Serum- and Glucocorticoid-Induced Kinase SGK1 Directly Promotes the Differentiation of Colorectal Cancer Cells and Restrains Metastasis

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Abstract

Background and Aims—The molecular events that determine intestinal cell differentiation are poorly understood and it is unclear whether it is primarily a passive event or an active process. It is clinically important to gain a greater understanding of the process, since in colorectal cancer (CRC), the degree of differentiation of a tumour is associated with patient survival. *SGK1* has previously been identified as a gene that is principally expressed in differentiated intestinal cells. In colorectal cancer (CRC), there is marked downregulation of *SGK1* compared to normal tissue.

Methods—An inducible *SGK1* viral overexpression system was utilised to induce re-expression of *SGK1* in CRC cell lines. Transcriptomic and phenotypic analyses of these CRC lines was performed and validation in mouse and human cohorts was performed.

Results—We demonstrate that *SGK1* is upregulated in response to, and an important controller of, intestinal cell differentiation. Re-expression of *SGK1* in CRC cell lines results in features of differentiation, decreased migration rates, and inhibition of metastasis in an orthotopic xenograft model. These effects may be mediated in part by SGK1-induced PKP3 expression and increased degradation of MYC.

Conclusions—Our results suggest that *SGK1* is an important mediator of differentiation of colorectal cells and may inhibit CRC metastasis.

Introduction

The unit structure of the normal large bowel is the crypt. Stem cells at the crypt base produce progeny that expand in number and differentiate as they pass to the lumen into which they are shed. Although the biology of the stem cell niche is relatively well understood, much less is known about the process of differentiation. The involvement of

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signalling pathways such as Wnt, TGFβ/BMP/Hedgehog, Notch and Ephrins is required for production of the main differentiated cell types (colonocyte, neuroendocrine, goblet, paneth). However, especially for colonocytes, it is unclear to what extent differentiation is a fundamentally passive process – for example, resulting from absence of microenvironmental Wnt activity, which declines as cells move up the crypt – or an active process, in which unknown pathways are switched on in order to drive the expression of genes involved in the functioning of the differentiated cells.

The degree of differentiation (usually termed "grade") of colorectal cancers (CRC) is a well-established tumour feature that is associated with patient prognosis, and its assessment forms part of the American Joint Committee on Cancer (AJCC) recommendations [1]. The five year cumulative survival for patients with well-differentiated CRCs is 80.9%, compared with 76.9% and 45.5% for moderately and poorly differentiated lesions respectively [2]. Since CRC patients rarely die from effects of the primary cancer, poor differentiation must ultimately increase the risk of developing metastatic disease.

The molecular pathways that determine CRC differentiation are incompletely described. There are two main theories to account for how an undifferentiated tumour might arise: (i) cancer cells are selected for their ability to down-regulate differentiation gene(s), the so-called *"de-differentiation"* hypothesis; or (ii) differentiation reflects the cell of origin of the cancer in the crypt [3] [4]. However, whilst it is evident that CRCs demonstrate histological and growth characteristics similar to undifferentiated or stem cells in normal tissue, neither hypothesis fully explains why CRCs vary in their degree of differentiation.

Serum/glucocorticoid-regulated kinase 1 (SGK1) is a highly conserved member of the AGC family of serine/threonine kinases. It was originally identified as being transcriptionally activated in response to glucocorticoid stimuli in a mammary epithelial cell line [5]. *SGK1* is also induced by various cell stresses and is involved in the cell survival response. Its transcription is influenced by different types of hormones, growth factors, cytokines and conditions of cellular stress [6]. *SGK1* also regulates a number of other ion channels, including K^+, Ca^{2+} and Cl^- channels, and glucose transporters such as *GLUT1* and *SGLT1*, thus controlling cell volume and osmolality [6]. In the colorectum, such proteins are expected to be important in the functioning of differentiated colonocytes, since these cells primarily have a water-absorbing role. In keeping with this notion, we have previously demonstrated that *SGK1* expression in normal intestinal epithelium is highest at the top of the crypt, corresponding to the positions of differentiated colonocytes, with reduced expression in intestinal adenomas [7].

Much remains unknown about *SGK1's* function, and it appears to have a number of tissue-specific effects in both normal cells and cancers. *SGK1* has been proposed to form an alternative arm of Pi3 kinase (*PI3K*) signalling to the canonical *AKT* arm. *AKT* and *SGK1* have very similar catalytic domains, recognising similar substrate motifs *in vitro*, and are both activated in a *PI3K*-dependent manner. This means that *SGK1* potentially provides a parallel pathway to *PI3K/AKT* signalling and an alternative player in tumorigenesis [8][9]. *SGK1* effects *PI3K* signalling through *mTORC2* [10]. SGK1 is phosphorylated, and therefore activated, by PDKs. SGK1 expression is also regulated at the mRNA level, for

example by Hippo pathway members *Yap* and *Taz* [11], and by glucocorticoid and mineralocorticoid receptors. Downstream, *SGK1* phosphorylates the E3 ubiquitin ligase *NEDD4*-2, thus tagging it for degradation and allowing sodium channel expression. Other *SGK1* targets include *Foxo3*, *NRDG1* and *MNK1*. Nuclear *SGK1* may also play a role in the epigenetic control of gene expression [12]. *SGK1* activation has been proposed as a mechanism of cancer resistance to PI3K inhibitors [13]. There have been few studies of *SGK1* in CRC. The kinase inhibitors SI113 and EMD638683 have anti-SGK1 activity and have been demonstrated to inhibit the growth of RKO and CACO2 CRC cells respectively [14], often in combination with genotoxic therapies [15]. Sgk1 knockout has also been reported to reduce intestinal tumour numbers in mice with germline *Apc* mutations [16].

In this study, we examine the role of *SGK1* in intestinal cell differentiation, both in the normal crypt and in colorectal cancers. We determine the consequences of *SGK1* re-expression for CRC behaviour in model systems *in vitro* and *in vivo*, and identify c-Myc inhibition and plakophilin 3 expression as important downstream mechanisms. Finally, we test for associations between *SGK1* levels and the clinico-pathological features of a set of sporadic CRCs. Our findings suggest that the level of *SGK1* expression is a strong determinant of CRC differentiation status, supporting the cancer "de-differentiation" hypothesis. We also demonstrate that the differentiation status of a cancer is strongly associated with its metastatic potential. Our findings potentially open new avenues for research into CRC metastasis.

Materials and methods

Cell lines and cell culture

Human CRC cell lines were obtained from collaborators, and are commercially available from the American Type Culture Collection (ATCC): HT29 (ATCC® HTB-38™), LS174T (ATCC® CL-188™), SW480 (ATCC® CCL-228™). Murine MC-38 cell line is commercially available from Kerafast (ENH204). All CRC cell lines were grown in DMEM. Media were supplemented with 10% foetal calf serum and 1% penicillin/streptomycin, and incubated at 37°C and 5% CO2. For sodium butyrate treatment, cells at 80% confluence were treated with 5mM NaBut diluted in medium. Mycoplasma status was tested prior to each experiment using the Lonza Mycoalert mycoplasma detection kit (LT07-218).

NaBut and expression of luciferase

A 1.7kb region upstream of the start codon of rat *Sgk1* was cloned into the pGL3-enhancer vector. Cells were transiently transfected with either of the *Sgk1* promoter-pGL3 constructs and the Renilla luciferase pGL4.75 vector *(Promega)*, as a control for transfection efficiency. Twenty-four hours after transfection, experimental cells were treated with 5mM NaBut and luciferase activity was measured 48 hours later with the Dual-Glo™ Luciferase Assay System *(Promega)*.

Generation of SGK1-expressing construct

For lentivirus production, a 3X-FLAG tag was added to the 3' end of the full coding sequence of human *SGK1*. Transcription of *SGK1* sequences was initiated by the CMV promoter, regulated by the Tet operon in a Tet-Off regulated manner.

Western Blotting

Cells were harvested in lysis buffer supplemented with protease and phosphatase inhibitors (Roche). Protein content was measured by BCA assay (Pierce) and equal amounts were run onto pre-cast 4-12% NuPage gels (Invitrogen). Blotted membranes were blocked in 5% skimmed milk in PBS and incubated with the indicated primary and secondary antibodies.

Immunocytofluorescence

Cells were grown in confocal dishes with cover glass bottom (PAA), fixed in 3.7% paraformaldehyde for 10 minutes at room temperature and permeabilised with 0.1% TritonX-100 in PBS for 5 minutes at room temperature. Fixed cells were incubated either with serum obtained from the species in which the secondary was raised or with 1% BSA to prevent non-specific binding. Cells were then incubated with primary antibodies for 1 hour, rinsed, incubated with fluorescently-labelled secondary antibodies for 1 hour in the dark, and finally washed and counterstained with DAPI (Sigma). Samples were analysed with the ZEISS 510 MetaHead confocal microscope.

Tissue Microarrays

TMAs (CO484a, CO485) were obtained from Insight Biotechnology Ltd, the UK suppliers of US Biomax. They comprised histological sections of colon cancer specimens with information on TNM and clinical stage, differentiation and pathological grade. Scoring for SGK1 protein expression was performed following IHC on a scale of 1-5 for each specimen.

Microarrays and SGK1 expression from villi and crypts

Endoscopic biopsies were taken from the descending colon of five normal control patients and were microdissected into crypt tops and bottoms. RNA was extracted, amplified and quantified using Illumina beadchip microarrays. Analysis was performed using the R package "limma".

RNA sequencing

RNA was isolated from pelleted cells using the Qiagen Rneasy MinElute clean-up kit and analysed using Tapestation. RNA sequencing libraries were prepared using the QuantSeq 3' mRNA library prep kits (*Lexogen)* and sequencing performed on an Illumina Hiseq 4000 with 75bp paired end reads. RNA sequencing for the CRC cell lines were performed in duplicate and for animal experiments in triplicate. Sequencing was performed to an average depth of 5 million reads. Reads were de-multiplexed and mapped using Hisat2 to the reference human genome GRCh37. Feature counting was performed using Htseq version 0.6.1 and read normalisation and differential expression, using DESeq version 1.10.1. JavaGSEA was used using the Molecular Signatures Database on pre-ranked lists to perform

gene set enrichment analysis. Curated signatures for cellular senescence, apoptosis and proliferation were obtained from published genesets [17].

Proliferation and apoptosis

Cell proliferation was measured with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Apoptosis was measured with the Caspase-Glo 3/7 Assay (Promega) under basal conditions and upon osmotic stress stimulus, achieved by treating the cells with 500mM sorbitol (Sigma) for 24 hours.

Anchorage-independent growth assay

Anchorage-independent growth was measured as the ability of cells to form colonies in soft agar. Briefly, a bottom layer consisting of 0.6% agar (Sigma, A9414) diluted in tissue culture medium was plated out in 6-well plates. Equal numbers of cells were diluted in 0.3% agar in tissue culture medium and plated onto the bottom layer at a concentration of 1000 cells per well, in triplicates. Cells were incubated for three weeks and fed with 200µl of medium twice weekly. Images were acquired with a scanner and the number of colonies formed was counted for each well.

Boyden transwell assay

8uM transwell assays were obtained from Corning and 300,000 cells were trypsinised and suspended in DMEM with 0% FBS and placed in the upper chamber. DMEM with 10% FBS was placed in the lower chamber. 16 hours later, cells were removed from the upper chamber and cells on the lower surface of the transwell assay were stained using Kwik-Diff stains (ThermoScientific). Automated cell counting was performed using the Nikon NIS Elements software package.

Stable isotope labelling of amino acids in culture (SILAC)

SILAC was performed with the SILAC Protein Quantitation Kit (ThermoScientific/Pierce) according to instructions. HT29-SGK1 cells were cultured in 'heavy' medium (13C6Lys + 13C6/15N4Arg) and HT29 parental cells in 'light' medium in the presence of doxycyline for 5 passages, after which incorporation of the heavy label was measured by tandem mass spectrometry. Resulting peptides were analysed by LC-MS/MS using an Ultimate3000™ HPLC system coupled on-line to a 3D high-capacity ion trap tandem mass spectrometer via a pneumatically assisted nano-electrospray source as described previously [18].

Mouse models

Animals were a mix of 5-10 week old male and female JAX™ NSG mice (NOD.Cg-Prkdcscid Il2rgtm1WjI/SzJ) obtained from Charles River Laboratories, UK. Animals were maintained under specific pathogen-free conditions, and food and water were supplied *ad libitum*. Housing and all procedures involving animals were performed according to protocols approved by the United Kingdom Home Office, in compliance with Animals (Scientific Procedures) Act and home office guidelines on animal welfare. Orthotopic implantation of tumours was performed on 5-10 week old mice under anaesthesia by

isofluorane inhalation, using a 30 gauge custom-made needle under direct endoscopy vision with the Coloview system (*Karl Storz*) [19].

TCGA human cohort data

The human cohort data was obtained from The Cancer Genome Atlas (TCGA) which is publically available for the scientific research community. Ethical approval for the data was obtained from the National Institute of Health (NIH).

Results

SGK1 is preferentially expressed in intestinal tissue compartments comprised of differentiated cells

In order to confirm that expression of *SGK1* alters along the crypt axis of the colon and small intestine, endoscopic biopsies from the terminal ileum and colon from 5 patients were obtained and microdissected into crypt tops and bottoms. RNA was extracted and global gene expression was quantified using microarrays. *SGK1* expression was 9.6-fold higher in small bowel villi than crypts (p=0.003) and 10.8-fold higher in colonic crypt tops than bottoms (p=0.1), consistent with previous data showing that SGK1 is expressed predominantly in differentiated enterocytes and colonocytes. Increased expression of *SGK1* was associated with increasing expression of *SHBG*, *CIDEC*, *DUSP5* and *MAF*, known drivers of differentiation in cell lines. *DUSP5* was especially interesting, as low expression is known to be associated with poor tumour differentiation, lymph node metastasis, distant metastasis and poorer overall survival [20]. Expression of *SGK1* was negatively associated with expression of *ASCL2*, *OLFM4*, *EPHB2*, *RETNLB*, *MSI1* and *SOX9*, genes associated with stem cell maintenance and function (Supp. Table 1).

Since CRCs are typified by reduced differentiation, we compared the levels of *SGK1* mRNA in CRCs with those in normal intestine (data from The Cancer Genome Atlas). *SGK1* expression was >10-fold lower in the former (Supp. Figure 1). In keeping with this, *SGK1* was recently identified as a "hub gene", the reduced expression of which is central to a set of gene expression changes in colorectal carcinogenesis (24).

Agents that cause differentiation of colorectal cells also induce SGK1 mRNA expression

Sodium butyrate (NaBut) is a short-chain fatty acid normally found in the colorectal lumen. NaBut promotes cellular differentiation *in vitro* in a number of CRC cell lines (26). Since our data showed *SGK1* to be upregulated in differentiated cells of the intestine, at the crypt tops, we investigated whether *SGK1* expression was induced by NaBut-driven differentiation in CRC lines. Two cell lines were selected, LS174T which expresses almost no endogenous *SGK1,* and HT29 which expresses modest levels [23]. Upon NaBut treatment, both HT29 and LS174T cells showed a significant increase in *SGK1* transcript levels over time, as measured by qRT-PCR (p<0.01) (Figure 1A). This increase was confirmed in luciferase reporter assays (3.9-fold for HT29, 1.7-fold for LS174T; data not shown).

SGK1 expression induces differentiation of CRC cells

The NaBut experiments demonstrated that *SGK1* is induced by differentiating agents. Based on these data and the *SGK1* expression pattern in the crypt, we pursued the hypothesis that SGK1 is a key player in signalling pathways that induce and directly control differentiation of colorectal cells. To determine experimentally if this could be the case, we developed a system to establish stable, inducible expression of *SGK1* using a lentivirus-based system. HT29 and LS174T CRC cell lines were used as models and comparisons made to their control counterparts that had been transduced with an empty lentivirus (HT29-*SGK1* vs. HT29-WT, LS174T-*SGK1* vs. LS174T-WT). This system resulted in levels of *SGK1* mRNA expression that were comparable to those in normal intestinal tissues (Supp. Figure 2) and in the production of SGK1 protein (Supp. Figure 3). Taking this approach, it was possible to demonstrate that functionally active SGK1 protein was expressed, based on reduced levels of the known *SGK1* phosphorylation target, NEDD4-2 (Supp. Figure 4).

The process of differentiation in colonocytes is characterised by several histological features, including the formation of junctional complexes that maintain cell shape and the establishment of basal-apical cell polarity, with the formation of actin filament caps that co-localize with apical markers in differentiated colonocytes [24]. Upon NaBut-induced differentiation, LS174T cells are known to undergo specific morphological changes, which include the appearance of mucin-like granules [21]. We found that similar morphological changes were induced by re-expression of SGK1 in the absence of NaBut, and there was an increased tendency for cells to grow as colonies. HT29 cells treated with NaBut or in which expression of SGK1 was induced, showed comparable morphological changes (Supp. Figure 5).

We investigated whether other features of differentiation could be identified in our CRC cell lines following re-expression of *SGK1*, specifically investigating the levels and distribution of proteins important for the formation of key junctional proteins, beta-catenin, ZO-1 and E-cadherin. *SGK1* caused an increase in total ZO-1 but not beta-catenin (Figure 1B, Supp. Figure 6). Immunofluoresence analysis of E-cadherin demonstrated increased membrane expression following *SGK1* re-expression, and this was confirmed following cell fractionation western blotting (Figure 1C). We then assessed markers of cell polarity, which is a feature of differentiated intestinal epithelial cells. We found that LS174T and HT29 displayed actin caps upon re-expression of *SGK1*, and these were not seen in control cells. Moreover, the apical marker villin, which showed diffuse cytoplasmic expression in control cells, co-localised with the actin caps (Figure 1D). Together these findings demonstrated the ability of *SGK1* re-expression to induce colonocyte differentiation.

We also transiently expressed *SGK1* in the HCT116 CRC cell line, which has very low endogenous *SGK1* and does not spontaneously differentiate in culture, perhaps having irreversibly lost the ability to do so. Following transient high-level SGK1 expression, HCT116 cells ruptured, with no evidence of differentiation.

SGK1 re-expressing CRC lines demonstrate reduced tumorigenicity, characterised by decreased anchorage-independent growth and cellular migration

To understand the phenotypic changes in cells that are induced by *SGK1*, we performed a number of functional assays using the re-expression cell lines, HT29-*SGK1* and LS174T-*SGK1*. Twenty-four hours after re-expression of *SGK1*, we observed a significant reduction of ~40% in apoptosis rates under basal conditions, and a more modest but significant reduction upon induction of osmotic stress with sorbitol (Figure 2A). Apoptosis rates subsequently increased over the time course of the experiment to reach levels comparable to or exceeding those of control cells (data not shown), suggesting that the *SGK1*-induced protection was only temporary, or that resistant clones had grown out. *SGK1* re-expression also led to modest, but significant, reductions in proliferation in the *SGK1* re-expressing cells compared to controls (Figure 2A), and this was maintained over time.

To probe the effects of *SGK1* on stem-like cell properties, colonosphere formation assays were performed. Upon re-expression of *SGK1*, both HT29 and LS174T cell lines showed dramatically impaired formation of colonospheres (Figure 2B), in keeping with *SGK1*-mediated reversion of the CRC cell lines to a more differentiated phenotype. To investigate the effects on anchorage-independent growth, soft agar assays were performed to assess characteristics observed in differentiated cells, anoikis (cell death) and the inability to proliferate, when there is loss of adhesion from the extracellular matrix. *SGK1* re-expression results in reduced colony formation (Figure 2C).

Finally, we performed a Boyden migration assay that assesses cellular motility across a transwell membrane towards a growth factor gradient. At 24 hours, re-expression of *SGK1* resulted in a significant reduction in rates of migration compared with cells that had been transduced with an empty lentiviral construct (Figure 2D). This observation was confirmed using a different approach, where a migration assay was performed on 11 CRC cell lines that naturally expressed different amounts of *SGK1*. This assay identified that lines with low *SGK1* mRNA expression, such as LS174T and SW480, exhibited the highest migration rate whereas cell line with higher *SGK1* (COLO205, SNU-C4, SW1222) demonstrated low cellular migration (Figure 2E).

The Cancer Cell Line Encyclopaedia (CCLE) was then utilised to obtain SGK1 expression for a total of 43 metastatic and primary colorectal cancer cell lines. Of the 42 cell lines, 9 were derived from metastatic sites. Primary CRC lines expressed significantly higher *SGK1* than lines derived from metastases (2.7 Transcripts Per Kilobase Million (TPM) vs 6.7 TPM, $p<0.05$), but expression was both low and variable (Supp. Table 2).

In summary, these findings point towards a link between increased *SGK1* expression and reduced proliferation, apoptosis, stem cell phenotype and motility.

SGK1 re-expression results in enrichment of gene networks involved in cell differentiation and reduction of MYC signalling

SGK1 could in principle influence numerous gene networks through protein phosphorylation, thus amplifying environmental or other triggers for differentiation. To gain clues about the gene expression changes induced by *SGK1*, it was re-expressed in 7 CRC

cell lines (GP2D, HT29, HCT116, LS174T, SW1222, SW480 and T84). Whole-transcriptome profiling was performed using 3'-RNA sequencing.

Three genes were found to be differentially expressed: *SGK1*, *NEFM*, and *RNA18S5* (p.adj all <0.05). To determine if there were more subtle changes in expression of genesets, GSEA using signatures from the MsigDB databases and curated genesets were performed. Re-expression of *SGK1* was associated with enrichment for gene networks involved not only in the "protein kinase cascade" and "stress-activated protein kinase signalling" as expected from existing data, but also for "cell polarity", "differentiation" and "cell matrix adhesion". A focussed analysis of cancer signalling pathways demonstrated that expression levels of genes in several pro-oncogenic pathways were significantly decreased following *SGK1* re-expression. This effect was most pronounced for the gene sets "MYC targets" and "retinoblastoma/E2F signalling" (NES=-26.1 and -1.56 respectively, p<0.001 for both) (Figure 3A, Table 1). Following re-expression of *SGK1*, expression of *MYC* mRNA was reduced by 23.6% (p=0.03) and expression of c-Myc target *PGK1* was reduced by 23.1% (p=0.03).

In CRC, c-Myc lies at the crossroads of many growth-promoting signal transduction pathways and plays a key role as a pro-metastatic transcription factor, regulating epithelial-to-mesenchymal transition, cell-cell matrix interactions and cellular invasion in cell and murine models. In order to confirm reduction in c-Myc protein, we transduced CRC lines HT29 and SW480 with *SGK1* re-expression lentivirus, a kinase dead *SGK1* re-expression lentivirus or an empty lentiviral vector. Upon re-expression of SGK1, there was a consistent and significant decrease in c-Myc protein expression only in the cell lines carrying the SGK1 re-expression vector (mean reduction=56% at day 4 (Figure 3B, Supp. Figure 7). Using antibodies specific to phospho-Ser62 and phospho-Thr58, we demonstrated that there was a corresponding decrease in phosphorylation at both c-Myc Ser62 and Thr58 in the *SGK1* re-expressing cells (Figure 3C).

SGK1-induced differentiation is mediated by plakophilin 3

We searched for proteins that were differentially expressed as a consequence of *SGK1* re-expression and hence were potential downstream effectors of SGK1-induced differentiation. We utilized stable isotope labelling of amino acids in culture (SILAC) on our HT29-SGK1 and parental cells, followed by mass spectrometry differential analysis. The experiment identified a small number of proteins showing differential expression upon SGK1 re-expression. Mass spectrometry results consistently indicated an increased abundance (~20-fold) of plakophilin 3 (PKP3) (Figure 4A), an essential component of desmosomes that has been linked to metastatic potential and differentiation in tumours [25] [26]. Western blotting (Supp. Figure 8A) confirmed higher levels of PKP3 expression in both HT29-SGK1 and LS174T-SGK1 cells compared to parental cells. Investigation of PKP3 expression by immunohistochemistry in normal human colon showed increased expression in differentiated cells, paralleling *SGK1* expression (Figure 4B).

To assess whether PKP3 mediates the effects of SGK1 on cell differentiation, we engineered lentiviral-based shRNA knockdown of *PKP3* in HT29-SGK1 and LS174T-SGK1 cells. When compared to control cells re-expressing SGK1 and transduced with a non-targeting

shRNA virus, abolition of PKP3 expression (Supp. Figure 8B) lowered membranous E-cadherin expression, and also restored colony-forming ability (Figure 4 C, D).

SGK1 suppresses cancer cell metastasis in an orthotopic xenograft model through inhibition of metastatic outgrowth

Our experiments demonstrated that *SGK1* exerted diverse effects on the cancer cell phenotype. Many of the processes affected – differentiation, migration, anchorage-independent growth, inter-cellular adhesion and c-Myc expression – are important in metastasis. We therefore developed an orthotopic xenograft mouse model of CRC metastasis, by endosocopically injecting genetically-modified CRC cells into the sub-mucosa of the mouse descending colon. We initially tested *Sgk1* knockout in MC38 CRC cells, using a CRISPR/Cas9 strategy as part of a whole genome screen in 8 mice. *Sgk1* knockout did not detectably change metastasis rates, as might be expected given the very low *Sgk1* levels in CRCs (details not shown). We therefore tested the effects on metastasis of over-expressing *SGK1*. LS174T-*SGK1* and LS174T-WT cells were labelled using a luciferase-expressing lentivirus (pHIV-Zsgreen-luc2) to enable *in vivo* imaging, and transplanted into Nod/Scid Gamma mice under endoscopic guidance. We found *SGK1* mRNA expression in the LS174T-SGK1 cell lines and primary xenografts to be at levels comparable to normal epithelium (data not shown).

All animals (n=16) developed tumours in the colonic lumen following successful implantation. By day 21, mice became symptomatic principally due to intestinal obstruction but also metastatic outgrowth. All mice developed lung metastases, but secondary spread to the liver was uncommon (4/16 mice) and lesions in bone and other distant sites were not detected. At day 21, mice were culled, lungs were resected and *ex vivo* analysis performed.

Ex vivo imaging demonstrated that there was a striking 25.8-fold decrease in lung metastasis burden (p<0.008) in the mice that received LS174T-*SGK1* compared to those that received LS174T parental cells. In a replication cohort (n=15), there was a similar (20.4-fold) decrease in lung metastasis burden (p<0.001; Figure 5A, B). By contrast, there was no significant difference in primary tumour size between the LS174T-*SGK1* and LS174T-WT groups (Supp. Figure 9A).

These findings are in keeping with *SGK1*-mediated differentiation *in vitro* not limiting primary tumour growth, broadly consistent with the modest decreases in proliferation found *in vitro* (see above), but acting potently as a suppressor of metastasis.

To determine the stage at which *SGK1* exerted its effect on metastasis, whether inhibiting the ability of cells to migrate away from the primary tumour, or inhibiting later stages in metastasis such as the ability of cells to survive in the circulation and enter metastatic tissues, a tail vein assay was performed. LS174T-*SGK1* and LS174T-WT cells were injected into the tail veins of 8 Nod/Scid Gamma mice. Bioluminescence *ex vivo* analysis confirmed a marked reduction in metastasis in the LS174T-*SGK1* cells, comparable in magnitude to the reduction seen in the orthotopic xenografts (p<0.001; Supp. Figure 9B).

SGK1 expression levels are reduced in poorly differentiated human CRCs, but not in lymphatic metastases

Having established that *SGK1* expression could induce features of differentiation in CRC cell lines, we examined this association in human CRCs. A tissue microarray of sporadic CRC resection specimens (n=86) was obtained comprising of cancers of principally stage II/III CRC and from patients with a mean age of 51.7 years (Supp. Table 3). Differentiation grading had been performed by accredited histopathologists according to AJCC guidelines. We examined SGK1 expression using immunohistochemistry (Supp. Figure 10). This demonstrated that there was a decrease in *SGK1* expression in poorly differentiated than well or moderately differentiated cancers (t-test, p<0.01; Figure 5C). This result validated our experimental findings suggesting an association between low *SGK1* expression and poor colorectal cancer differentiation in both CRC lines and tumour resections specimens.

Low primary SGK1 expression is a biomarker of poor prognosis in different stages of human CRC

Poor differentiation (high grade) is a well-known poor prognostic feature of CRC. Undifferentiated CRC are associated with increased cellular motility and features such as tumour and epithelial-mesenchymal transition (EMT) [27]. Patients with poorly differentiated CRCs tend to be younger, have larger, right-sided tumours with lymphovascular invasion, a higher frequency of MSI, a significantly poorer 4 year overall survival [28] and a higher propensity to develop metastasis [29]. As we have demonstrated the expression of *SGK1* to be closely associated with CRC differentiation/grade, we sought to determine whether *SGK1* expression could be utilised as a similar marker of poor prognosis.

The Cancer Genome Atlas sample set comprised an annotated set of US-based patient genomes with CRC of various stages [30]. A set of cohort of 284 patients has detailed follow-up information and RNAseq had been performed on all specimens, enabling an analysis of the effect of *SGK1* expression. *SGK1* expression was expressed as quintiles as it was non-normally distributed and quintiles used as a quantitative variable. This analysis found a significant association between lower *SGK1* expression and poorer survival (p=0.023) (Figure 5D). There was no correlation between *SGK1* expression and tumour stage and the association of low expression of *SGK1* with poorer prognosis was observed across all stages of CRC except the small number of patients with stage 1 disease (Supp. Figure 11).

Discussion

SGK1 is a serine/threonine kinase that shares homology with proteins of the *AKT* family. To date, the role of *SGK1* has been most clearly elucidated in regulation of ion channels [31], neuronal function [32] and T helper cell differentiation [33]. More recently, there has been a growing realization that *SGK1* lies at the junction of, and could mediate the effects of, several oncogenic signalling networks. The effects of *SGK1* are variable, depending on the tissues or cell lines analysed and the model systems used. A number of studies have highlighted the pro-proliferative effects of *SGK1*: in hepatocyte regeneration, *SGK1*

mediated the phosphorylation of ERK2 and the activation of the Ras-MAPK kinase pathway [34]; and in breast cancer, activation of *SGK1* sustained mTOR activation in the context of AKT inhibition through direct phosphorylation of TSC2 [35]. *SGK1* is also thought to act in conjunction with PHLPP1 to enable Ras-mutant cancers survive anoikis during metastasis [36]. By contrast, in HEK293 cells, *SGK1* inhibited the Ras-MAPK kinase pathway through phosphorylation of B-Raf, which is likely to exert an anti-proliferative effect [37].

In terms of regulation of SGK1 expression, in CRC, mutations in *SGK1* may be observed in up to 2% of cases, although the mutations are usually missense and not isolated to the kinase domain [38]. Our previous work has identified that down-regulation in CRCs appears to be independent of promoter hypermethylation [39] and these observations suggest that control is likely to be transcriptionally regulated.

Our focus in this work was on the potential role of *SGK1* in actively promoting differentiation, based on our validated finding that in the colorectum, *SGK1* is expressed predominantly in the terminally differentiated epithelial cell compartment, in which cells have little proliferative potential, but are required to survive and function in a potentially hostile osmolar environment. We initially showed that *SGK1* is induced by the extrinsic differentiating agent NaBut, consistent with NaBut acting, at least in part, through *SGK1*. Based on this preliminary evidence, we directly pursued the hypothesis that *SGK1* re-expression induces features of differentiation. For this, we used both cellular and molecular read-outs in CRC cell lines that retain some differentiation capability. We found that *SGK1* induces not only a gene expression profile of differentiation, but also several specific features of differentiation, including cell polarity, increased tight junction protein expression, and relocation of adherens junction proteins to the membrane. Stem cell-like features, proliferation, apoptosis, growth in soft agar and migration were correspondingly decreased. Our data showed that mechanisms of *SGK1* action plausibly include reduction of c-Myc signalling, and increased expression of the desmosome protein plakophilin 3. In a CRC cell line orthotopic xenograft model, we found *SGK1* re-expression to reduce the growth of lung metastases. The results of this assay suggested that *SGK1*-mediated differentiation of CRC lines inhibits the later stages of metastasis, perhaps functioning by limiting the ability of cells to migrate into the lung parenchyma from the endothelial cells.

Some caveats must be applied to our data. First, SGK1 expression can be hard to assess using western blots/IHC, at least in part because the protein has multiple isoforms and there are few antibodies with good specificities. We therefore relied on mRNA expression using a pan-isoform probe or total isoform expression by sequencing as a read-out for many of our assays. We did, however, perform western blot analysis of tagged SGK1, a limited analysis of native SGK1, and also showed that functional SGK1 protein was produced based on assessment of NEDD4-2 levels. In combination, these experiments strongly supported the existence of active SGK1 in our experiments. Second, given the low *SGK1* levels in CRC cell lines (including those with differentiation capacity) and the potential redundancy between *SGK1* and related SGKs, our experiments necessarily relied on *SGK1* re-expression rather than knock-down. Whilst we appreciate that over-expression of kinases can lead to non-physiological effects, *SGK1* re-expression in our systems led to near-physiological levels of mRNA. Finally, we have utilised an endoscopic transplantation model to determine

effect on metastasis. The limitation of this approach is that as the lumen of the descending colon is narrow and mice become symptomatic relatively early. By contrast, the alternative, a caecal transplantation model allows mice to survive for a relatively longer period and often leads to increased levels of liver metastasis. We utilised the endoscopic transplantation model as it does not involve the creation of a track made from the external surface of the bowel and believe that, anatomically, the caecum of the mouse is markedly different from human.

One largely unanswered question in the field is the differences and similarities between the *AKT* and *SGK1* arms of the *PI3K* pathway. We speculate that whilst both arms promote cell survival, which may explain some of the data showing that loss of SGK1 is deleterious to cells (see above), *SGK1* does this in the context of its additional function of promoting differentiation, particularly in the intestine. In human CRCs, features of differentiation are not necessarily lost, with well and moderately differentiated cancers retaining some ability to form glandular units. Tumour differentiation is an important histological feature that is associated with poor cancer prognosis. The mechanisms through which the differentiation status of a tumour occurs and affects its behaviour and patient prognosis have, however, been somewhat overlooked. Our findings are consistent with the reduced expression of *SGK1* being one important means by which tumours may *"de-differentiate",* and acquire other malignant phenotypes such as migration and invasion. We conclude that a renewed focus on therapeutic agents to differentiate tumours, rather than simply to target cell survival, is justified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Statement of Translational Relevance

The degree of differentiation (usually termed "grade") of colorectal cancers (CRC) is a well-established tumour feature that is associated with patient prognosis, and its assessment forms part of the American Joint Committee on Cancer (AJCC) recommendations. Serum/glucocorticoid-regulated kinase 1 (SGK1) is a highly conserved member of the AGC family of serine/threonine kinases. We have previously demonstrated that *SGK1* expression in normal intestinal epithelium is highest at the top of the crypt, corresponding to the positions of differentiated colonocytes. In this study, we examine the role of *SGK1* in intestinal cell differentiation, both in the normal crypt and in colorectal cancers. Our findings suggest that the level of *SGK1* expression is a strong determinant of CRC differentiation status, identify c-Myc inhibition and plakophilin 3 expression as important downstream mechanisms and demonstrate that increased expression of *SGK1* in an orthotopic xenograft model reduces metastases. In human CRCs, lower *SGK1* expression is associated with poorer differentiation and worse prognosis. Strategies to increase SGK1 pathway activity have potential use as anti-metastatic agents, perhaps in the neo-adjuvant setting.
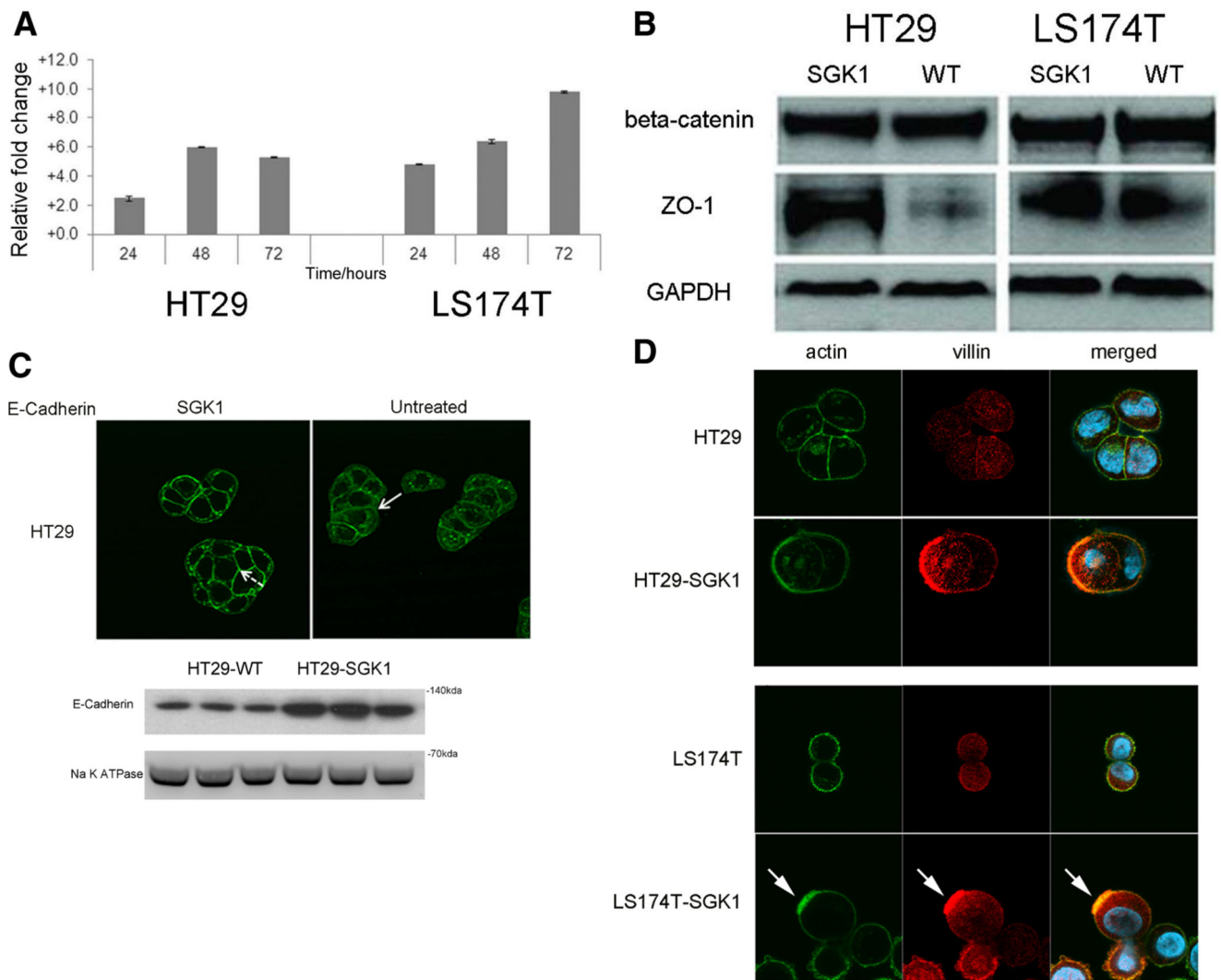
Figure 1. SGK1 causes colorectal cancer cell differentiation.
A. *SGK1* transcription is increased upon sodium butyrate (NaBut) treatment in colorectal cancer cells. The column chart plots fold-change increases in *SGK1* transcript levels, as measured by qRT-PCR, in HT29 and LS174T colorectal cancer cells upon treatment with NaBut, normalized to untreated control and relative to levels at time=0 hrs. Both cell lines showed a significant increase in *SGK1* between 0 and 24 hours, and between 24 and 48 hours (t-test, p<0.01 in all cases, experiments in triplicate). *SGK1* levels plateaued after 48 hours in HT29, whereas a further significant increase was found in LS174T.
B. Representative western blots of junctional proteins in HT29-SGK1 and LS174T-SGK1 cells and untreated wild-type controls. There are essentially unchanged levels of beta-catenin and an increase in ZO-1 abundance upon SGK1 re-expression. A similar ZO-1 increase was also found for cell line HCT116 (not shown).
C. Representative immunofluorescence images suggest re-localisation of E-cadherin from the cytoplasm in parenteral controls (solid arrows) to the membrane in SGK1 re-expressing HT29 (dashed arrows). The cellular fractionation blot confirms increased expression of E-

cadherin in the membrane. Note that LS174T cells carry bi-allelic mutations of the *CDH1* gene, resulting in protein absence.

D. Immunofluorescence analysis on HT29-SGK1 and LS174T-SGK1 and parental controls illustrates the formation of actin 'caps' (green, arrowed), co-localised with the apical marker villin (red), in *SGK1*-transduced cells only. This indicates the establishment of cell polarity owing to SGK1 re-expression.
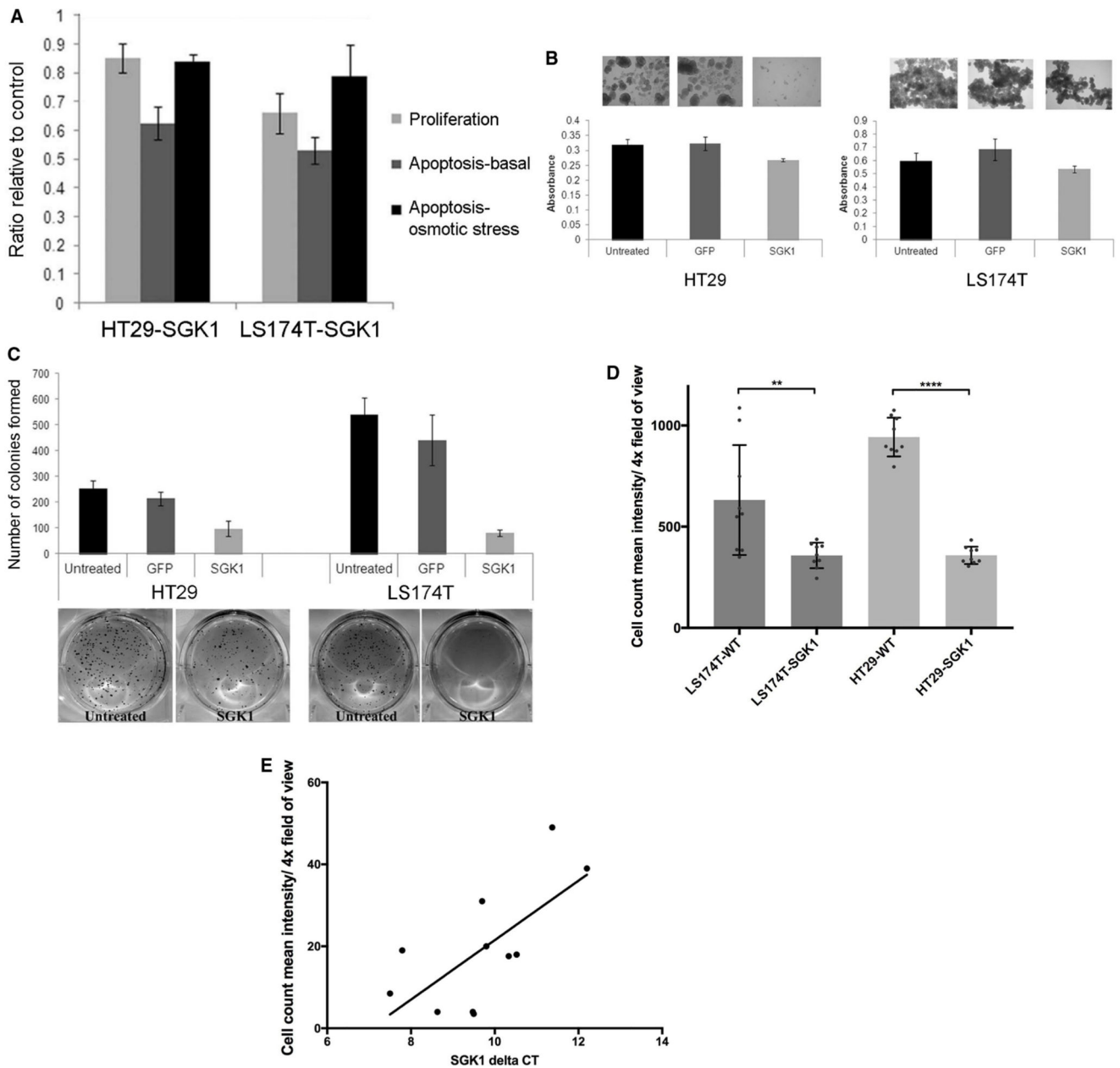
Figure 2. Effects of SGK1-induced differentiation on colon cancer cell proliferation and tumorigenicity.

A. The column chart displays ratios of HT29-SGK1 and LS174T-SGK1 proliferation (measured by tetrazolium compound assay) and apoptosis (basal and upon osmotic stress stimulation) 24 hours after removal of doxycycline, relative to controls with doxycycline maintained. Proliferation and apoptosis were significantly reduced in SGK1-expressing cells (all t-test p-values <0.05, experiments done in triplicate).

B. Representative images show colonospheres formed by untransduced, GFP-transduced (GFP) and SGK1-transduced (SGK1) cells for HT29 and LS174T colorectal cancer cell lines. Images were acquired following 10 days of cell culturing conditions that favour stem

cell growth and colonosphere formation. The column charts represent absorbance measurements from an endpoint tetrazolium compound assay (day 10), showing decreased proliferation rates of the stem cell populations upon re-expression of *SGK1* (t-test p-values<0.01 in all cases; experiments done in triplicate).

C. The column charts show numbers of colonies formed in soft agar by untreated, GFP-transduced (GFP) or SGK1-transduced (SGK1) cells. Results are an average of three experiments (t-test p-values<0.005 in all cases). Below the chart are representative images displaying macroscopic differences between untreated controls or SGK1-transduced cells.

D. Cellular migration is reduced on SGK1 re-expression (reduction in migration rate 43% for LS174T and 62% for HT29; t-test, p<0.01 and p<0.0001 respectively.

E. SGK1 mRNA expressions and CRC baseline migration rate. There is a significant negative correlation (Pearson correlation=0.68, p=0.02) between native *SGK1* expression levels and migration in a panel of CRC lines (COLO205, GP2D, HCT116, HT29, LS174T, LS180, SNU-C4, SW1222, SW48, SW480, T84). Note that higher deltaCt corresponds to lower SGK1 expression.
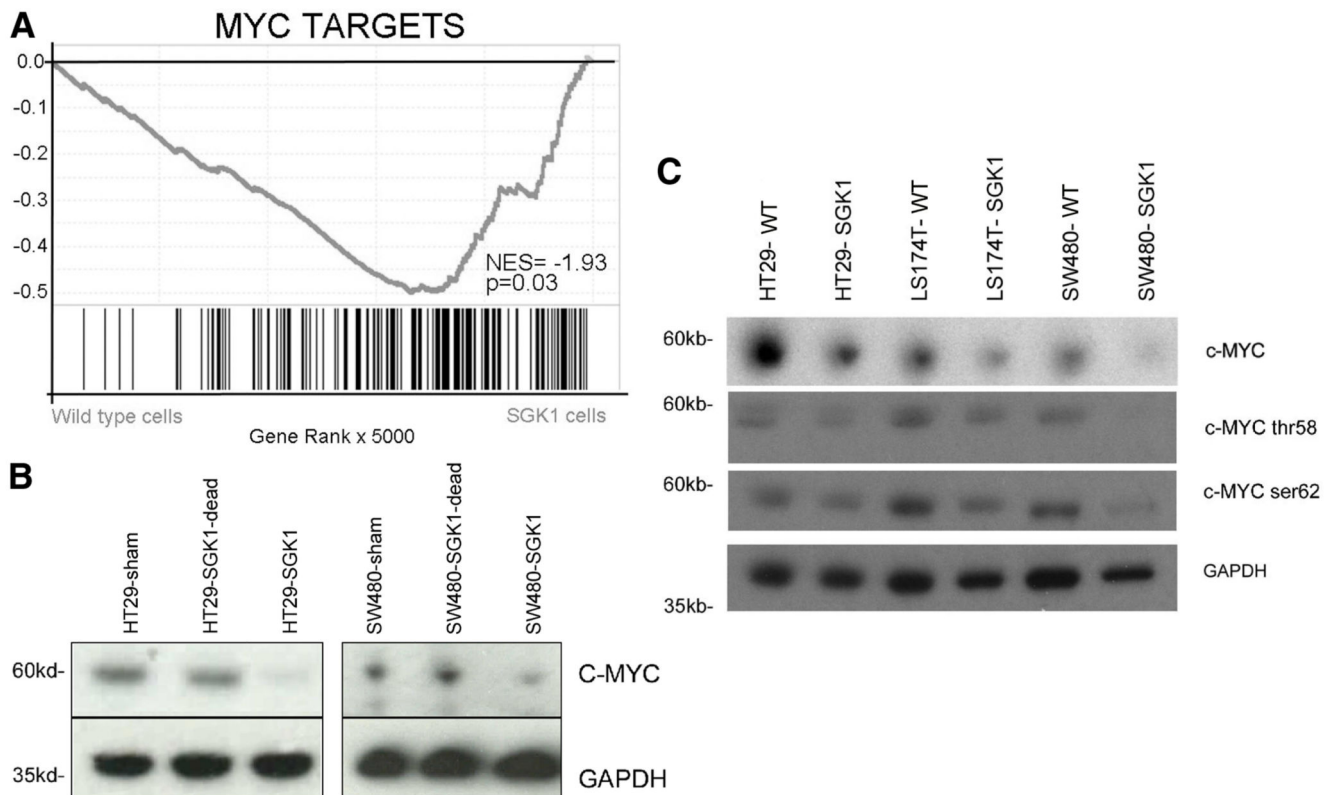
Figure 3. Reduced expression of MYC and its targets after SGK1 re-expression.
A. Gene set enrichment analysis of RNA sequencing data shows greatly reduced expression of c-Myc target genes following *SGK1* re-expression.

B. Representative western blots demonstrating reduced MYC protein expression following *SGK1* re-expression in HT29 and SW480, but not after re-expression of a kinase-dead SGK1.

C. Representative western blots demonstrating decreased total MYC and total phospho-MYC (Ser62 and Thr58) following *SGK1* re-expression in HT29, LS174T and SW480 cell lines.
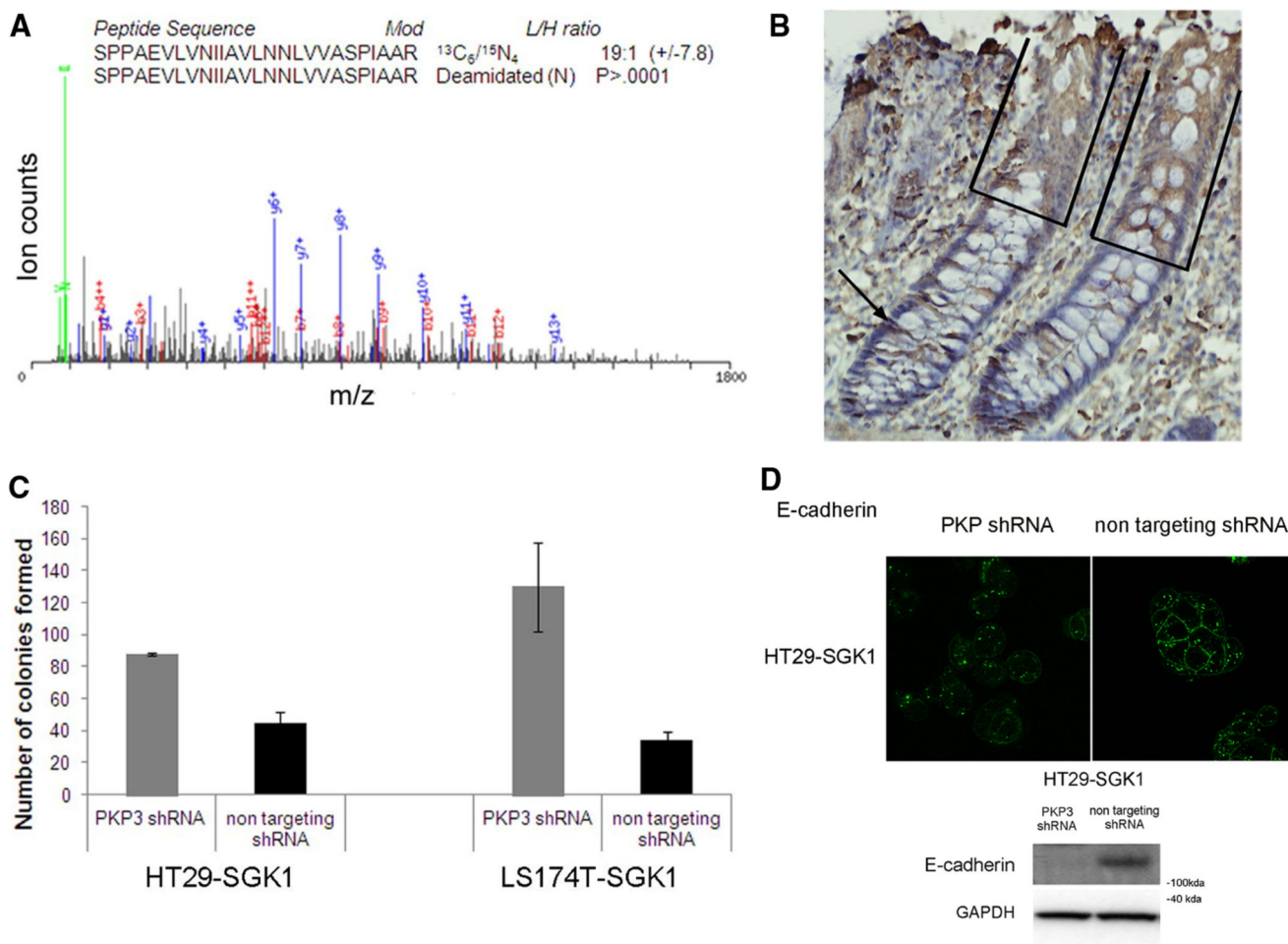
Figure 4. Plakophilin 3 is induced by SGK1 and in turn mediates features of differentiation.

A. Quantitative SILAC mass spectrometry reveals elevated PKP3 in SGK1 re-expressing cells. MS/MS spectrum shows PKP3-derived peptide 727-752, [M+3H]+ 884.2 (calculated MW 2649.5Da), where the fragment ions are indicated (green: immonium ions; blue: y-ions; red: b-ions). The light (SGK1 expressing cells) versus heavy (control cells) ratio as well as the significance (P) are indicated.

B. Immunohistochemistry on paraffin sections derived from normal human colonic tissue shows that expression of PKP3 is stronger in the differentiated compartment of the colonic crypt (boxed), where SGK1 is also expressed, that in the proliferative compartment (arrowed).

C. The bar chart represents the number of colonies formed in soft agar for HT29-SGK1 and LS174T-SGK1 cells with PKP3 knock-down (grey bars) or non-targeting shRNA (black bars), showing that SGK1-induced impaired anchorage-independent growth is reversed upon PKP3 knock-down (t-test p-values<0.005 in all cases).

D. Immunofluorescence shows that the *SGK1*-induced re-localisation of E-cadherin to the cell membrane is impaired upon PKP3 knockdown by shRNA compared with non-targeting shRNA controls. Representative western blots demonstrating decreased total E-Cadherin when *SGK1* re-expressing cells are subjected to PKP3 knockdown by shRNA compared

with non-targeting shRNA controls (results for LS174T cells not shown due to E-cadherin deficiency).
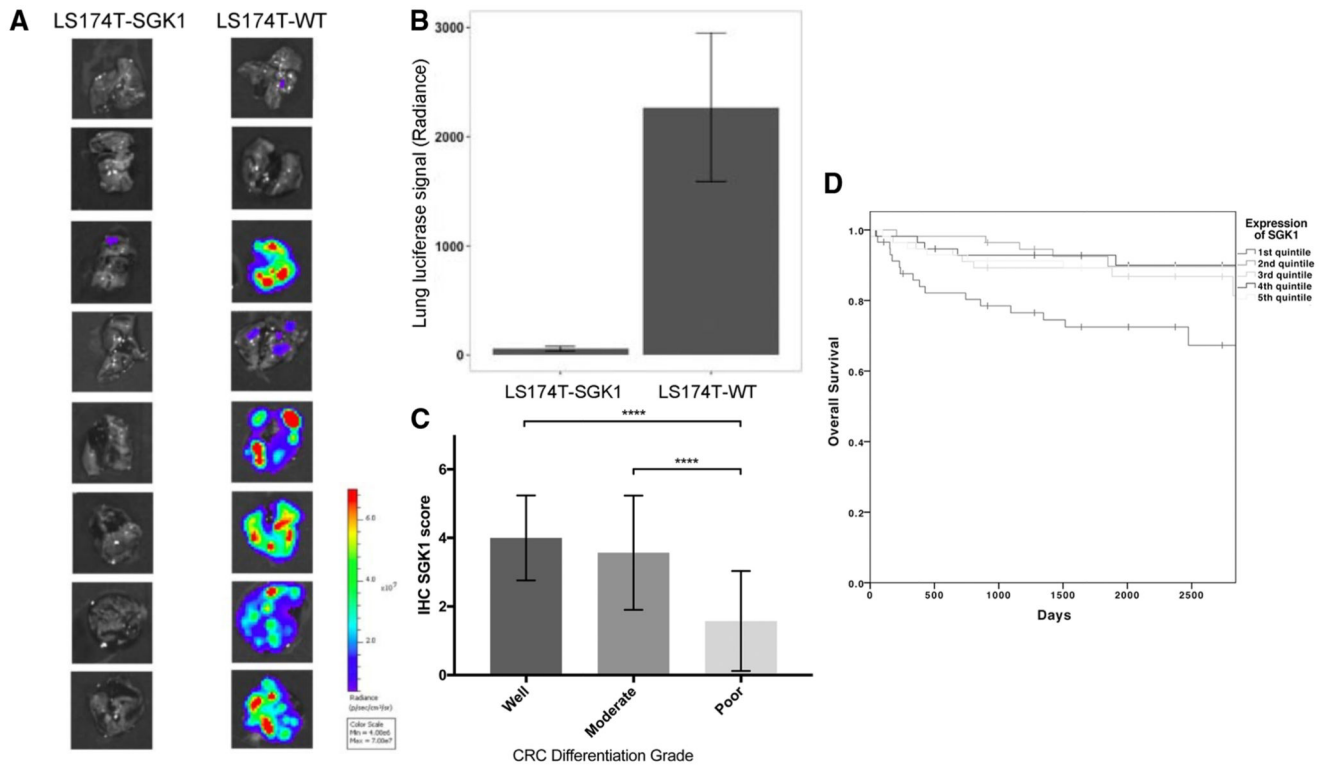
Figure 5. SGK1 re-expression reduces lung metastases in an orthotopic xenograft model of CRC and is associated with poor differentiation and inferior prognosis in human CRCs.

A. *Ex vivo* analysis of luminescence from lung metastases of LS174T-SGK1 and parental cells, demonstrates only very small lesions in the former.

B. Lung metastasis burden after orthotopic delivery of LS174T-SGK1 is much lower than in parental cells.

C. Immunohistochemical detection of SGK1 in a human CRC tissue microarray shows significantly less expression in poorly differentiated CRCs (p<0.01).

D. Data from TCGA demonstrate that patients with the lowest expression of SGK1 have significantly poorer overall survival (p<0.001).

Table 1

Top dysregulated pathways in cell lines re-expressing SGK1 compared with parental controls.

The normalised enrichment core (NES) shows that SGK1 re-expressing cell lines have significantly less expression of gene sets such as Rb/E2F signalling, MYC targets and EMT, with increased expression of gene sets including differentiation, cell polarity and adhesion.

Gene Set	NES	p-value
Protein kinase cascade	1.34	0.004
Cell polarity	1.70	0.010
Regulation of cell differentiation	1.54	0.014
Cell substrate adhesion	1.45	0.031
Stress activated protein kinase signalling pathway	1.39	0.040
Cell matrix adhesion	1.43	0.044
Regulation of cell adhesion	1.47	0.047
Epithelial morphogenesis	-1.38	0.005
Epithelial-mesenchymal transition	-1.69	0.013
MYC targets	-2.61	<0.001
mTORC1 signalling	-2.61	<0.001
Rb/E2F signalling	-1.56	<0.001