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ORIGINAL ARTICLE

Insulin resistance in vascular endothelial cells promotes intestinal tumour formation

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The risk of several cancers, including colorectal cancer, is increased in patients with obesity and type 2 diabetes, conditions characterised by hyperinsulinaemia and insulin resistance. Because hyperinsulinaemia itself is an independent risk factor for cancer development, we examined tissue-specific insulin action in intestinal tumour formation. *In vitro*, insulin increased proliferation of intestinal tumour epithelial cells by almost two-fold in primary culture of tumour cells from *Apc*$^{Min/+}$ mice. Surprisingly, targeted deletion of insulin receptors in intestinal epithelial cells in *Apc*$^{Min/+}$ mice did not change intestinal tumour number or size distribution on either a low or high-fat diet. We therefore asked whether cells in the tumour stroma might explain the association between tumour formation and insulin resistance. To this end, we generated *Apc*$^{Min/+}$ mice with loss of insulin receptors in vascular endothelial cells. Strikingly, these mice had 42% more intestinal tumours than controls, no change in tumour angiogenesis, but increased expression of vascular cell adhesion molecule-1 (VCAM-1) in primary culture of tumour endothelial cells. Insulin decreased VCAM-1 expression and leukocyte adhesion in quiescent tumour endothelial cells with intact insulin receptors and partly prevented increases in VCAM-1 and leukocyte adhesion after treatment with tumour necrosis factor-α. Knockout of insulin receptors in endothelial cells also increased leukocyte adhesion in mesenteric venules and increased the frequency of neutrophils in tumours. We conclude that although insulin is mitogenic for intestinal tumour cells *in vitro*, impaired insulin action in the tumour microenvironment may be more important in conditions where hyperinsulinaemia is secondary to insulin resistance. Insulin resistance in tumour endothelial cells produces an activated, proinflammatory state that promotes tumorigenesis. Improvement of endothelial dysfunction may reduce colorectal cancer risk in patients with obesity and type 2 diabetes.

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INTRODUCTION

Obesity and type 2 diabetes are independent risk factors for the development of colorectal cancer and other malignancies.[1–3] Hyperinsulinaemia is a central feature of these metabolic diseases and is itself an independent risk factor for tumour formation,[4] including colorectal cancer[5] and colorectal adenomas.[6] Since a mitogenic action of insulin is well described,[4,7–9] it has been proposed that the link between obesity and cancer can be explained by a growth-promoting effect of increased insulin action directly on tumour cells.[4,10,11] However, this hypothesis has not been directly tested in the context of intestinal tumour formation.

Chronic inflammation has often been proposed as another possible contributor to the relationship between obesity and cancer.[12] Diet-induced obesity changes the immune cells resident in the intestine[13] and myeloid cells can promote tumour growth by releasing cytokines like tumour necrosis factor-α (TNF-α) or interleukin-6 or by production of reactive oxygen or nitrogen species.[14] We previously found that suppression of the proinflammatory factor prostaglandin E_2 suppressed the stromal inflammatory response and reduced intestinal tumour formation.[15] Anti-inflammatory treatment has been effective in clinical translation of such findings as shown by prevention of colorectal adenomas with the cyclo-oxygenase-2 inhibitor celecoxib[16] and reduction of colorectal cancer risk with long-term aspirin treatment.[17]

Intriguingly, impaired insulin signalling may contribute to the chronic inflammation observed in obesity. We previously demonstrated that endothelial-specific deletion of the insulin receptor results in a marked increase in the burden of atherosclerosis in the *Apoe* null mouse.[18] Loss of vascular endothelial cell insulin signalling also resulted in a pronounced increase in leukocyte rolling and adhesion in the intestinal microcirculation observed during intravital microscopy of mesenteric venules.[18] This supports a pro-inflammatory effect of endothelial cell insulin resistance in the intestine akin to that observed in atherosclerotic plaques. Importantly, endothelial cell insulin resistance occurs early in the development of diet-induced obesity in animal models[19,20] and is present in humans with obesity or type 2 diabetes.[21–24] Therefore, impaired insulin signalling in endothelial cells could contribute to the increased risk of colon cancer in obesity by promoting chronic inflammation.

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In this study, we examined the contribution of epithelial and endothelial insulin signalling to the development of endogenous intestinal tumour formation. Tumour-prone *Apc*$^{Min/+}$ mice were modified by tissue-specific knockout of the insulin receptor in intestinal epithelium or in vascular endothelial cells. Remarkably, tumour burden was not affected by loss of epithelial cell insulin signalling in lean animals or in the context of hyperinsulinaemia induced by high-fat diet feeding. In contrast, loss of the endothelial insulin receptor enhanced intestinal tumour formation. Moreover, vascular cell adhesion molecule-1 (VCAM-1), a key mediator of vascular inflammation and immune cell recruitment, was upregulated by loss of the insulin receptor in primary tumour endothelial cells. We conclude that insulin resistance in vascular endothelial cells promotes vascular inflammation and intestinal tumouriogenesis.

RESULTS

Insulin has been shown to promote proliferation in a range of cancer cell lines.[4,7–9] To determine whether insulin has this effect in primary tumour cells from mice with the multiple intestinal neoplasia (Min) mutation (*Apc*$^{Min/+}$ mice), we enzymatically dissociated polyps from the small intestine of *Apc*$^{Min/+}$ mice and maintained mixed tumour cells in short-term culture. Tumour cells were serum-starved and treated with 10 nM insulin for 16 h, then labelled with 5-ethynyl-2′-deoxyuridine (EdU) and analysed by flow cytometry. An antibody against EpCAM, a marker of epithelial cells, stained 70.1 ± 7.8% of the cell population cultured from polyps (Figure 1). In EpCAM$^+$ tumour epithelial cells, insulin treatment increased EdU incorporation by 1.9 ± 0.3-fold, a considerable increase given that treatment with FBS increased EdU incorporation by 3.4 ± 0.3-fold (Figure 1). Therefore, insulin clearly increased DNA synthesis in transformed epithelial cells from *Apc*$^{Min/+}$ mice during serum-starved conditions in culture.

Whole-body glucose tolerance is not altered by Insr knockout in intestinal epithelial cells

Despite the well-known mitogenic effect of insulin on tumour cells it has not been directly assessed whether insulin action on normal or transformed epithelial cells contributes to intestinal tumour formation *in vivo*. We therefore generated *Apc*$^{Min/+}$ mice with or without knockout of the insulin receptor gene (*Insr*) specifically in intestinal epithelial cells. In these *Vil1*-cre *Insr*$^{lox/lox}$ *Apc*$^{Min/+}$ (VILIRKO-Min) mice, insulin receptor mRNA was reduced by 97% and 93% in lysate of normal epithelium and intestinal tumours, respectively, compared to *Insr*$^{lox/lox}$ *Apc*$^{Min/+}$ controls (Figure 2a).

VILIRKO-Min and control mice were fed a high-fat or control diet with 60% or 22% of calories provided by fat, respectively. An oral glucose load administered by gavage showed that animals with diet-induced obesity had glucose intolerance compared to lean animals (Figure 2b). However, glucose tolerance was not different between VILIRKO-Min and control mice within the same diet group (Figure 2b). Similarly, plasma insulin was 2.4-fold higher in obese versus lean control mice but not different between the genotypes (Figure 2c).

Loss of insulin signalling in intestinal epithelial cells does not change tumour formation

Surprisingly, loss of insulin signalling in intestinal epithelial cells did not change tumour formation in lean animals (Figure 2d) and tumour size distribution was unchanged (Figure 2e). Consistent with this result, proliferation was similar in tumours from mice of either genotype with similar values for the relative frequency of

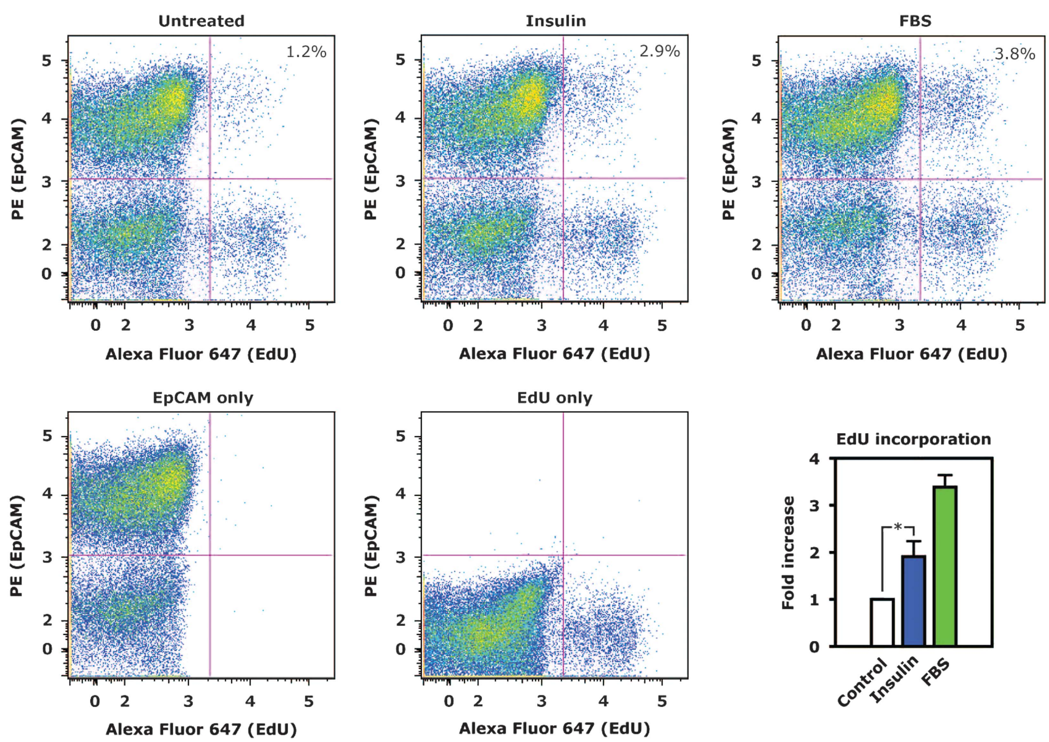


Figure 1. Insulin increases proliferation of serum-starved primary tumour epithelial cells in culture. Polyps were isolated from *Apc*$^{Min/+}$ mice and tumour cells dissociated by enzymatic digestion were grown in culture. Cells were serum-starved overnight, then stimulated with insulin for 16 h. In the final 4 h of this period, cultures were labelled with EdU. The proportion of cells double-positive for EpCAM, an epithelial cell marker, and EdU were analysed by flow cytometry. Scatter plots show representative data from flow cytometry. The graph shows mean values from independent experiments using primary culture from four different animals. *$P < 0.05$.

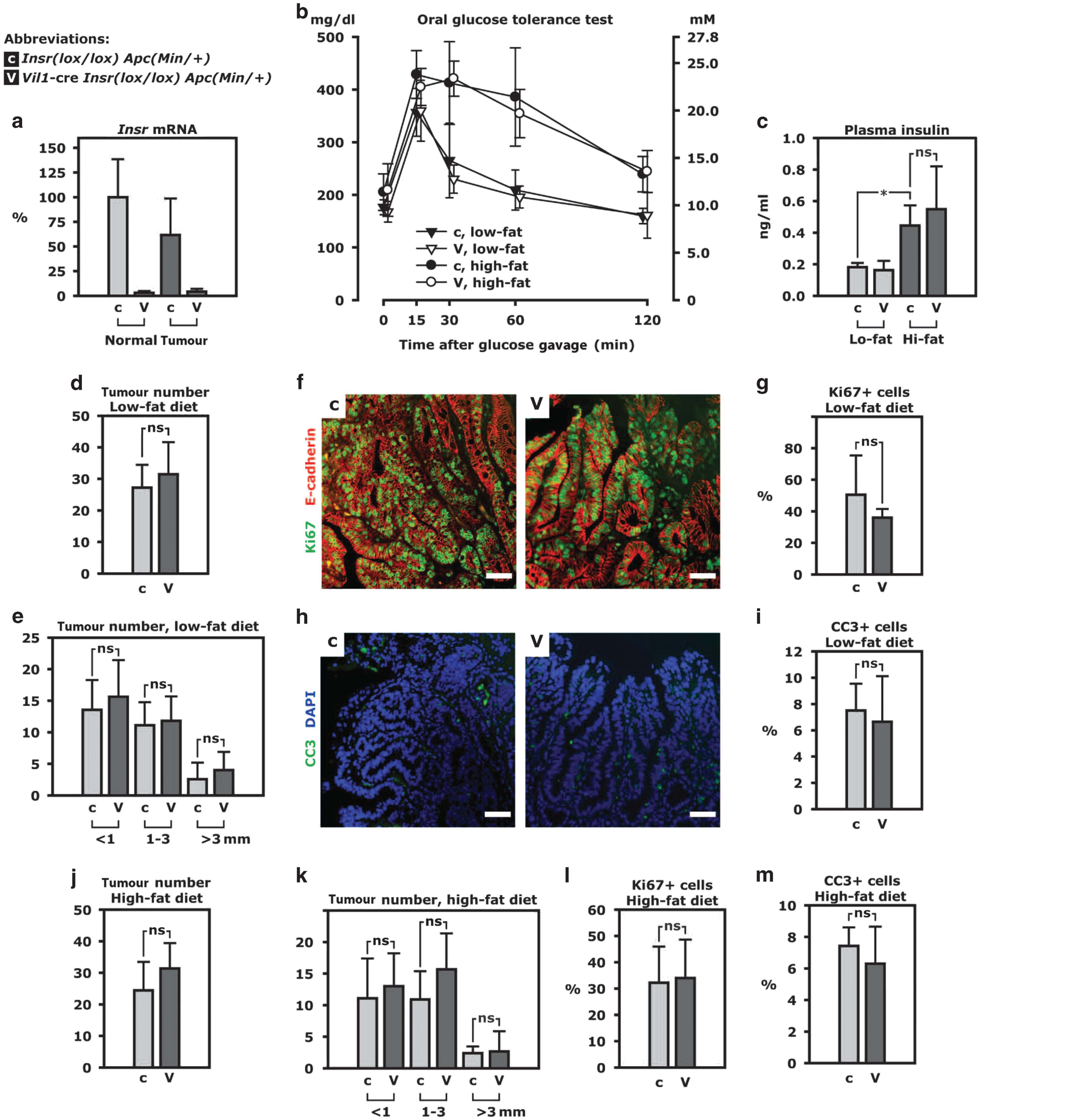
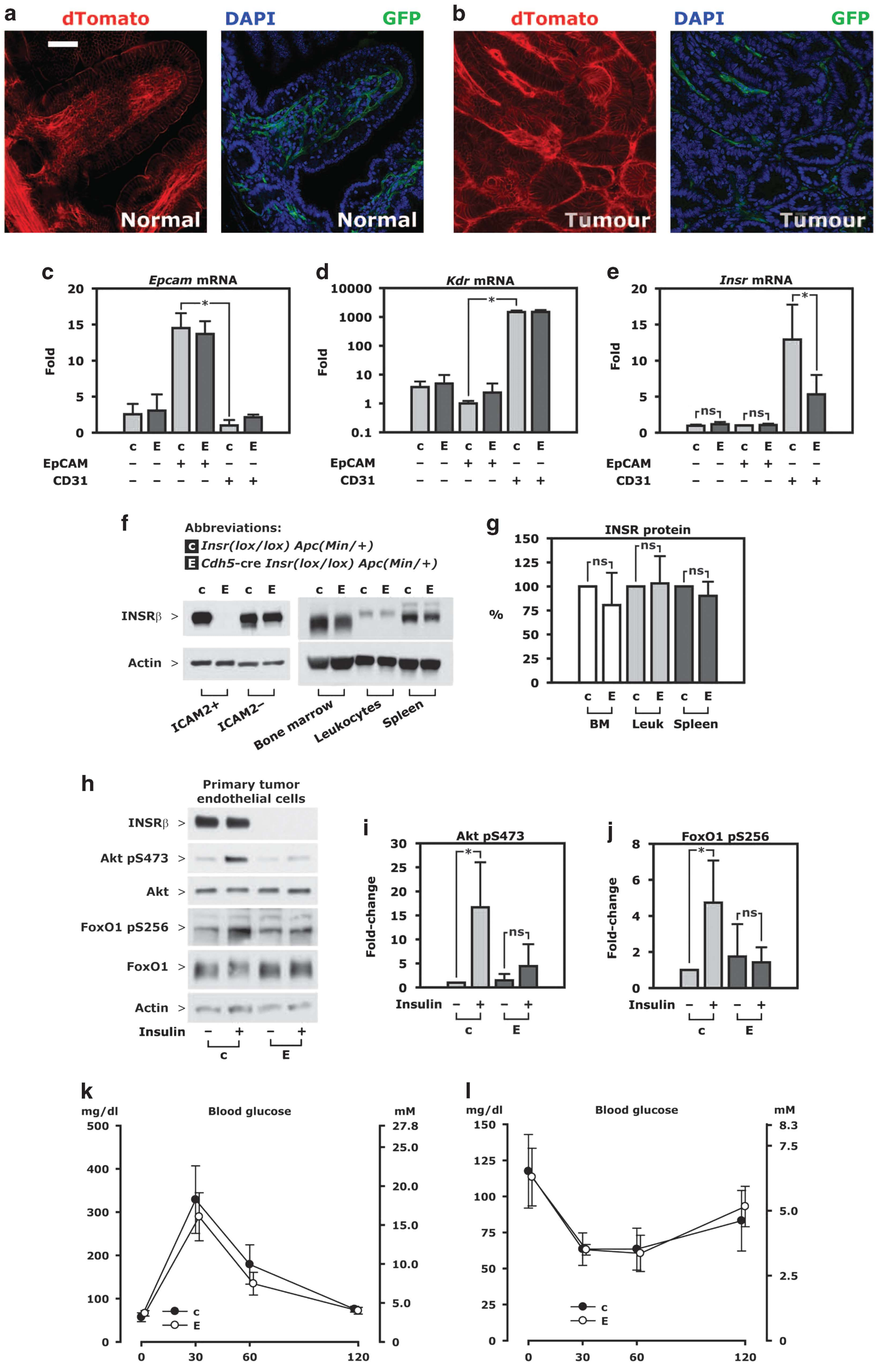


Figure 2. Loss of the insulin receptor in intestinal epithelial cells does not change tumorigenesis. $Apc^{Min/+}$ mice with insulin receptor knockout targeted to intestinal epithelial cells were studied (genotype *Vil1*-cre $Insr^{lox/lox}$ $Apc^{Min/+}$, abbreviated VILIRKO-Min and labelled 'V' in this figure). They were compared to littermate controls (genotype $Insr^{lox/lox}$ $Apc^{Min/+}$ and labelled 'c' in this figure). (**a**) *Insr* mRNA was measured by real-time PCR in lysate of tumours or normal jejunum. (**b**) Mice were fed a low-fat or high-fat diet for 8 weeks and subjected to an oral glucose tolerance test at 12 weeks of age. For the test, mice fasted overnight and were given 2 mg glucose per g body weight by gavage. Glucose concentration was measured in plasma from tail blood. (**c**) Insulin was measured in plasma in fasted animals. (**d**) Tumour number was counted in the entire small intestine of 9 control mice and 11 VILIRKO-Min mice fed a low-fat diet. (**e**) Tumour number in three categories of tumour size. (**f**) Representative microphotos of Ki67 (green) and E-cadherin (red) immunohistofluorescence performed on sections of paraffin-embedded tissue. Scale bars represent 50 μm. (**g**) Frequency of Ki67+ cells in tumour tissue. (**h**) Representative microphotos of cleaved caspase-3 (CC3) immunohistofluorescence (green) and DAPI (blue). Scale bars represent 50 μm. (**i**) Frequency of CC3+ cells in tumour tissue. (**j**) Tumour number in the entire small intestine of 9 control mice and 11 VILIRKO-Min mice fed a high-fat diet. (**k**) Size distribution of small intestinal tumours in mice fed a high-fat diet. (**l**) Frequency of Ki67+ cells in tumour tissue mice fed a high-fat diet. (**m**) Frequency of CC3+ cells in tumour tissue in mice fed a high-fat diet. All panels: $*P < 0.05$.

tumour cells positive for Ki67 (Figures 2f and g). In addition, the relative frequency of cells positive for cleaved caspase 3, a measure of apoptosis, was similar in the two groups (Figures 2h and i).

Diet-induced obesity and hyperinsulinaemia failed to reveal any difference in tumour number and size distribution between VILIRKO-Min and control mice (Figures 2j and k). Again, frequency of cells positive for Ki67 or cleaved caspase 3 in tissue sections of tumours



was similar in VILIRKO-Min and control mice when both groups were fed a high-fat diet (Figures 2l and m). Thus, insulin does not affect tumour cell proliferation or apoptosis in these intestinal tumours. We therefore concluded that even though insulin increases proliferation of adenoma epithelial cells during serum-starved conditions *in vitro*, insulin action on tumour epithelium plays a minor role *in vivo* where it is only one of many growth factors.

Loss of insulin signalling in vascular endothelial cells does not change systemic glucose tolerance

We wondered whether insulin resistance might be more closely related to tumorigenesis than hyperinsulinaemia and whether tumour stroma could mediate an effect of abnormal insulin signalling. We have previously demonstrated that insulin resistance in vascular endothelial cells produces an activated endothelium with increased leukocyte adhesion and accelerated recruitment of leukocytes to atherosclerotic plaques.[18] We therefore hypothesized that endothelial cell insulin resistance can promote tumour progression by facilitating chronic inflammation.

We generated *Apc*$^{Min/+}$ mice with insulin resistance in vascular endothelial cells by use of mice with a cre recombinase transgene under control of the VE-cadherin (*Cdh5*) promoter.[25] To characterise the specificity of cre-mediated recombination we bred mice carrying the *Cdh5*-cre transgene with *Apc*$^{Min/+}$ mice and double-fluorescent (mT/mG) cre reporter mice.[26] These reporter mice express a red fluorescent protein (membrane-targeted tandem dTomato) in most cells. In cells expressing cre, however, the transgene is recombined and expression of dTomato is replaced by a green fluorescent protein (membrane-targeted enhanced green fluorescent protein).[26] In normal intestine (Figure 3a) and in polyps (Figure 3b), green fluorescent protein fluorescence was clearly confined to vascular structures whereas epithelial cells in villi and polyps had uniform red fluorescence (Figures 3a and b). As *Cdh5*-cre transgene activity was specific for our tissues of interest, we then bred *Cdh5*-cre mice with *Insr*$^{lox/lox}$ and *Apc*$^{Min/+}$ mice to generate *Cdh5*-cre *Insr*$^{lox/lox}$ mice (EndoIRKO) and their *Insr*$^{lox/lox}$ controls as well as *Cdh5*-cre *Insr*$^{lox/lox}$ *Apc*$^{Min/+}$ (EndoIRKO-Min) mice and their *Insr*$^{lox/lox}$ *Apc*$^{Min/+}$ controls.

To validate that insulin receptor knockout was restricted to endothelial cells in polyps, we used flow-activated cell sorting (FACS) to separate endothelial cells and epithelial cells from enzymatically dissociated tumours and measured insulin receptor expression in the sorted cells. Sorting was efficient. Thus, in control animals, mRNA expression of the epithelial cell marker *Epcam* was 14.5 ± 2.1-fold higher in EpCAM+ cells than CD31+ cells (Figure 3c). In contrast, *Kdr* mRNA, another marker for endothelial cells, was 1465 ± 206-fold higher in CD31+ cells than EpCAM+ cells (Figure 3d). *Epcam* and *Kdr* mRNA was not different between EndoIRKO-Min mice and controls in the same category of cell marker (Figures 3c – e). Although not a substitute for quantitating the number of insulin receptors per cell it is interesting that *Insr* mRNA, normalised to *Rplp0,* which encodes a ribosomal RNA, was 12.5-fold higher in CD31+ (endothelial) cells than EpCAM+ (epithelial) cells (Figure 3e). *Insr* mRNA was reduced by 57% in CD31+ cells in EndoIRKO mice but not different in EpCAM+ cells or double-negative cells (Figure 3e), demonstrating that *Cdh5*-cre mediated recombination had no effect on tumour epithelial cells or, as a group, other tumour cell types.

We then evaluated the efficiency of the targeted gene deletion at the protein level by culturing the mixed tumour cell population and isolating primary endothelial cells by immunomagnetic selection. We have previously shown that this method yields cultures with a very high purity of endothelial cells.[18] In primary tumour endothelial cells cultured from control animals with intact *Insr*, insulin receptor protein was clearly present whereas no protein band was detected in cultures from EndoIRKO-Min mice (Figure 3f). In contrast, insulin receptor expression was similar in tumour cells negatively selected by Dynabeads conjugated with ICAM-2 antibody (Figure 3f). Insulin receptor expression was also unchanged in bone marrow, peripheral blood leukocytes and spleen from EndoIRKO-Min and control mice (Figures 3f and g). These data show that the insulin receptor was efficiently deleted in vascular endothelial cells without signs of reduction in hematopoietic lineages where endothelial-specific cre promoters can have off-target activity.[18] In cultured primary tumour endothelial cells from control mice with intact insulin receptors, treatment with 10 nM insulin increased Akt phosphorylation by 17-fold (Figures 3h and i) and FoxO1 phosphorylation by 4.7-fold (Figures 3h and j). By contrast, insulin had no significant effect in primary tumour endothelial cells from EndoIRKO-Min mice (Figures 3h – j).

Endothelial cell *Insr* knockout did not change measures of systemic insulin regulation of glucose metabolism. Plasma insulin concentrations were not different in EndoIRKO-Min and control mice, 0.60 ± 0.20 and 0.78 ± 0.36 ng/ml, respectively. There was no difference in whole-body glucose tolerance (Figure 3k) or insulin tolerance (Figure 3l).

Endothelial cell Insr knockout causes increased intestinal tumour formation

At 16 weeks of age, the total tumour number in the small intestine was 42% higher and the combined tumour area 60% higher in EndoIRKO-Min than in control mice (Figures 4a – c). There were 67.8 ± 14.6 and 47.7 ± 13.8 tumours in EndoIRKO-Min and control

Figure 3. Characterisation of EndoIRKO-Min mice. (**a**–**b**). *Cdh5*-cre *Apc*$^{Min/+}$ mice were crossed with mT/mG mice, a double-fluorescent reporter of cre recombinase activity. Offspring have ubiquitous expression of a red fluorescent protein, that is membrane-targeted tandem dTomato (dTomato), except in cells with cre-mediated recombination of the mT/mG transgene where dTomato expression is replaced with expression of membrane-targeted enhanced green fluorescent protein. 80-μm cryosections of formalin-fixed tissue was stained with DAPI only and imaged with confocal microscopy. Scale bar, 50 μm. (**a**) Normal intestine. (**b**) Polyp. (**c**–**e**) Tumours from three EndoIRKO-Min mice and three controls were enzymatically digested and cells sorted by FACS using antibodies against EpCAM or CD31; status as double-negative or single-positive for these antigens is indicated below graphs. mRNA expression was measured by real-time PCR and normalised to expression of *Rplp0*, a ribosomal RNA. (**c**) Enrichment of epithelial cells in EpCAM+ CD31 − fraction shown by expression of *Epcam* mRNA. (**d**) Enrichment of endothelial cells EpCAM − CD31+ fraction shown by expression of *Kdr* mRNA. Please note log scale. (**e**) Expression of *Insr* mRNA. (**f**) Tumour cells from *Cdh5*-cre *Insr*$^{lox/lox}$ *Apc*$^{Min/+}$ mice (EndoIRKO-Min, 'E') or their littermate *Insr*$^{lox/lox}$ *Apc*$^{Min/+}$ controls ('c') were grown in a mixed culture and endothelial cells isolated by magnetic selection using ICAM-2 conjugated Dynabeads at the first and second passage. ICAM-2-negative cells were collected at the first of these immuno-magnetic sortings while tumour endothelial cells were used after passage 3. Bone marrow, peripheral blood leukocytes and spleen were isolated and lysed. Representative western blots of lysate are shown. (**g**) Quantitation based on densitometry of western blots using material from three pairs of animals. (**h**) Isolated tumour endothelial cells were serum-starved overnight, then treated with 10 nM insulin for 5 min. Representative western blots are shown. (**i** – **j**) Quantitation based on lysate from four independent experiments. (**k**) Glucose tolerance test. Plasma glucose concentrations were measured after intraperitoneal injection of 2 mg/g glucose in five EndoIRKO-Min mice and five control animals. (**l**) Insulin tolerance test. Plasma glucose concentrations after intraperitoneal injection of 0.75 mU/g Humulin R in five EndoIRKO-Min mice and five control animals. All panels: *$P < 0.05$.

mice, respectively (Figure 4b, $P = 0.002$). This difference was manifest without changes in tumour size distribution (Figure 4d), indicating an effect on tumour initiation or early tumour progression. Specifically, 52 ± 21 and 47 ± 17% of tumours were > 1.0 mm in diameter in EndoIRKO-Min and control mice, respectively; 8 ± 6 and 7 ± 5% were ⩾ 2.0 mm (both $P > 0.4$).

Loss of insulin signalling in vascular endothelial cells can impair angiogenesis.[27] Therefore, endothelial cell *Insr* knockout, as in EndoIRKO-Min mice, would be expected to decrease tumour angiogenesis and impair tumour growth whereas we in fact found increased tumour formation in EndoIRKO-Min mice. Vascular density in tumours measured by CD31 immunohistochemistry revealed no changes between EndoIRKO-Min and control mice (Figures 4e and f). Therefore, although tumour angiogenesis could be limited by endothelial cell *Insr* knockout, such a mechanism is unlikely to cause the phenotype of EndoIRKO-Min mice.

Loss of endothelial cell insulin signalling increases VCAM-1 expression in tumour endothelial cells and increases neutrophil recruitment to tumours

Using *in vivo* microscopy of postcapillary venules in the intestinal mesentery, we have previously shown an increase in leukocyte adhesion to endothelial cells in mice with knockout of the *Insr* gene in endothelial cells.[18] The increase in leukocyte adhesion to endothelial cells *in vivo* was completely reversed by a VCAM-1 blocking antibody. We therefore hypothesized that a similar mechanism could increase VCAM-1 expression and promote chronic inflammation and tumour formation in EndoIRKO-Min mice. In primary tumour endothelial cells from EndoIRKO-Min mice, VCAM-1 protein expression was 56% higher than in cultures from control mice (Figures 5a and b). Conversely, in quiescent cultures from control mice insulin treatment decreased VCAM-1 expression by 18% (Figures 5c and d). Treatment with TNF-α, on the other hand, induced VCAM-1 expression by 3.8-fold and 10 nM insulin prevented 56% of this induction (Figures 5c and d). Therefore, insulin decreases VCAM-1 expression in quiescent primary tumour endothelial cells and partly prevents VCAM-1 induction when these cells are activated.

In our previous study, *Insr* knockout in endothelial cells was achieved using a *Tie2*-cre transgene and was complicated by reduction of insulin receptor expression in hematopoietic cells.[18] *Insr* knockout using a *Cdh5*-cre transgene in the current study is more specific in this regard (Figures 3f and g). We therefore characterised leukocyte adhesion in EndoIRKO mice. During intravital microscopy, rolling of leukocytes on the endothelium of mesenteric venules in EndoIRKO mice was increased by 2.2-fold compared with their controls (Figures 5e and f). This result extends the finding of a proinflammatory activation of insulin resistant endothelium to the mice generated for the current study.

We then measured the frequency of tumour-associated neutrophils and macrophages. In tumours from EndoIRKO-Min mice the frequency of Ly-6G+ cells was 5.6 ± 1.1% compared to 3.3 ± 1.6% in control mice (Figures 5g and h, $P = 0.02$). The frequency of F4/80+ cells, 4.3 ± 1.3 and 3.7 ± 1.3%, respectively,

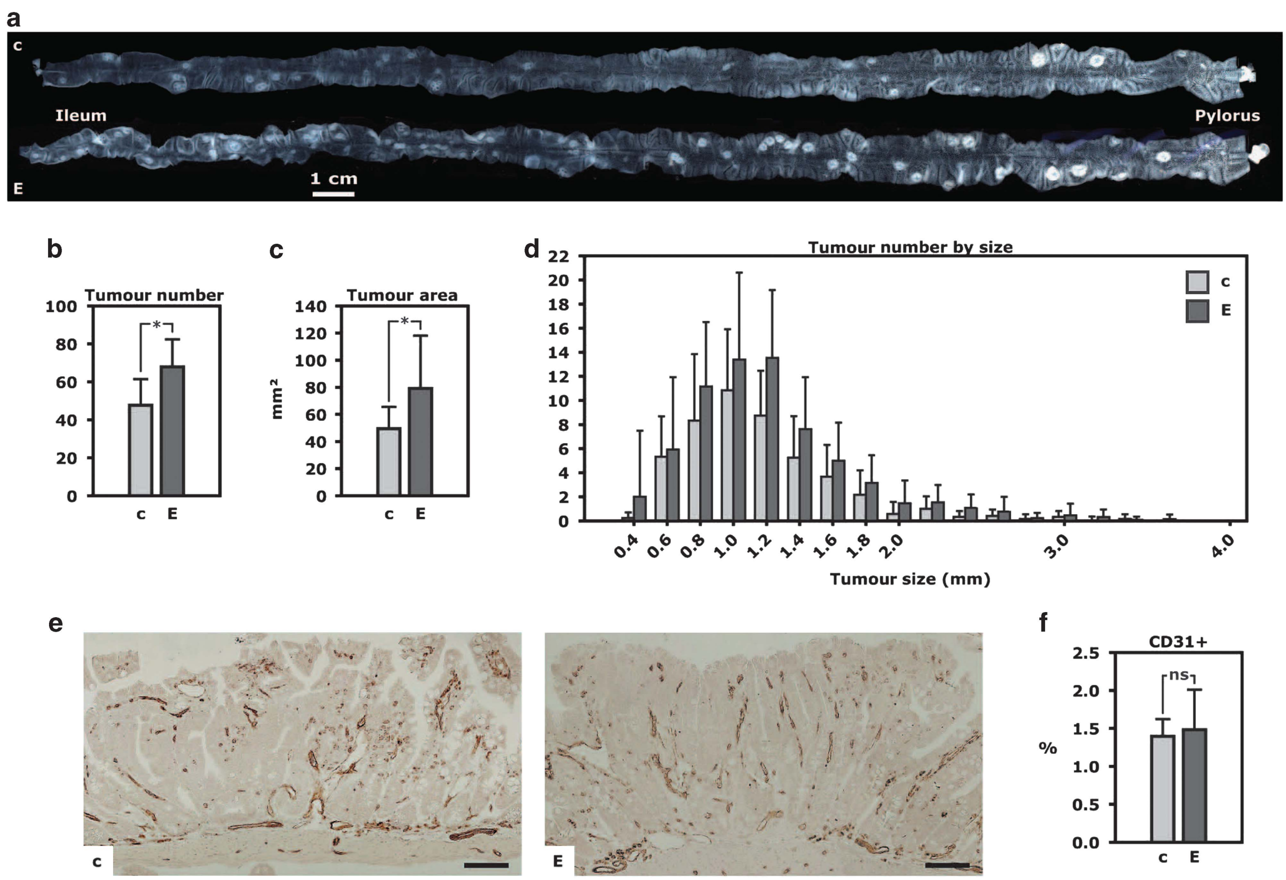


Figure 4. Loss of the vascular endothelial cell insulin receptor enhances tumour formation. EndoIRKO-Min mice ('E') and their littermate controls ('c') were fed a regular chow diet and killed at 16 weeks of age. (**a**) The entire small intestine from two littermates. Adenomas are clearly visible as pale, circular lesions. (**b**) Total number of tumours in the small intestine from EndoIRKO-Min mice ($n = 12$) and controls ($n = 13$). *$P = 0.002$. (**c**) Total tumour area. *$P = 0.02$. (**d**) Distribution of tumour number by diameter. (**e**) Representative microphotos of CD31 immunohistochemistry in small intestinal tumours. Scale bar, 100 µm. (**f**) Quantitation of tumour vascular density based on CD31 immunohistochemistry.

was not different (Figures 5g and i, $P > 0.4$). These data suggest that insulin resistant endothelium promotes recruitment of neutrophils to tumours where they may promote tumorigenesis.

DISCUSSION

Insulin resistance and ensuing compensatory hyperinsulinaemia are central features of obesity and type 2 diabetes. Hyperinsulinaemia has been implicated in the pathogenesis of a range of cancers that are more common in these metabolic diseases.[4] However, in the present study we demonstrate that tumour progression is unaffected by hyperinsulinaemia via insulin action on intestinal epithelial cells. Our data further show that insulin resistance in vascular endothelial cells can promote tumour formation, possibly through mechanisms involving chronic inflammation.

In 1924, three years after the discovery of insulin, George Gey published that this novel hormone could promote proliferation of cells in culture.[28] Since then, his suggestion that the observed effect could have relevance for malignant growth has been supported by numerous publications reporting that insulin increases proliferation of cancer cells *in vitro*.[4,7,9,29–31] This has led to the widely held belief that hyperinsulinaemia in obesity or type 2 diabetes promotes tumour progression by acting directly on tumour cells. We extend these observations by showing that insulin increases proliferation of primary adenoma cells in culture. However, we find no evidence that the mitogenic action of insulin is relevant to intestinal tumour formation *in vivo*. Indeed, deletion of the insulin receptor in normal intestinal epithelium and in tumour cells did not limit tumour formation, proliferation or apoptosis on a regular chow diet or in the presence of hyperinsulinaemia caused by high-fat feeding. Thus, we expect that the role of insulin is minor in the context of a growth factor replete tumour microenvironment. An alternative explanation is that hyperinsulinaemia may not actually result in enhanced insulin signalling in normal or transformed epithelial cells because of insulin resistance associated with a high-fat diet. Indeed, impaired insulin signalling has been demonstrated in enterocytes from obese individuals.[32,33]

How our finding relates to other cancers associated with obesity or hyperinsulinaemia remains to be determined. DeRoith and colleagues have implicated insulin in the progression of breast cancer using mice expressing a dominant-negative insulin-like growth factor-1 (IGF-1) receptor in skeletal muscle (MKR mice). These animals have marked hyperinsulinaemia in the absence of obesity. In mice with breast tumours induced by transgenic expression of the polyomavirus middle T oncogene, the MKR mutation causes accelerated tumour growth.[34] In addition, enhanced growth of tumours implanted in MKR mice was inhibited by insulin receptor knockdown in tumour cells.[35] Therefore, the role of insulin action on tumour cells may be different in tumours from different tissues and at different stages of malignant transformation.

It is recognized that neutrophils participate in tumour initiation and progression in many cancers.[36,37] In $Apc^{Min/+}$ mice, depletion

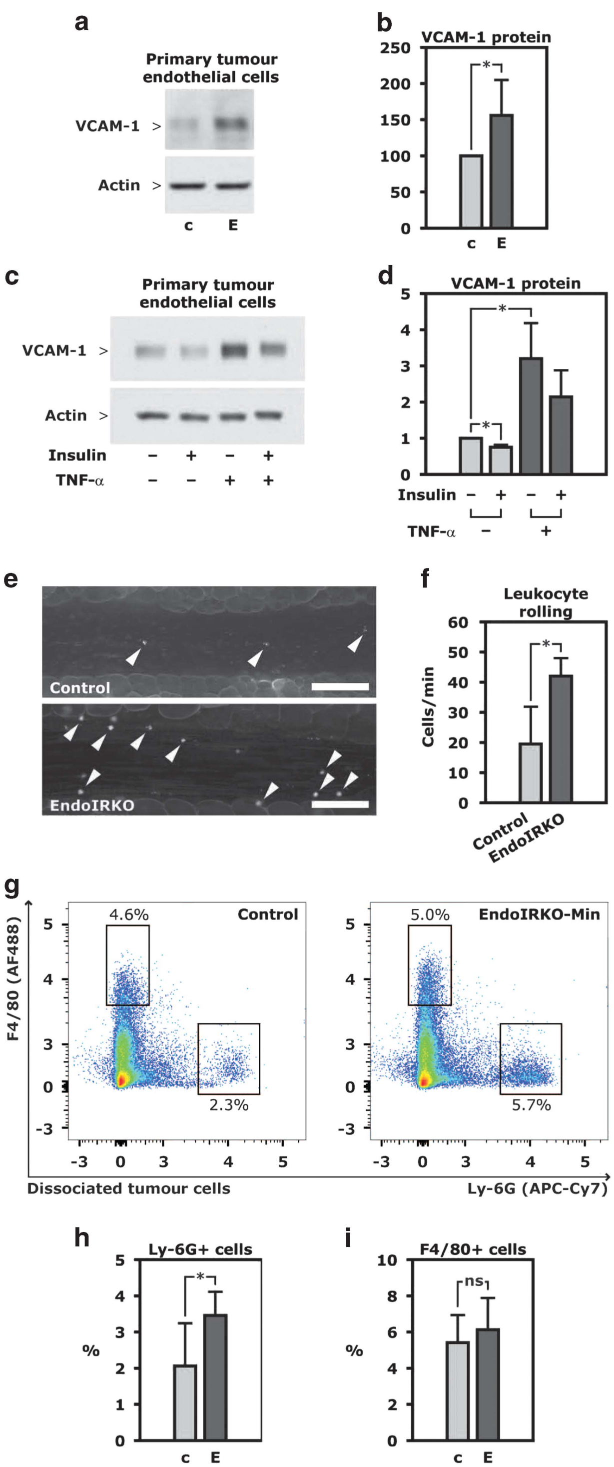


Figure 5. VCAM-1 expression and leukocyte recruitment associated with insulin-resistant tumour endothelium. Primary tumour endothelial cells were isolated by immuno-magnetic selection from a mixed culture of tumour cells. Cultures were used at passage 3 or 4. After overnight serum starvation, cells were treated with 10 nM insulin for 24 h and in some experiments with or without 1 ng/ml TNF-α. (**a**) Representative western blot from tumour endothelial cells cultured from EndoIRKO-Min or control mice. (**b**) Quantitative analysis based on densitometry from three independent experiments. (**c**) Representative western blot from tumour endothelial cells cultured from control animals with intact insulin receptors. (**d**) Quantitative analysis based on densitometry from three independent experiments. (**e**) Representative image frames from video of intravital microscopy of mesenteric venules. Leukocytes were labelled by intravenous injection of rhodamine 6G. Arrows point to rolling leukocytes. Scalebar, 100 μm. (**f**) Quantitation of leukocyte rolling in three EndoIRKO mice and four controls. (**g**) Results from a representative experiment using flow cytometry of enzymatically dissociated tumour cells. (**h**) Quantitation of the frequency of neutrophils (Ly-6G+ cells) in six tumours per genotype, analysed by flow cytometry. (**i**) Quantitation of the frequency of macrophages (F4/80+ cells) in six tumours per genotype. All panels: $^{*}P < 0.05$.

of neutrophils by anti-Ly-6G antibody reduced tumour formation.[38] It is possible that upregulation of VCAM-1 in insulin resistant tumour endothelium changes homing of other immune cells to the tumour microenvironment and future studies should further characterise how endothelial cells activated by insulin resistance affects interaction with different leukocyte subpopulations. It will also be important to provide evidence for causality in this relationship by showing that recruitment of neutrophils or other immune cells is responsible for accelerated tumour progression in EndoIRKO-Min mice or models of obesity or type 2 diabetes, for example by showing that treatment with anti-VCAM-1 antibody inhibits excess tumorigenesis.

To date, vascular biology has made a profound contribution to our understanding of cancer. Tumour angiogenesis is now understood as a key driver of tumour progression and vasculostatic agents have been employed as successful clinical therapies.[39] In contrast, relatively little is known about how endothelial cell recruitment of immune cells contributes to tumorigenesis.[40,41] Vascular inflammation is present in a number of chronic inflammatory diseases[42] and inhibiting immune cell adhesion to the endothelium has shown promise as a treatment for inflammatory bowel disease, suggesting that vascular inflammation is a primary event in intestinal inflammation.[43,44] Blocking α4 integrin, the VCAM-1 ligand on leukocytes, is efficacious in the treatment of inflammatory bowel disease[45] consistent with the notion that endothelial VCAM-1 upregulation due to endothelial cell insulin resistance promotes intestinal inflammation. To further understand this mechanism it will be important to evaluate whether insulin-resistant endothelial cells selectively recruit certain immune cell populations and characterise how such recruitment is regulated by molecules expressed on or secreted from endothelial cells.

Loss-of-function mutations in the adenomatous polyposis colon (*APC*) gene cause familial adenomatous polyposis. Similar mutations are present in >80% of sporadic colorectal cancer and *APC* mutations are a very frequent if not mandatory event in intestinal tumour formation in humans. Loss of *Apc* in intestinal stem cells is sufficient to initiate tumour formation in mice[46] and restoration of *Apc* leads to sustained regression of intestinal tumours caused by loss of *Apc*.[47] Therefore, $Apc^{Min/+}$ mice are a widely used model of intestinal tumour formation. Removal of adenomas can reduce risk of colorectal cancer,[48] supporting the notion that in most patients colorectal carcinoma develops from adenoma. The increased tumour formation in EndoIRKO mice has relevance for patients with obesity, as this patient group has an increased risk of developing colorectal adenomas.[49–51] However, to test whether endothelial cell insulin resistance also affects malignant disease, experiments similar to the ones presented in the current manuscript should be performed in animal models of colon adenocarcinoma. Future studies should also characterise insulin sensitivity in different types of tumour cells in metabolic disease. Impaired insulin signalling is present in intestinal tumours in mice with diet-induced obesity, including in $Apc^{Min/+}$ mice, when measured in tumour lysate.[52] Insulin signalling is impaired in large[53] and small[54] vessels in animal models of obesity and in both arteriolar[23] and venous endothelial cells[24] in insulin-resistant humans. Regardless, it will be important to directly demonstrate insulin resistance in intestinal or tumour-associated endothelial cells in diet-induced obesity or other models of insulin resistance.

Our findings demonstrate that insulin resistance in endothelial cells, a characteristic of endothelial dysfunction in obesity and type 2 diabetes,[21–24] can promote tumour development. In addition, we find little effect of intestinal epithelial cell insulin signalling on intestinal tumorigenesis. These findings provide an impetus to re-examine the dogma of insulin as a tumour promoter in obesity and type 2 diabetes and draw attention to the fact that important regulatory actions of insulin may be lost in obesity-induced insulin resistance.[55] We propose that insulin resistance, not increased insulin stimulation, may drive tumour development in certain tissues in these metabolic diseases. Improving endothelial function could decrease cancer risk in obesity.

MATERIALS AND METHODS

Animals

LIRKO mice[56] were backcrossed >10 times to the C57BL/6 background strain. $Insr^{lox/lox}$ mice negative for cre from this colony were cross-bred with $Apc^{Min/+}$ mice[57] (Jackson Labs, Bar Harbor, ME, USA, stock number 002020) and with mice harbouring either a cre transgene under control of the villin-1 (*Vil1*) promoter[58] (Jackson Labs stock number 004586) or the VE-cadherin (*Cdh5*) promoter[25] (Jackson Labs stock number 006137). $Apc^{Min/+}$ mice had been backcrossed 95 generations to C57BL/6, *Vil1*-cre mice at least six generations and *Cdh5*-creat least 13 generations (although a single nucleotide polymorphism panel analysis of *Cdh5*-cre at Jackson Labs indicates less extensive backcrossing, see www.jax.org). *Cdh5*-cre and $Apc^{Min/+}$ mice were also cross-bred with mT/mG mice[26] (Jackson Labs stock number 007676), backcrossed to the C57BL/6 strain for at least five generations.

Mice were fed either a control chow diet with 9.0% fat by weight (22% of calories provided by fat, Mouse Diet 9F, LabDiet, St Louis, MO, USA) or a high-fat rodent diet containing 34.9% fat by weight (60% of calories provided by fat, Research Diets cat. no. D12492). *Insr* knockout animals used for experiments were caged with littermate controls from birth to sacrifice. All protocols for animal use and killing were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with NIH guidelines.

Tumour number and size

Animals were sacrificed by CO_2 inhalation and perfused with 4% paraformaldehyde in PBS (formalin) through the left ventricle. The intestine was flushed with formalin and mesenteric fat removed by dissection. The intestine was opened longitudinally and flatmounted in formalin under a strip of transparency copy film (Staples, Framingham, MA, USA) for 30 min. The tissue was subsequently fixed in formalin for 24 h at room temperature, then stored at 4 °C in PBS. The small intestine was photographed through a stereomicroscope producing overlapping images, 15–20 per intestine, which were stitched together (Adobe Photoshop version CS4). Circles were drawn to fit tumour outline and the number and size circles were quantified automatically (ImageJ version 1.43 u). In the images shown in Figure 4, for presentation purposes only, the image area representing the intestine was selected in PhotoShop and placed on a uniformly black background.

Cell culture

Primary tumour cells were isolated and cultured using methods described previously.[18] Briefly, polyps were removed from a single animal by microdissection, then incubated for up to 1 h at 37 °C in DMEM containing 500 U/ml collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ, USA), dispase 3 mg/ml Dispase (Roche, Indianapolis, IN, USA) and 10% FBS. The tissue digest was passed through a 70-µm strainer and centrifuged at 200 × g. Pelleted cells were cultured on collagen-coated tissue culture dishes in DMEM with 10% (v/v) FBS. If the culture was used for EdU incorporation, the medium was supplemented with 50 ng/ml mouse epidermal growth factor (Gold Biotechnology, St Louis, MO, USA) and used without subculture. If the culture was used for isolation of tumour endothelial cells, the medium was instead supplemented with 50 µg/ml endothelial cell growth substance (Alfa Aesar, Ward Hill, MA, USA) and 100 µg/ml heparin. After trypsin treatment in the first and second passages cultures were sorted with Dynabeads conjugated with sheep anti-rat IgG (Thermo Fisher Scientific, Waltham, MA, USA) and complexed to ICAM-2 rat anti-mouse monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA) in DMEM containing 0.1% bovine serum albumin for 30 min at 4 °C. These primary endothelial cells were used at passage 2 – 4. Lung endothelial cells were isolated similarly to tumour endothelial cells as described previously.[18]

EdU incorporation

Tumour cells were grown to subconfluence, typically for 4 days, then starved in DMEM containing 0.1% bovine serum albumin overnight before treatment with 10 nM insulin or 10% FBS for 16 h. Cultures were labelled with 10 µM of 5-ethynyl-2-deoxyuridine (EdU) (Thermo Fisher Scientific) for 4 h. A single-cell suspension was made by trypsin treatment and stained by Click-iT EdU Alexa Fluor 647 Kit (Thermo Fisher Scientific) and with an epithelial cell adhesion molecule (EpCAM) antibody conjugated to PE (Biolegend, San Diego, CA, USA). Stained cells were analysed by flow cytometry using an LSRII instrument (BD Biosciences).

Intravital microscopy of leukocyte – endothelial cell interaction

Animals were anaesthetised by intraperitoneal injection of avertin 480 mg/kg, supplemented as necessary. Circulating leukocytes were fluorescently labelled by intravenous injection of 0.3 mg/kg rhodamine 6G (Sigma-Aldrich, St Louis, MO, USA). The abdomen was opened in the midline, a loop of ileum exteriorised and the mouse placed on a heated stage (Harvard Apparatus WP-10). Second- or third-order mesenteric venules were observed by fluorescence microscopy using an Axio Observer D1 microscope with inverted configuration and a ×20 objective, resulting in a final magnification of ×200. Images were acquired at 60 frames per second at high resolution by a digital video camera (C11440-22CU CMOS Camera, Hamamatsu, Bridgewater, NJ, USA) and Zeiss software (ZEN Pro 2012). Rolling leukocytes were measured as the total number of leukocytes crossing a 100-μm venular segment in 60 s at a velocity significantly lower than the centreline velocity.

Flow cytometry and fluorescence-activated cell sorting of dissociated tumour cells

Intestinal tumours were isolated by dissection under a stereomicroscope, minced by scalpels and digested for 1 h in DMEM containing 2 mg/ml (560 U/ml) collagenase I (Worthington Biochemical Corporation) and 3 mg/ml Dispase (Roche). Cell suspensions were washed and passed through a 40-μm filter and Fc receptors were blocked using TruStain fcX (anti-mouse CD16/32) antibody (Biolegend cat. no. 101320), then stained with EpCAM antibody conjugated to Brilliant Violet 421 (BioLegend cat. no. 118225), F4/80 antibody conjugated to Alexa Fluor 488 (BioLegend cat. no. 123119), and Ly-6G antibody conjugated to APC-Cy7 (BioLegend cat. no. 127623). Dead cells were stained with propidium iodide immediately before analysis. Filtered cells were stained with antibodies and sorted on a Moflo Legacy (Cytomation, Fort Collins, CO, USA) or analysed on an LSRII instrument (BD Biosciences). For FACS, 100 000–300 000 cells were sorted directly into TRI Reagent (Molecular Research Center). For all analysis, populations were first gated for cells (as opposed to debris) in an FSC-A vs SSC-A graph, for single cells (as opposed to doublets/aggregates) in an FSC-A vs FSC-W graph and for live (propidium iodide-negative) cells. Compensation was performed using single-stained cells and gating was aided by fluorescence minus one controls.

Other analyses

Real-time PCR, western blotting, glucose and insulin tolerance tests, insulin ELISA and immunostaining were performed as described previously.[18]

Statistical analysis

Comparisons were made using paired *t* test for the cell culture studies and unpaired *t* test when comparing variables in mice with $P<0.05$ considered statistically significant. In text and graphs, data are presented as the mean ± s.d.

CONFLICT OF INTEREST

Thomas Rathjen is an employee of Novo Nordisk A/S as a participant in the company's 'STAR Programme' for postdoc training. Christian Rask-Madsen receives research support from Novo Nordisk as part of this programme. The company had no role in the design of this study, collection and analysis of data or decision to publish. The remaining authors disclose no financial, professional or personal conflict of interest.

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