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# **Globular adiponectin, acting via adiponectin receptor-1, inhibits leptin-stimulated oesophageal adenocarcinoma cell proliferation**

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## **Running title**

Adiponectin in oesophageal adenocarcinoma

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## **Key words**

adiponectin, AMP-activated kinase, cell proliferation, leptin, obesity, oesophageal  
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**Abstract**

Obesity increases the risk of developing several cancers including oesophageal adenocarcinoma (OAC). Obesity is characterized by hyperleptinaemia and hypoadiponectinaemia: we have hypothesized that these hormonal factors may contribute to the progression of OAC. We have examined the effects of leptin and adiponectin on proliferation of OAC cells. Leptin stimulated proliferation in 4 different OAC lines (OE33, OE19, BIC-1 and FLO) and this was inhibited by globular but not full length adiponectin. All 4 OAC lines expressed both adiponectin-receptor isoforms (AdipoR1 and AdipoR2). Globular adiponectin also inhibited leptin-induced proliferation in rat IEC-18 cells which only expressed AdipoR1. Specific inhibitors of 5'-AMP-activated protein kinase (Compound C) and serine/threonine phosphatases (okadaic acid) and a specific siRNA to AdipoR1 blocked the anti-proliferative effects of adiponectin. Adiponectin inhibited leptin-induced Akt phosphorylation; this action was sensitive to okadaic acid but not to Compound C. Adiponectin deficiency may contribute to the promotion of OAC in obesity.

**Abbreviations used:** adipo, adiponectin; adipoR, adiponectin receptor; AMP, adenosine monophosphate; AMPK, 5'-AMP-activated protein kinase; BrdU, bromodeoxyuridine; Compound C, 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]-pyrimidine; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; OAC, oesophageal adenocarcinoma; RT-PCR, reverse transcription polymerase chain reaction.

## Introduction

Obesity is becoming an increasingly important health problem in the developed world. Obesity is associated with an increased risk of developing several cancers including oesophageal adenocarcinoma (OAC)(Lagergren et al. 1999), as well as the adverse metabolic effects which predispose to diabetes mellitus and atherosclerosis. It is likely that the relationship between body composition and morbidity is multifactorial. Lifestyle, diet and genetic factors probably contribute to the adverse health effects of obesity, and fat tissue may theoretically act as a storage reservoir for toxins that could contribute to the pro-carcinogenic effects of obesity (Calle and Thun, 2004). However it has now become recognised that adipose tissue is an active contributor to the inflammatory and metabolic consequences of obesity. Fat cells secrete a wide range of mediators (collectively called adipocytokines), including leptin, tumor necrosis factor- $\alpha$ , IL-6 and resistin which can have an influence far from the adipose tissue itself (Baratta, 2002). These may provide a means whereby body composition influences the development and progression of cancers at distant sites.

Serum levels of leptin increase in proportion to body fat mass and most overweight men and women have significantly increased levels; leptin deficiency is a very rare cause of human obesity (Dieudonne et al. 2006; Considine et al. 1996). The metabolic effects of leptin in relation to body weight and energy balance have been well-described, although the mechanisms underlying the relative central resistance to leptin in obesity remain unclear (Fam et al. 2007; Wilsey et al. 2003; Friedman and Halaas, 1998). In addition, it has recently become apparent that leptin may be an important endocrine factor in driving the promotion of several obesity-related cancers. We have shown that leptin increases proliferation and inhibits apoptosis in OAC cells (Ogunwobi et al. 2006) and leptin also stimulates proliferation in other obesity-related cancer cell lines (pancreas, breast, prostate, colon) (Somasundar et al. 2003; Frankenberry et al. 2004; Ogunwobi and Beales, 2007; Ogunwobi et al. 2006). In keeping with this, leptin levels have been shown to be an independent risk factor for colon and endometrial cancers (Petridou et al. 2002; Stattin et al. 2004).

Adiponectin is another recently described adipocytokine: in contrast to leptin secretion of adiponectin and serum levels are inversely related to body fat mass (Arita et al. 1999; Dieudonne et al. 2006).

Adiponectin has beneficial effects on metabolism; in general these are opposite to the effects of leptin: it enhances glucose uptake and fatty acid oxidation and acts as an insulin sensitizer (Combs et al. 2004; Yamauchi et al. 2003) and relative adiponectin deficiency may itself contribute to the adverse consequences of obesity.

Adiponectin is abundant in serum (circulating levels are approximately 1 – 15  $\mu\text{g} / \text{ml}$ ), one study showed that levels in obese subjects were  $3.7 \pm 3.2 \mu\text{g} / \text{ml}$  compared to  $8.9 \pm 5.4 \mu\text{g} / \text{ml}$  in normal body weight individuals (Arita et al. 1999). Other studies have given similar results although the exact values within this general range have varied depending on the assays used; levels in obese patients are typically only 50 % of those seen in those of lean body weight.

There are 2 forms of adiponectin; the majority circulates as the full length form (fAdipo), which consists of an N-terminal sequence, a complement C1q-like domain and a C-terminal globular domain (Kadowaki and Yamauchi 2005). A truncated form containing only the C-terminal portion (globular adiponectin, gAdipo) is also produced by proteolytic cleavage and is biologically active, although the relative serum levels of the 2 forms in different disease states have not been investigated to any great extent and fAdipo may be cleaved within inflamed tissues to gAdipo by elastase (Waki et al. 2006).

As adiponectin is regulated inversely to leptin and the two appear to have opposing metabolic actions, we have hypothesized that adiponectin may oppose the growth promoting effects of leptin in OAC cells. There are some preliminary data suggesting that adiponectin may have anti-cancer actions (Dieudonne et al. 2006) and a low adiponectin level has been reported to be an independent risk factor for cancers of the stomach, colon, breast and endometrium (Dal Maso et al. 2004; Ishikawa et al. 2005; Kadowaki and Yamauchi, 2005). To our knowledge the specific relationship between serum adiponectin levels and OAC has not yet been investigated.

Therefore we have examined the effects of adiponectin on leptin-induced proliferation in OAC cells. This is an obesity-related cancer and we have previously shown that leptin induces a complex sequence of signalling reactions (involving Akt and all 3 mitogen-activated protein kinases)(Beales and Ogunwobi, 2007; Beales et al. 2007; Ogunwobi et al. 2006) leading to proliferation and adiponectin may block signalling via these pathways. We initially determined the presence of the 2 known adiponectin receptors. Some data have shown that some of the metabolic effects of adiponectin are mediated via the 5'-AMP-activated protein kinase (AMPK pathway)(Yamauchi et al. 2003) and there are preliminary data suggesting this pathway may have anti-cancer effects (although the exact mechanisms are unclear)(Hardie, 2004), therefore we have examined the involvement of AMPK using the specific AMPK inhibitor Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]-pyrimidine)(King et al. 2006). As many of the signalling steps activated by leptin involve serine/threonine phosphorylation we also hypothesized that adiponectin may interfere with leptin-induced signalling by activating phosphatases and we have examined the effect of the serine/threonine phosphatase inhibitor okadaic acid (Todisco et al. 1995). We have also utilized the rat IEC-18 non-tumorigenic cell line as model to test the pathways involved.

## Methods and methods

### *Cell culture and proliferation*

The human oesophageal adenocarcinoma cell lines OE33, OE19 (both obtained from the European Collection of Cell Cultures (Wiltshire, UK)) and BIC-1 and FLO (kindly provided by Dr David Beer, University of Michigan, USA) and the IEC-18 non-tumorigenic rat normal small intestinal cell line (ECACC) were cultured and seeded into 48 well (for MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, Poole, UK) assay) and 96 well (for BrdU incorporation) plates as described previously (Ogunwobi et al. 2006). Forty eight hours after subculturing, cells were serum starved for 24 hours and then treated as appropriate with recombinant human leptin (Bachem, St Helens, UK), recombinant human full length adiponectin (RandD Systems, Abingdon, UK) or recombinant human globular adiponectin (Peprotech, London, UK). In co-treatment experiments adiponectin was added 30 minutes prior to the leptin. A leptin concentration of 10 nM was used as we have previously shown this to reliably maximally stimulate proliferation. After 24 hours relative cell numbers were assessed using the MTT and BrdU incorporation assays as described (Ogunwobi et al. 2006). The BrdU assay kit was obtained from Roche (Burgess Hill, UK). In order to examine the effect of AMPK, the specific AMPK inhibitor Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]-pyrimidine)(40  $\mu$ M)(Merck, Nottingham, UK)(King et al. 2006) was added to cells 60 minutes prior to the addition of any other substances. Where appropriate cells were also pretreated for 60 minutes with the serine/threonine phosphatase inhibitor okadaic acid (10 nM)(Merck) (Todisco et al. 1995).

### *RT-PCR*

Total RNA was isolated from cell lysates and RT-PCR performed and products visualised as described previously (Ogunwobi et al. 2006). The primers used were (all 5'  $\rightarrow$  3'): human AdipoR1 sense ACGTTGGAGAGTCATCCCGTAT, anti-sense TCTTGAAGCAAGCCCGAAAG, human AdipoR2 sense AGCCTCTATA-TCACCGGAGCTG, anti-sense GCTGATGAGAGTGAAACCAGATGT, rat AdipoR1 sense CTTCTACTGCTCCCC-ACAGC, anti-

sense TCCCAGGAACACTCC-TGCTC' rat AdipoR2 sense CCACACAA-CACAAGAATCCG, anti-sense CCCTTCT-TCTTGGGAGAATGG (Oana et al. 2005; Caminos et al. 2005)(all synthesized by Invitrogen, Paisley, UK).

#### *Western blotting*

Western blotting and detection were performed on whole cell lysates as described previously (Ogunwobi et al. 2006). Primary polyclonal antibodies to AdipoR1 and AdipoR2 (Abcam, Cambridge, UK), and leptin receptor isoforms (H-300, Santa Cruz Biotechnology, CA, USA, which recognises both Ob-Ra and Ob-Rb) were used at 1:200 and antibodies to total and phospho-AMPK (threonine-172) (Cell Signalling Technology, Hitchin, UK) were used at 1:1000, where appropriate equal loading was confirmed by reprobing with an antibody against  $\beta$ -actin (Santa Cruz). Detection was with horseradish peroxidase-conjugated secondary antibodies (1:2000) (Santa Cruz).

#### *Adiponectin receptor RNA interference*

OE33 cells were seeded at  $1 \times 10^4$  cells per well into 48 well plates and 24 hours after subculture were transfected with 60 pmol AdipoR1 siRNA using Oligofectamine<sup>®</sup> (Invitrogen) following the manufacturer's instructions. The AdipoR1 siRNA sequence was 5'-GCUAGAAGAGAAGGGCAAATT -3' and the control scrambled siRNA sequence was 5'-UUUGCCCUUCUCUUCUAGCTT -3'. Both were obtained from Invitrogen and the sequences were designed using the Invitrogen BLOCK-iT<sup>™</sup> software. Cells were then cultured for 72 hours and serum-starved before assessing responses to adiponectin.

#### *Akt phosphorylation*

OE33 cells were serum-starved for 24 hours and then pretreated with either okadaic acid or compound C for 60 minutes before treating with gAd for 30 minutes and then stimulating with leptin. Serine-473 phosphorylation of Akt was quantified as previously described in formalin-fixed cells

using a commercially available ELISA (Akt FACE™ kit, Active Motif, Rixensart, Belgium) (Beales and Ogunwobi, 2006).

*Statistical analysis*

All proliferation experiments were carried out in triplicate or quadruplicate wells. Akt phosphorylation was quantified in triplicate wells; all were compared to untreated control cell on the same tissue culture plate. The mean of the results from one experiment was regarded as N=1 and each experiment was repeated 3-6 times. Analysis of variance was used to analyse dose-response curves and Student's paired t-test to compare the effects of individual treatments. A *P* value of 0.05 was regarded as significant.

## Results

Using reverse transcriptase polymerase chain reaction we detected mRNA expression for both AdipoR1 and AdipoR2 in all 4 oesophageal adenocarcinoma cell lines (fig 1a). Consistent with this, protein expression of both receptor isoforms in all 4 oesophageal cell lines was confirmed with western blotting (fig 1b). The rat IEC-18 ileal cell line expressed only the AdipoR1 isoform which was detectable with both RT-PCR (fig 1a) and western blotting (fig 1b); no expression of AdipoR2 was seen at either the mRNA or protein level. Western blotting with two other different anti-AdipoR2 antibodies (Phoenix Europe GmbH, Karlsruhe, Germany and Alpha Diagnostic, TX, USA) also failed to detect AdipoR2 in IEC-18 cells (data not shown). All 4 OEC cells lines and the IEC-18 cell line expressed both the long (Ob-Rb) and short (Ob-Ra) receptor forms as assessed by western blotting (fig 1c).

Neither full length nor globular adiponectin alone ( $0.1 - 10 \mu\text{g} / \text{ml}$ ) had any significant positive or negative effect on relative cell numbers of unstimulated OE33, OE19 or IEC-18 cells ( $N=4$ , data not shown).

In contrast globular adiponectin dose-dependently reduced the proliferative response of OE33 cells to  $10 \text{ nM}$  leptin (fig 2a). A similar inhibition of the proliferative response to leptin was seen when both OE19 (fig 2b) and IE-18 cells (fig 2c) were co-treated with globular adiponectin. Both  $1$  and  $10 \mu\text{g} / \text{ml}$  gAdipo significantly ameliorated leptin-induced proliferation and the higher concentration essentially abolished leptin-driven proliferation in OE19 and IEC-18 cells. Full length adiponectin had no effect on leptin-stimulated proliferation over the concentration range  $0.1 - 10 \mu\text{g} / \text{ml}$  in any cell type (data not shown).

To confirm that the reduction in relative cell numbers seen in leptin-stimulated adiponectin-treated cells was due to inhibition of proliferation we carried out complementary experiments using a BrdU incorporation assay as a read-out of new DNA synthesis. In all 4 oesophageal adenocarcinoma cell lines and the IEC-18 rat ileal cell line gAd  $10 \mu\text{g} / \text{ml}$  significantly blocked leptin-induced

proliferation (fig 3). The degree of inhibition varied slightly between the cell lines, being greatest (essentially abolishing leptin-induced proliferation) in the OE33 and IEC-18 cells and least in the FLO cells (57% inhibition).

Transfection of a specific siRNA for human AdipoR1 into OE33 cells was effective at reducing protein expression of AdipoR1 (fig 4a) and also prevented the inhibitory effect of gAd on leptin-induced proliferation (fig 4b). The control siRNA sequence had no effect on either AdipoR1 protein expression detected by western blotting or on the inhibitory effect of gAd on proliferation.

Pretreatment of OE33 cells with globular adiponectin at the proliferation-inhibitory concentration of 10  $\mu\text{g} / \text{ml}$  did not affect overall leptin receptor protein levels (fig 4c).

Globular adiponectin significantly increased the activation of AMPK, as assessed by western blotting against the active phosphorylated form; no changes in total AMPK levels were detected. Increased AMPK phosphorylation was detectable 5 minutes after the addition of adiponectin and levels appeared to have reduced to basal levels after 30 minutes. Leptin itself did not increase AMPK activation (fig 5a). Pretreating the cells with the AMPK inhibitor compound C prevented the activation of AMPK by globular adiponectin without affecting basal AMPK phosphorylation (fig 5b). Consistent with this, compound C also significantly ameliorated the inhibitory effect of gAdipo on leptin-induced proliferation (fig 5c) but compound C alone had no significant effects on basal or leptin-induced relative cell numbers.

The serine/threonine phosphatase inhibitor okadaic acid also abolished the growth inhibitory effects of gAdipo without itself significantly altering basal or leptin-stimulated cell numbers (fig 6a).

Leptin potently increased serine-473 phosphorylation of Akt, increasing phosphorylation by 193 % above unstimulated basal (fig 6b). Globular adiponectin (10  $\mu\text{g} / \text{ml}$ ) significantly blunted leptin-induced Akt phosphorylation (reduced to 55% above basal as quantified by the specific ELISA).

Pretreatment of OE33 cells with okadaic acid abolished this inhibitory action of gAd but pre-

treatment with compound C did not ameliorate the effect of gAd in reducing Akt phosphorylation (fig 6b).

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## Discussion

In this study we have shown that gAdipo inhibits leptin-induced cell proliferation via adiponectin receptor-1 and requires activation of AMPK and serine/threonine phosphatase activity. This suggests that relative adiponectin deficiency may contribute to the increased proliferation that predisposes to malignant transformation and the progression of cancers in obesity.

These effects against leptin-induced proliferation were mediated by AdipoR1: an inhibitory effect of gAdipo was seen in the IEC-18 cell line which only expressed the type 1 receptor and the specific siRNA against AdipoR1 abolished the effect in the OE33 cells. We only showed an inhibitory effect with gAdipo and our data are consistent with previous data showing that AdipoR1 is the main functional receptor for gAdipo and the effects of fAdipo are predominantly mediated via AdipoR2, this presumably explains why different effects have been attributed to the two forms of the hormone (Waki et al. 2003; Ogunwobi and Beales, 2006; Kadowaki and Yamauchi, 2005). Some effects of adiponectin may be mediated by non-receptor mechanisms via the C1q-like domain (Kadowaki and Yamauchi, 2005) and as adiponectin has structural similarity to TNF- $\alpha$  it has been suggested that receptor cross-reactivity could occur (Pajvani et al. 2003). However our AdipoR1 RNA interference data suggest that the effects demonstrated here are mediated via the specific AdipoR1 receptor. One study has shown that both AdipoR1 and AdipoR2 were required for the inhibition of cell proliferation by adiponectin in AZ521 and HGC27 gastric cancer cells, but that study did not explore the intracellular mechanisms responsible and used full length adiponectin at a much higher, supra-physiological concentration of 30  $\mu\text{g}/\text{ml}$  to inhibit epidermal-growth factor driven proliferation (Ishikawa et al. 2007). Further recent studies have shown that full length adiponectin (the only form tested) inhibited the growth of MCF7 breast cancer cells, these cells expressed both AdipoR1 and AdipoR2, but the specific involvement of each receptor isoform was not investigated (Dieudonne et al. 2006).

All of the OAC cell lines studied here expressed both AdipoR1 and AdipoR2: our data suggest that AdipoR1 mediates the proliferation inhibitory effects of globular adiponectin and that the presence of AdipoR1 alone was sufficient for this effect. We have not yet examined the specific function(s) of AdipoR2 in OAC cells but it is conceivable that this receptor may contribute to other anti-cancer effects such as regulation of apoptosis or invasion. Our data suggest that the inhibition of leptin-induced proliferation resides in the truncated globular adiponectin form; we have not specifically examined the effects of high-molecular weight adiponectin complexes. Native (and recombinant) full length adiponectin has a multimeric structure: polypeptides form homotrimers and these can link into hexamers and larger complexes (Pajvani et al. 2003). In some systems the different high molecular weight forms have different biological actions (Waki et al. 2003; Haugen and Drevon, 2007): however it appears that only full length adiponectin forms into such multimers and such complexes would not be expected to be directly relevant to our results with globular adiponectin. Whether these effects of adiponectin are cell line or tissue specific will require further investigation. Similarly although considerable published data now support the concept of leptin as a growth factor for benign and malignant cells (Attoub et al. 2000; Hardwick et al. 2001), further studies examining the function of the receptors for both leptin and adiponectin in response to the prolonged alterations in circulating peptide levels characteristic of obesity will be informative.

The concentrations of adiponectin that were effective were equivalent to those seen in normal body weight individuals suggesting that our data may be physiologically relevant. We have shown similar effects in 4 different OAC lines and a non-tumorigenic intestinal cell line. In this study we have utilised the IEC cell line as a convenient model for AdipoR signalling (although oesophageal cancers derived from metaplastic oesophageal epithelium do have many intestinal features)(Fitzgerald et al. 1997), and it is important to note that the inhibitory effects of gAdipoR were seen in both tumorigenic and non-tumorigenic cell lines: we propose that adiponectin deficiency may promote the progression of OAC but further studies examining AdipoR expression *in vivo* will be required to explore this further.

Although 2 adiponectin receptor isoforms have been described, relatively little is known about the signal transduction mechanisms responsible for the effects (Kadowaki and Yamauchi, 2005). The anti-proliferative effects of gAdipo involved separate activation of AMPK and serine/threonine phosphatase activity. The anti-proliferative action of gAdipo was abolished by specific inhibition of either AMPK or serine/threonine phosphatases. Compound C has been used as selective inhibitor of AMPK, concentrations of 20 – 60  $\mu$ M have been effective in cell-based studies (Haugen and Drevon, 2007; King et al. 2006). In this study 40  $\mu$ M blocked both the activation of AMPK and the inhibition of proliferation by gAdipo. Although leptin has been reported to activate AMPK in some systems (Kadowaki and Yamauchi, 2005), we did not detect any increased AMPK phosphorylation consequent on leptin stimulation in OE33 cells.

To further characterise the mechanisms underlying the inhibitory action of adiponectin we examined the effect on leptin-induced Akt phosphorylation. We have previously shown that leptin-induced Akt activation is essential for the cell proliferation and anti-apoptotic actions in OAC cells (Beales and Ogunwobi, 2007; Ogunwobi et al. 2006). Adiponectin significantly reduced leptin-induced phosphorylation of Akt at the serine-473 position and this effect was blocked by okadaic acid but not by compound C, suggesting that although the anti-proliferative action of adiponectin requires AMPK, this pathway is activated in parallel to activation of phosphatase activity and has different intracellular targets. This would also be consistent with the time course of activation of AMPK: gAdipo activation of AMPK had reduced to basal by the time of experimental stimulation of the cells with leptin, yet gAdipo pre-treatment clearly blocked Akt phosphorylation. Suggesting that AMPK activation is not directly involved in the inhibition of Akt phosphorylation and that other downstream targets of AMPK are also important in the growth inhibitory actions of globular adiponectin. Such parallel activation has been described using phenformin in SH-SY5Y neuroblastoma cells and rat hippocampal neurones: phenformin activated AMPK and reduced Akt phosphorylation but inhibitor

studies demonstrated that AMPK was not implicated in the inhibition of Akt phosphorylation (King et al. 2006).

At present the specific serine/threonine phosphatase(s) activated by gAdipo are unknown but in view of the potential for anticancer actions, we believe this will be a fruitful avenue of further investigation. Similarly in this study we have only specifically examined the effect of adiponectin on Akt phosphorylation, it is possible that by activating serine/threonine phosphatases adiponectin inhibits leptin-induced signalling via the mitogen-activated protein kinases and protein kinase C cascades (Ogunwobi et al. 2006).

In this study we showed that leptin stimulated proliferation in 4 different OAC cell lines and the non-neoplastic rat ileal cell line, all the cell lines expressed both the long and short forms of the leptin receptor. The long (Ob-Rb) isoform is usually held as responsible for leptin-mediated signalling as it contains all the elements necessary for downstream signalling, however the truncated Ob-Ra form has been reported to be capable of activating some downstream signalling cascades (Tartaglia, 1997). Globular adiponectin pre-treatment did not alter overall protein levels of the short and long leptin receptor isoforms; of course this does not exclude more subtle changes in leptin receptor function, such as ligand-binding, cellular localisation or early signalling being influenced by gAdipo.

The intracellular target of the anti-proliferative action of AMPK in OAC cells is also unclear. Some data have shown that AMPK may reduce cell proliferation by inhibiting mTOR or via a p53-dependant pathway (Hardie, 2004), however we have previously shown that leptin-induced proliferation in OE33 cells is mTOR-independent (Ogunwobi et al. 2006) and here we were able to demonstrate inhibitory effects in cells with both mutant p53 (FLO, BIC-1) and wild-type (OE33) cells as well as the normal rat ileal IEC-18 cell line (Mahidhara et al. 2005), suggesting that there must be alternative targets.

In conclusion, we have shown that globular adiponectin inhibits leptin-induced proliferation in oesophageal adenocarcinoma cells via AdipoR1 and activation of AMPK and serine/threonine phosphatases. The phosphatase activity but not AMPK kinase leads to blunting of leptin-induced Akt activation. Adiponectin may function as a physiological inhibitor of the growth promoting effects of leptin. Relative adiponectin deficiency combined with hyperleptinaemia may contribute to the progression of oesophageal adenocarcinoma and other obesity-associated cancers.

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**Figure legends**

**Figure 1: Expression of adiponectin receptors in human oesophageal adenocarcinoma cell lines and the rat IEC-18 intestinal cells line. (a)** RT-PCR analysis of total RNA extracted from 4 human oesophageal cancer cells line (OE33, OE19, BIC-1, FLO) and the rat IEC-18 intestinal cell line using specific primers for AdipoR1 and AdipoR2. **(b)** Protein expression of AdipoR1 and AdipoR2 in the same 5 cell lines. Whole cell lysates were subjected to Western blot analysis using specific antibodies as described under Materials and methods. A single representative experiment (of 3) is shown for each. **(c)** Protein expression of Ob-Ra and Ob-Rb leptin receptors in the same 5 cell lines. Whole cell lysates were subjected to Western blot analysis using a specific antibody as described under Materials and methods. A single representative experiment (of 3) is shown.

**Figure 2: Dose response of effect of globular adiponectin on leptin-induced cell proliferation.**

**(a)** OE33, **(b)** OE19 and **(c)** IEC-18 cells were serum starved for 24 hours and then treated with increasing concentrations of globular adiponectin and then stimulated with 10 nM leptin. After 24 hours relative cell numbers were quantified using a MTT assay. Results expressed as mean  $\pm$  SEM as % of unstimulated control cells, N = 6, \*  $P < 0.05$ , \*\*  $P < 0.01$  vs leptin-stimulated.

**Figure 3: Globular adiponectin inhibits leptin-induced proliferation in OAC and IEC-18 cells.**

OE33, OE19, BIC-1, FLO and IEC-18 cells were serum starved for 24 hours and then treated with 10  $\mu$ g/ml of globular adiponectin (gAD) and then stimulated with 10 nM leptin. After 24 hours DNA synthesis was quantified using a BrdU incorporation assay. Results expressed as mean  $\pm$  SEM as % of unstimulated control cells, N = 3, \*  $P < 0.05$  vs unstimulated basal, \*\*  $P < 0.05$  vs leptin-stimulated.

**Figure 4: Globular adiponectin inhibits leptin-induced proliferation via AdipoR1.**

**(a)** 50% confluent OE33 cells in 6 well plates were transfected with 100 nM specific AdipoR1 siRNA or control siRNA, after 72 hours cells were lysed and AdipoR1 and  $\beta$ -actin protein expression

were examined by Western blotting. **(b)** OE33 cells in 48 well plates were transfected with 100 nM siRNA and after 72 hours cells were serum-starved for 24 hours and then treated with of globular adiponectin (gAD) and then stimulated with 10 nM leptin. Relative cell numbers were quantified after 24 hours using the MTT assay. Results expressed as mean  $\pm$  SEM as % of unstimulated control cells, N = 3, \*  $P < 0.05$  vs leptin-stimulated **(c)** OE33 cells were stimulated for 30 minutes with 10  $\mu$ g/ml globular and cells were lysed and leptin receptor expression examined by western blotting. Western blots are representative of 3 experiments.

**Figure 5: Adiponectin inhibits leptin-induced proliferation via AMPK.**

**(a)** Serum-starved OE33 cells were treated with 10  $\mu$ g/ml globular adiponectin (gAdipo) or 10 nM leptin and cells lysed at the indicated times (0 – 30 mins or 24 hours). Phosphorylated and total AMPK were examined using western blotting. **(b)** Serum-starved OE33 cells were pre-treated with the AMPK inhibitor Compound C (Com C, 40  $\mu$ M) for 60 minutes and then treated with globular adiponectin (gAdipo, 10  $\mu$ g/ml). Subsequently cells were lysed and total and phosphorylated AMPK examined by western blotting. Western blots are representative of 3 preparations. **(c)** Serum-starved OE33 cells were pretreated with Compound C (40  $\mu$ M), then treated with globular adiponectin (gAD, 10  $\mu$ g/ml) for 30 minutes before stimulating with 10 nM leptin. After 24 hours relative cell numbers were measured using the MTT assay. Results expressed as mean  $\pm$  SEM as % of unstimulated control cells, N = 3, \*  $P < 0.05$  vs leptin-stimulated.

**Figure 6: Adiponectin inhibits leptin-induced proliferation via serine/threonine phosphatase mediated inhibition of Akt activation.**

**(a)** Serum-starved OE33 cells were pretreated with the serine/threonine phosphatase inhibitor okadaic acid (10 nM) for 60 minutes and then treated with globular adiponectin 10  $\mu$ g/ml (gAD) and / or 10 nM leptin. After 24 hours relative cell numbers were measured using the MTT assay. Results expressed as mean  $\pm$  SEM as % of unstimulated control cells, N = 3, \*  $P < 0.05$  vs leptin-stimulated. **(b)** Serum-starved OE33 cells were pretreated with Compound C (40  $\mu$ M) or okadaic acid (10 nM)

for 60 mins, then treated with globular adiponectin 10  $\mu\text{g}/\text{ml}$  for 30 minutes before stimulating with 10 nM leptin. After 10 minutes cells were formalin fixed and total Akt and serine-473 phosphorylated Akt were quantified using a cell-based ELISA. Subsequent crystal violet staining was used to normalise results for cell number. Results expressed as phospho-Akt / total Akt relative to untreated control cells. Mean  $\pm$  SEM, N = 3, \*  $P < 0.05$  vs leptin-stimulated.

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