogen, Cat. No. P/N LC5602)
and were added to the gel well.

Following gel electrophoresis, proteins were electrophoretically transferred to
polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Cat. No. 162-0177) in ice cold
transfer buffer at 100 Volt for 1 hour. Then membranes were blocked with the blocker
solution that was appropriate for each antibody (Table 2.1) for 1 hour at room temperature.
Specific immuno-detections were carried out by incubation with relevant primary antibodies.

The PVDF membranes were incubated with relevant primary antibodies overnight at
4°C. After three washes with TBS-T buffer, the membranes were incubated for 90 minutes
with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse
immunoglobulin G) (Table 2.1), diluted 1:10,000 in TBS-T at room temperature. Antigens
were revealed using ECL developing solution (Amersham, ECL Prime Western Blotting
Detection Reagent, ge-9466588; GE Healthcare, Little Chalfont, UK).

The presence of proteins was imaged and captured using UVITEC Cambridge / Alliance
4.7 device and measured by UV band software programme.
LEPTIN AND ADIPONECTIN MODULATE PROLIFERATION AND VEGF SECRETION IN ENDOMETRIAL CANCER CELLS

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Introduction
Obesity is a significant risk factor in the development of endometrial cancer. Furthermore, morbid obesity is associated with a marked increased risk of and death from endometrial cancer. The mass of adipose tissue is different in obese and lean individuals. Leptin and adiponectin are derived from adipose tissue (Fig. 1), and high levels of leptin and low levels of adiponectin are associated with endometrial cancer growth. In this study we investigated the effects of leptin and adiponectin, on cultured cells of an endometrial adenocarcinoma cell line (Ishikawa with PTEN mutation).

Hypothesis

- Leptin has tumorigenic effects on endometrial cancer cells and adiponectin has antitumorigenic effects on endometrial cancer.

Method
Endometrial adenocarcinoma cell line (Ishikawa) was treated with single drug of leptin 50ng/ml, adiponectin 100ng/ml, adiponectin 20ng/ml, and combination of leptin with high and low concentrations of adiponectin respectively. The results in figure 2 and 3 show the effects of these adipokines on Ishikawa cell proliferation and VEGF secretion.

Results

Graphs A, B, and C show significant increase in endometrial cancer cell number for Ishikawa cells exposed to leptin 50 ng/ml, low adiponectin concentration 20 ng/ml and combination of leptin 50 ng/ml and adiponectin 20 ng/ml respectively compared to control.

Graph D shows a significant decline in cancer cell number for Ishikawa cells treated with high concentration of adiponectin 100 ng/ml. However, an elevation in cancer cell number occur when cell exposed to a combination of leptin 50 ng/ml and adiponectin 100 ng/ml up to control level (graph E).

Graphs F, leptin 50 ng/ml reduces VEGF secretion significantly. However, graph G and I show that both adiponectin concentrations increased VEGF secretion significantly.

Graph H, presence of leptin in combination with low adiponectin concentration 20 ng/ml increased VEGF secretion but not additive increase compared to control. That in graph I, the combination of leptin with high concentration of adiponectin 100 ng/ml stoped the effect of adiponectin and VEGF secretion level remained within the control level.

CONCLUSION

Leptin:
Increases cell number but does not increases VEGF secretion.

Adiponectin:
Decreases cell number at high concentrations but stimulates cells proliferation at low concentrations. However, both concentrations increase VEGF secretion.