

Merkel cell polyomavirus T antigens promote cell proliferation and inflammatory cytokine gene expression

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Merkel cell polyomavirus (MCV) is clonally integrated in over 80 % of Merkel cell carcinomas and mediates tumour development through the expression of viral oncoproteins, the large T (LT) and small T antigens (sT). Viral integration is associated with signature mutations in the T-antigen locus that result in deletions of C-terminal replicative functions of the LT antigen. Despite these truncations, the LT LXCXE retinoblastoma (Rb) pocket protein family binding domain is retained, and the entire sT isoform is maintained intact. To investigate the ability of MCV oncoproteins to regulate host gene expression, we performed microarray analysis on cells stably expressing tumour-derived LT, tumour-derived LT along with sT, and tumour-derived LT with a mutated Rb interaction domain. Gene expression alterations in the presence of tumour-derived LT could be classified into three main groups: genes that are involved in the cell cycle (specifically the G1/S transition), genes involved in DNA replication and genes involved in cellular movement. The LXCXE mutant LT largely reversed gene expression alterations detected with the WT tumour-derived LT, while co-expression of sT did not significantly affect these patterns of gene expression. LXCXE-dependent upregulation of cyclin E and CDK2 correlated with increased proliferation in tumour-derived LT-expressing cells. Tumour-derived LT and tumour-derived LT plus sT increased expression of multiple cytokines and chemokines, which resulted in elevated levels of secreted IL-8. We concluded that, in human fibroblasts, the LXCXE motif of tumour-derived LT enhances cellular proliferation and upregulates cell cycle and immune signalling gene transcription.

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INTRODUCTION

The study of tumour viruses has uncovered a large number of protein signalling networks involved in carcinogenesis. Experimental models have defined gene elements required for human cell transformation by using viral oncogenes as tools to target specific combinations of intracellular pathways *in vitro* (Hahn *et al.*, 1999; Hanahan & Weinberg, 2000, 2011). During carcinogenesis, normal cells acquire mutations that allow them to replicate indefinitely (immortalization), escape apoptosis and promote cell cycle progression. Merkel cell carcinoma (MCC) is an aggressive human skin cancer, typically affecting elderly and immunocompromised individuals (Becker, 2010; Gould *et al.*, 1985). Merkel cell polyomavirus (MCV) was

the first human pathogen discovered by undirected digital transcriptome subtraction (Feng *et al.*, 2008). MCV is causally linked to a majority of MCC cases, with the virally encoded T antigens contributing to transformation (Becker *et al.*, 2009; Feng *et al.*, 2008; Rodig *et al.*, 2012). While many polyomavirus family members can induce transformation of cells *in vitro*, MCV is currently the only polyomavirus identified as a carcinogenic agent of a human tumour (Bouvard *et al.*, 2012).

The large T antigen (LT) produced in tumour cells is universally truncated at the C terminus, eliminating ATPase/helicase functions, while preserving the DnaJ, Vam6p and LXCXE domains (Feng *et al.*, 2011; Houben *et al.*, 2015; Liu *et al.*, 2011; Shuda *et al.*, 2008). The small T (sT) antigen isoform remains intact (Feng *et al.*, 2011; Liu *et al.*, 2011; Shuda *et al.*, 2008). Functional studies on MCV T-antigen function and the detection of sT and LT in MCC tumours indicate a role for both proteins in promoting tumour formation and maintaining the tumour phenotype (Feng *et al.*, 2008; Guastafierro *et al.*, 2013; Houben *et al.*, 2010, 2012; Shuda *et al.*, 2008, 2009). Since the discovery of

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MCV in 2008, many key features of the viral life cycle and functions of viral proteins in tumorigenesis have been investigated; however, the impact of tumour-derived LT on host transcription is not well understood (Chang & Moore, 2012; Spurgeon & Lambert, 2013; Wendzicki *et al.*, 2015). The ability to monitor global mRNA responses to viral oncoproteins provides a powerful tool for investigating mechanisms underlying tumour development (Chang & Laimins, 2000; Duffy *et al.*, 2003; Guan *et al.*, 2003; Pyeon *et al.*, 2007; Rathi *et al.*, 2010). For MCV, digital transcriptome analysis has revealed regulation at the gene expression level of the host-cell protein survivin, which is required for MCV-positive MCC survival (Arora *et al.*, 2012; Dresang *et al.*, 2013). Several other studies have examined transcription profiles of MCV-positive and -negative MCC tumours and cell lines (Daily *et al.*, 2015; Harms *et al.*, 2013; Mouchet *et al.*, 2014). Here, we performed a genome-wide analysis using targeted genomic mutants, as well as comparing the combinatorial effects of tumour-derived LT and sT to examine the functional effect of MCV T-antigen expression on signalling pathways that may contribute to tumorigenesis.

To investigate the mechanisms of MCV-mediated tumorigenesis at the transcriptional level, hTERT-immortalized BJ human foreskin fibroblast (BJ-hTERT) cells were generated to express MCV full-length LT, tumour-derived LT, tumour-derived LT lacking the retinoblastoma (Rb) interaction (by mutating LFCDE to LFCDK) or tumour-derived LT plus sT. We showed that tumour-derived MCV LT enhanced human fibroblast proliferation in an LXCXE-dependent manner. In addition, our gene expression analysis indicated that global transcriptional changes are dependent on the LXCXE domain. We observed upregulation of genes involved in the G1/S-phase transition, DNA replication and the inflammatory response. Regulation was comparable in cells simultaneously expressing tumour-derived LT and sT, indicating that the LXCXE domain of tumour-derived LT is necessary and largely sufficient for transcriptional regulation in BJ-hTERT cells.

RESULTS

BJ-hTERT stable cell lines expressing MCV T antigens exhibit distinct cellular proliferation patterns

Established BJ-hTERT stable cell lines were used to monitor the effect of MCV T antigens on cellular proliferation. LT₃₃₉ isolated from MCC tumour 339 was used to represent tumour-derived LT and has been described previously (Guastafierro *et al.*, 2013; Shuda *et al.*, 2009). Production of LT and sT protein was confirmed by immunoblotting (Fig. 1b). Full-length LT protein was present at equivalent levels post-transduction; however, following selection, it was maintained at levels barely detectable by immunoblotting. Quantitative reverse transcriptase PCR (qRT-PCR) showed a corresponding

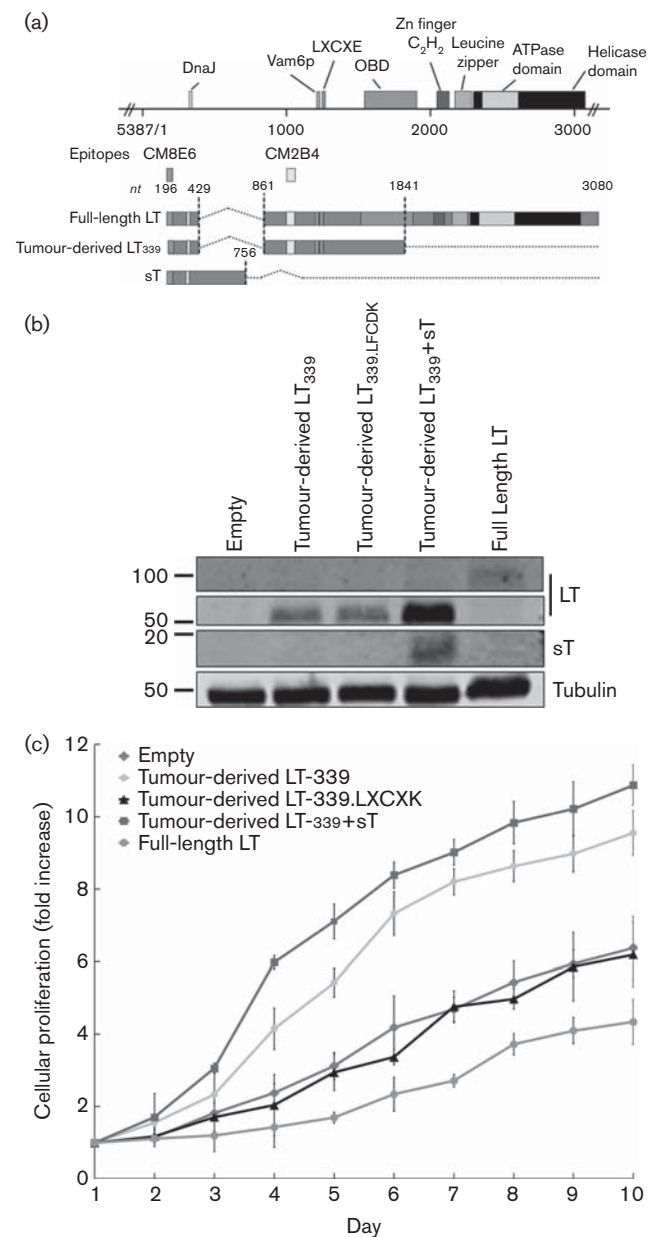


Fig. 1. Characterization of BJ-hTERT fibroblast stable cell lines expressing various MCV T antigens. (a) The early coding region of MCV expresses multiple gene products as a result of alternative RNA splicing. A diagram of full-length LT, tumour-derived LT₃₃₉ and sT cDNA constructs used for generation of stable BJ-hTERT cell lines showing functional domains and antibody epitopes maintained in full-length LT, tumour-derived LT and sT gene products. (b) Whole-cell lysates of stable cell lines expressing MCV T antigens were tested for T-antigen protein production using mouse mAbs CM2B4 (LT) and CM8E6 (sT), with α -tubulin serving as a loading control. (c) BJ-hTERT cellular proliferation was measured for 10 days with the Cell Counting kit-8 reagent. Values were normalized to day 1 and proliferation is represented as fold increase. Proliferation data shown are the means of two independent experiments performed with technical triplicates; error bars represent the SEM of interexperimental variation.

decrease in full-length LT transcript levels following selection, suggestive of selective pressure against high-level full-length LT expression (Fig. 1b and data not shown). In agreement with previous studies, the addition of sT stabilized protein levels of tumour-derived LT (Kwun *et al.*, 2013), which was not due to increased transcript levels (Fig. 1b and data not shown), and full-length LT-expressing cells displayed reduced cellular proliferation (Cheng *et al.*, 2013; Li *et al.*, 2013). In contrast, tumour-derived LT₃₃₉ increased cellular proliferation to significant levels over control cells (Fig. 1c). Cellular proliferation levels between control cells and tumour-derived LT₃₃₉-LFC_{DK} expressing cells were indistinguishable (Fig. 1c). The addition of sT to tumour-derived LT₃₃₉ cells increased initial proliferation over control cells (Fig. 1c).

Hahn *et al.* (1999) used simian virus 40 (SV40) LT and sT, together with human RAS (hRAS) and human telomerase catalytic subunit (hTERT), to fully transform human BJ fibroblasts in the first example of defined oncogene transformation of human cells. We sought to extend these studies to MCV by expressing tumour-derived LT₃₃₉, or tumour-derived LT₃₃₉ plus sT in combination with hTERT and hRAS in BJ fibroblasts. Anchorage-independent growth in a soft-agar colony formation assay was not observed in the presence of tumour-derived LT alone or in combination with sT (data not shown). This indicated that MCV T antigens may have weaker oncogenic activity in human cells compared with SV40 T antigens. Alternatively, MCV T antigens may require additional factors to obtain anchorage-independent growth or exploit an oncogenic programme that does not require hRAS.

Analysis of gene expression perturbations in the presence of MCV T antigens reveals upregulation of cell cycle, DNA replication and immune signalling pathways

To gain insight into the growth-promoting effect of tumour-derived LT₃₃₉, global gene expression changes induced by the expression of MCV T antigens were examined using microarray analysis. Global gene expression analysis was performed on mRNA isolated from three biological replicates of BJ-hTERT cell lines expressing constructs described in Fig. 1(a).

Tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ + sT produced comparable gene expression changes in host cells as determined by hierarchical clustering analysis on genes regulated at a significance level of $P < 0.01$ for any gene class (Fig. 2a). In contrast, results from clustering of tumour-derived LT₃₃₉-LFC_{DK} were indistinguishable from those of the empty vector control. Overall, 2971, 217 and 3490 genes were regulated significantly by tumour-derived LT₃₃₉, tumour-derived LT₃₃₉-LFC_{DK} and tumour-derived LT₃₃₉ plus sT, respectively (Fig. 2b). The overlap of significant gene expression regulation for the established cell lines is shown in Fig. 2(c). Genes showing significant regulation

were further trimmed to those with more than twofold regulation by any sample class: 1068 and 1345 genes were regulated by tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT, respectively (Fig. 2b). A table comparing the expression levels of common genes at a significance level of $P < 0.01$ for tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉-LFC_{DK} is shown in Fig. S1 (available in the online Supplementary Material).

Tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT cells shared more than twofold regulation of 885 genes. Genes regulated in both classes were imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID). Significantly regulated genes corresponded to a large number of gene ontologies that represented pathways such as DNA replication and repair, the cell cycle, cellular proliferation and cellular movement (e.g. taxis, chemotaxis) (Fig. 2d). Similar to the lack of complete overlap of genes in Fig. 2(c), DAVID analysis of over-twofold-regulated gene lists from tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT revealed a large number of genes that were not significantly regulated in both samples (Fig. 2c). Genes uniquely regulated by tumour-derived LT were further enriched for cell-cycle pathways, while addition of sT regulated genes in extracellular region and immune signalling gene ontologies (Fig. 2e).

Tumour-derived LT regulates levels of S-phase and DNA replication genes in an LXCXE-dependent manner

To examine the regulation of cell-cycle gene expression, significantly regulated genes were compared with a list of cell-cycle genes. The heatmap in Fig. 3(a) depicts the hierarchical clustering of significantly ($P < 0.01$) regulated cell-cycle genes, and underscores the importance of an intact LXCXE motif for cell-cycle gene perturbation. Tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT regulated approximately 100 cell-cycle-related genes by more than twofold, while tumour-derived LT₃₃₉-LFC_{DK} was not responsible for any gene regulation of more than twofold (Fig. 3b). Similar to global gene expression, the entire gene set for tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT did not completely overlap (Fig. 3c). Of the outlying genes, only two, PIM2 and EDN1, were regulated differentially. Details of tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT differentially regulated cell-cycle genes are provided in Fig. S2.

A subset of cell-cycle genes was highly regulated by tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT (Fig. 4a). Regulators of S-phase entry as well as DNA replication were altered in the presence of tumour-derived LT₃₃₉ with no significant change in sT-co-expressing cells. Tumour-derived LT₃₃₉ increased expression of multiple E2F1 targets such as CCNE, CDC25A, TYMS, RNA-SEH2A, CDT1 and members of the MCM complex, consistent with LT functions in inactivating the G1/S

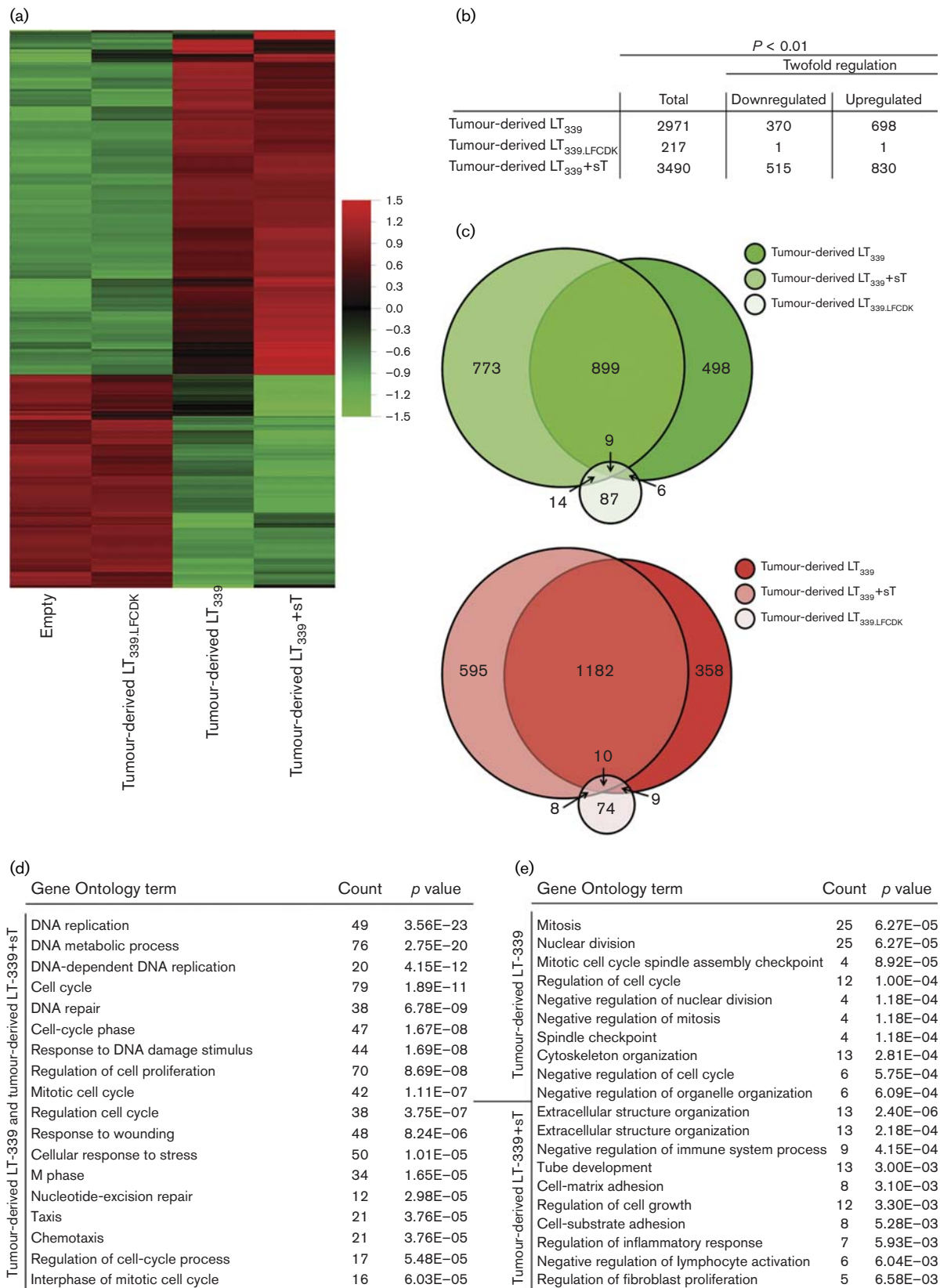


Fig. 2. Global gene expression profile of BJ-hTERT cells expressing MCV T antigens. (a) Heatmap generated from the results of gene expression profiling by microarrays performed on cells expressing tumour-derived LT₃₃₉, tumour-derived LT₃₃₉.LFCDK

and tumour-derived LT₃₃₉ + sT. The gene list used for heatmap generation corresponds to any gene significantly ($P < 0.01$) regulated across all sample classes. (b) Total numbers of genes regulated at $P < 0.01$ and those also regulated by more than twofold. (c) Venn diagrams representing the overlap of significantly regulated genes between tumour-derived LT₃₃₉, tumour-derived LT₃₃₉.LFC_{CDK} and tumour-derived LT₃₃₉ + sT (green, downregulated; red, upregulated). Gene lists generated from the overlapping or non-overlapping regions of the Venn diagram were imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID). (d, e) Gene Ontology results for genes regulated more than twofold by tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ + sT (d) or genes not showing similar regulation by tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ + sT (e).

checkpoint Rb protein and enhancing S-phase entry (Fig. 4a). CDKN2C, an inhibitor of CDK4, is capable of inducing cell-cycle arrest at G1 and was downregulated in the presence of tumour-derived LT₃₃₉. Cell-cycle genes involved in S-phase and DNA replication, such as DNA replication licensing factors CDT1 and GMNN, were positively regulated by tumour-derived LT₃₃₉. ID2, an inhibitor

of DNA replication, was downregulated by tumour-derived LT₃₃₉ alone and with sT (Fig. 4a).

To confirm the microarray results, we performed qRT-PCR (Fig. 4b). In agreement with the array data, CCNE1, CDK2 and GMNN were upregulated and CDKN2C was downregulated by tumour-derived LT₃₃₉. Activation of CDK2 and CCNE1 gene expression correlated with increased protein

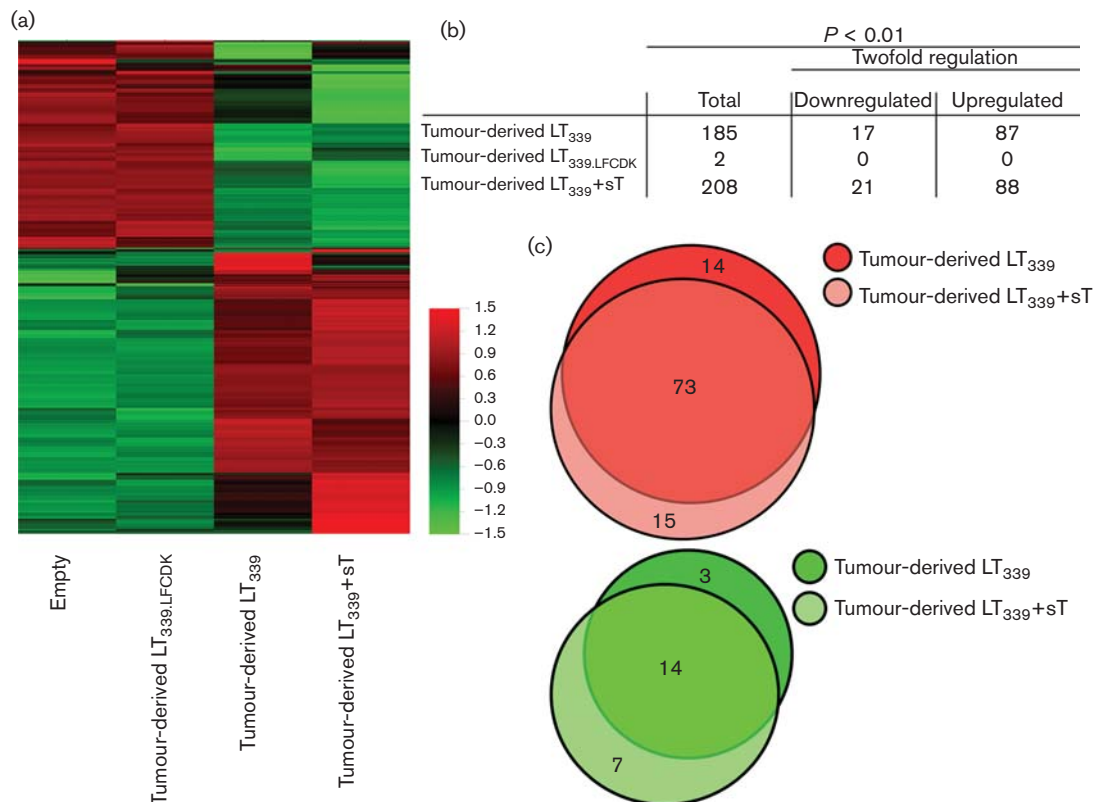


Fig. 3. Regulation of cell-cycle gene expression in the presence of MCV T antigens. (a) Heatmap generated from microarray gene expression profiling performed on cells expressing tumour-derived LT₃₃₉, tumour-derived LT₃₃₉.LFC_{CDK} and tumour-derived LT₃₃₉ + sT. The gene list used for heatmap generation corresponds to a list of genes known to be involved in regulation, progression or inhibition of the cell cycle and DNA replication. (b) Total numbers of cell-cycle genes up- or downregulated at $P < 0.01$ and those also regulated by more than twofold. (c) Venn diagrams of genes from tumour-derived LT₃₃₉- and tumour-derived LT₃₃₉ + sT-expressing cells that were regulated more than twofold at $P < 0.01$ (green, downregulated; red, upregulated).

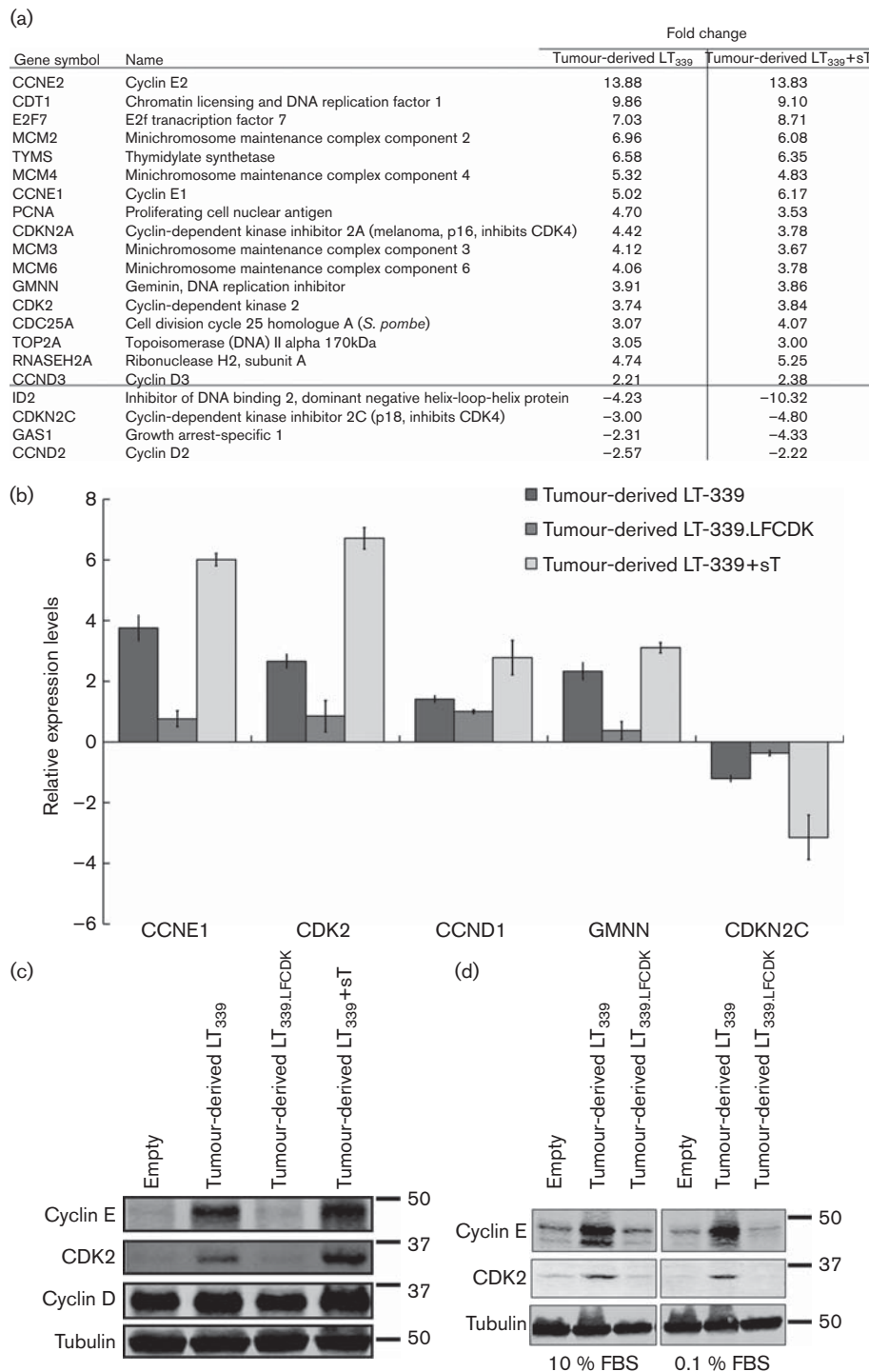


Fig. 4. LXCXE-dependent upregulation of genes involved in cell-cycle progression. (a) Cell-cycle genes significantly regulated in response to tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ + sT expression. Fold change values are the means of biological triplicates. (b) Relative expression levels of cell-cycle-related genes as determined by qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase expression was used as a control and expression levels were calculated using the $\Delta\Delta C_t$ method. Expression values were compared with empty vector cells. (c) Whole-cell lysates of stable cell lines expressing MCV T antigens were probed for cyclin E1, CDK2 and cyclin D1 proteins. Detection of α -tubulin was used as a loading control. (d) Lysates from cells expressing T antigens that were incubated with either 10 % or 0.1 % FBS were used to detect cyclin E1 and CDK2, with α -tubulin as a loading control.

levels (Fig. 4c). As levels of cyclin proteins are temporally regulated during the cell cycle, we tested cyclin E and CDK2 protein levels during serum starvation. After growth of LT₃₃₉-expressing cells in 0.1 % FBS for 3 days, upregulation of cyclin E and CDK2 protein levels was observed (Fig. 4d). To extend this finding, tumour-derived LT from MCC 350 (LT₃₅₀; the shortest identified truncated tumour LT protein), LT from MKL-1 cell line (LT_{MKL-1}) and a Δ LFCDE mutant LT truncated just N-terminal to the LFCDE domain (LT $_{\Delta$ LFCDE}) were examined (Arora *et al.*, 2012). Cells were tested for LT protein production and all MCV LT antigens having an intact LXCXE domain increased cyclin E protein levels (Fig. 5).

Chemokine and cytokine gene expression is upregulated in the presence of tumour-derived LT and is further enhanced by sT

Forero *et al.* (2014) recently described a novel immune activation pathway dependent on ATR signalling that is generated by SV40 LT expression. In our microarray analysis, an enrichment of deregulated genes involved in biological activities, such as chemotaxis and enhanced movement of leukocytes, in cells expressing MCV T antigens revealed a similar activation of immune response pathways by MCV LT (Fig. 6a). IFN-induced genes *OAS1*, *OAS2* and *ISG20* were upregulated by tumour-derived LT₃₃₉ and tumour-derived LT₃₃₉ plus sT; however, the most enriched pathways involved activation of chemokines and cytokines such as IL-8, CXCL1, IL-6, IL-1 β and CXCL6 (Fig. 6b). Increased expression of cytokines and chemokines is associated with cellular proliferation, activation of cells, movement/chemotaxis and the inflammatory immune response (Li *et al.*, 2003; Mantovani *et al.*, 2008; Waugh & Wilson, 2008). qRT-PCR confirmed increased *CXCL1*, *MMPI* and *IL-8* gene expression by tumour-derived LT₃₃₉, which was further enhanced in the presence of sT (Fig. 6c). Expression levels of IL-1 β were also increased when measured by qRT-PCR, but levels of expression in tumour-derived LT₃₃₉ plus sT cells did not mirror the microarray results (Fig. 6c). In order to confirm upregulation of IL-8 at the protein level, we monitored IL-8 levels in the supernatant. Tumour-derived LT₃₃₉ plus sT significantly increased the production of IL-8; however, secreted IL-8 in tumour-derived LT₃₃₉ expressing cells did not correlate with increased *IL-8* transcripts. Interestingly, upregulated genes enriched in immune signalling pathways were regulated by tumour-derived LT₃₃₉ with an intact LXCXE motif, linking the Rb-E2F signalling axis to the regulation of chemokine and cytokine gene expression. Addition of sT to tumour-derived LT enhanced regulation of immune response genes by as much as sevenfold (CSF2) but did not significantly regulate any genes differentially (Fig. 6b).

DISCUSSION

Tumour-derived LT alters several gene expression programmes in host cells that correlate with increased

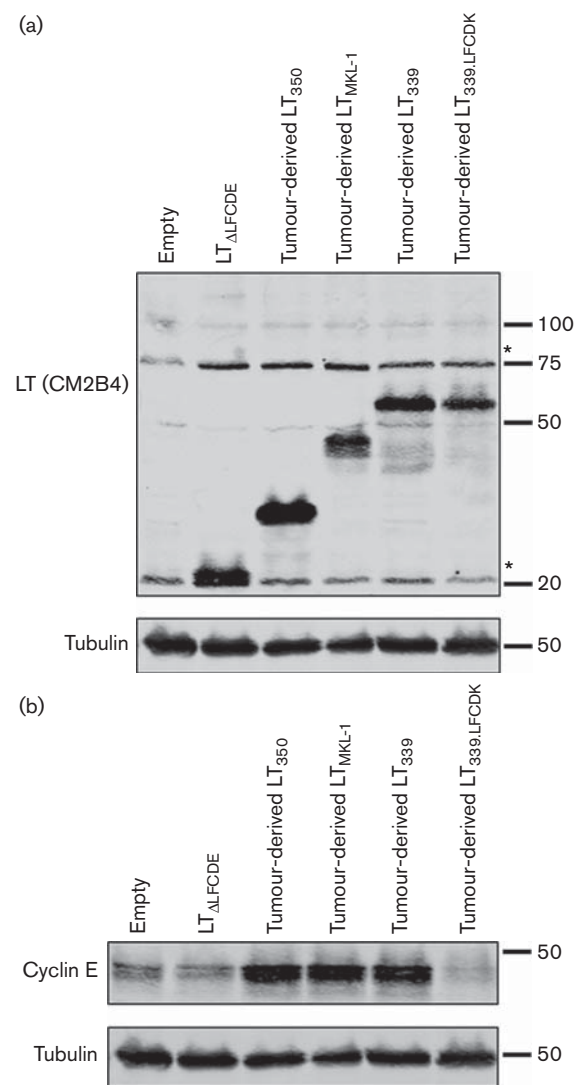


Fig. 5. Tumour-derived T antigens with varying truncation sites increase cyclin E protein levels. (a) Additional stable cell lines were generated to express MKL-1-LT, the 350-LT or LT truncated prior to the LFCDE motif. Cell lysates were immunoblotted for LT expression using CM2B4 mAb, as well as α -tubulin as a loading control. *, Non-specific. (b) BJ-hTERT stable cells from (a) were immunoblotted for cyclin E1, with α -tubulin as a loading control.

cell-cycle protein levels, cellular proliferation and secretion of IL-8. The tumour-derived LT gene expression alterations are quantitatively enhanced when sT is co-expressed. Interestingly, we found tumour-derived LT with a mutated LXCXE motif to be inert for gene expression changes and the associated enhanced cellular proliferation and chemokine secretion. MCV LT has numerous other biologically active domains including a DnaJ domain, a Vam6p sequestration domain, p300 interaction domains, and a DNA-binding and helicase domain (Houben *et al.*, 2015; Wendzicki *et al.*, 2015). These viral motifs are known to

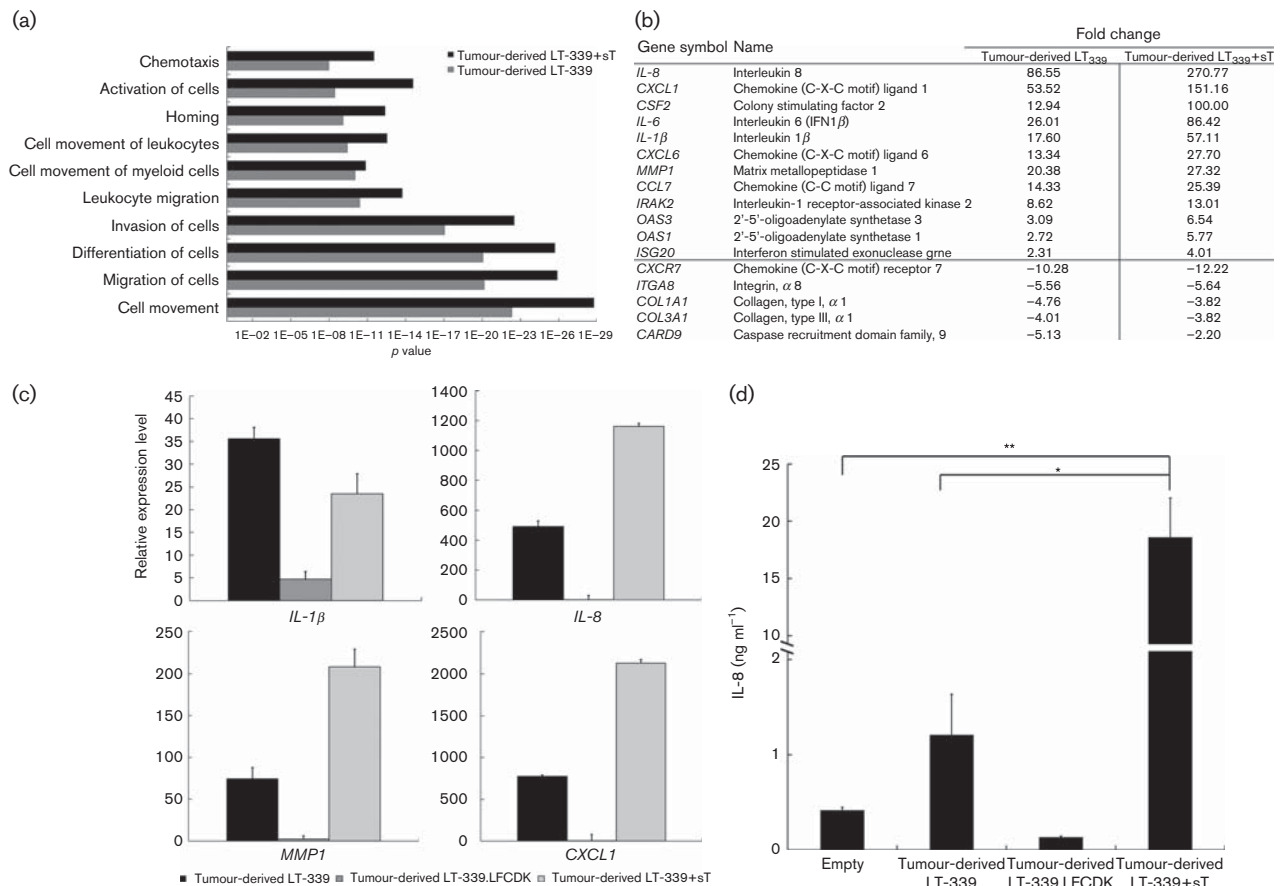


Fig. 6. Additional pathways upregulated by MCV T antigens. (a) Significantly regulated biological functions excluding cell-cycle/DNA replication functions from Ingenuity Pathway Analysis (see Methods). (b) Fold changes of highly regulated genes contributing to the regulation of biological functions in (a). Fold change values are the means of biological triplicates. (c) Relative expression levels of chemokine/cytokine-related genes in BJ-hTERT cells expressing tumour-derived LT₃₃₉, tumour-derived LT₃₃₉.LFCDK and tumour-derived LT₃₃₉ + sT as determined by qRT-PCR. Glyceraldehyde 3-phosphate dehydrogenase expression was used as a control and expression levels were calculated using the $\Delta\Delta C_t$ method. Expression values were compared with empty vector cells. (d) Concentration of IL-8 (ng ml^{-1}) in the supernatant of MCV T-antigen expressing cells. IL-8 levels were normalized against the total protein content of the same samples. ** $P < 0.001$; * $P < 0.005$ (Student's *t*-test).

regulate post-transcriptional responses that may not be reflected in microarray analyses. Under the conditions of these experiments, we failed to find evidence for non-LXCXE regulation of gene expression during tumour-derived LT expression. Involvement of the SV40 LT DnaJ domain in regulating host transcription is observed only in the presence of an intact LXCXE domain, a point our current analysis did not address (Rathi *et al.*, 2009; Stubdal *et al.*, 1997; Sullivan *et al.*, 2000). Co-expression of tumour-derived LT and sT also failed to qualitatively change cellular mRNA expression patterns, although sT enhances LT stability by inhibiting Fbw7 (Kwun *et al.*, 2013). MCV sT is a promiscuous inhibitor of E3 RING ligases, which target the anaphase-promoting complex cdc20, resulting in prometaphase arrest and activation of CDK1 (Shuda *et al.*, 2015). While sT is a potent inducer of mitosis, less than 0.1–1.0 % of BJ cells are in mitosis at any one time,

making it unlikely that sT effects on gene expression through this pathway can be detected by microarray analysis. Furthermore, MCV sT-induced CDK1 activation leads to phosphorylation and inactivation of 4E-BP1, resulting in increased cap-dependent translation without changes in mRNA levels that would be detected in our assay (Shuda *et al.*, 2015).

In this report, we further investigated the effect that tumour-derived MCV LT has on human fibroblast growth. While MCV T antigens are not capable of substituting for SV40 T antigens in a human cell transformation assay, tumour-derived LT does enhance cellular proliferation. Growth stimulation is dependent on an intact Rb interaction domain and is not significantly affected by sT expression. The growth-promoting effect of tumour-derived MCV T antigen in human BJ-hTERT cells as well

as mouse embryonic fibroblasts was reported previously; however, the contribution of sT antigen, as well as LXCXE dependence, was not addressed (Cheng *et al.*, 2013). In addition, a recent report by Li *et al.* (2013) indicated that truncated LT does not have a growth-promoting effect in U2OS cells, which may be due to the fact that U2OS is a transformed cancer cell line with oncogenic signalling already in place. MCV LT expression therefore may not further increase the growth rate of this highly proliferative cell line. Our results showed LXCXE dependence for tumour-derived LT₃₃₉ enhancement of cellular proliferation, which is in line with a previous study indicating loss of MCV-positive MCC xenograft growth upon LXCXE mutation (Houben *et al.*, 2012). In agreement with previous reports, we observed a decrease in proliferation in cells expressing full-length LT (Cheng *et al.*, 2013; Li *et al.*, 2013).

By microarray analysis, we observed two main gene expression programmes, cell-cycle/DNA replication and chemokine/cytokine activity, which were altered in the presence of tumour-derived LT with an intact LXCXE domain. Additional expression of sT enhanced the regulation observed for tumour-derived LT. As we observed increased tumour-derived LT protein levels when sT was present, increased gene expression regulation in co-expressing cells is not surprising. We used NextBio (Illumina) to compare previous MCC and MCV gene expression studies with our results for tumour-derived LT and tumour-derived LT plus sT (Kupersmidt *et al.*, 2010). An analysis by Harms *et al.* (2013) offered evidence that MCV regulation of Rb is required for the establishment of tumorigenesis in MCV-positive MCCs, providing confirmation of the importance of an intact LXCXE motif in MCV-mediated gene expression changes. Comparison of MCV-positive MCC cell lines with MCV-negative cell lines showed a similar enhancement of cell-cycle and DNA replication pathways, while not corroborating our observed immune signalling upregulation; however, the MCV-negative cell lines are reported to be more closely related to breast cancer cell lines and may not be a suitable negative control for such comparisons (Daily *et al.*, 2015).

The regulation of cell-cycle pathways is not unexpected for a DNA tumour virus, as the ability of the main oncoproteins of papillomaviruses and adenoviruses to manipulate host cell-cycle processes is well characterized. More specifically, the significant regulation of CDK2 and CCNE1/CCNE2 transcript levels indicates that tumour-derived LT₃₃₉ facilitates the G1/S transition and stimulates S-phase entry. This is supported by the fact that CDC25A, which functions to regulate G1/S transition by dephosphorylating CDK2, is upregulated in tumour-derived LT₃₃₉-expressing cells. In addition, the G1/S transition inhibitor CDKN2C (p18) is downregulated in BJ-hTERT tumour-derived LT₃₃₉ cells. Increased cyclin E/CDK2 is capable of inducing the S phase and also possesses the ability to further activate E2F-regulated transcription (Hwang & Clurman, 2005). The lack of consistent upregulation of D-type cyclins in

our analysis in cells expressing MCV T antigens may be due to cyclin redundancy. Similar induction of cyclin E/CDK2-mediated S-phase entry in the absence of cyclin D1 activation is accomplished by SV40 LT, human papillomavirus (HPV) E7 and adenovirus E1A (Lukas *et al.*, 1994; Spitkovsky *et al.*, 1994; Zerfass *et al.*, 1995). Cyclin E can regulate multiple cellular proteins, including cdc6, which allows assembly of the pre-replication complex during the G0/S transition (Coverley *et al.*, 2002). This correlates with our observed increase in cell-cycle and DNA replication gene transcription following T-antigen expression. MCV T antigens also regulate various members of host DNA replication pathways, such as CDT1, geminin (GMNN), proliferating cell nuclear antigen (PCNA), replication protein A (RPA) and multiple members of the MCM complex. Interestingly, CDT1 and GMNN upregulation would not normally be indicative of DNA replication, as GMNN is known to block formation of the pre-replication complex. However, evidence suggests that overexpression of GMNN does not suppress cellular proliferation and is instead a mechanism for controlling abnormal Cdt1 association with origins in the S and G2 phases of the cell cycle (Wohlschlegel *et al.*, 2002). In cancer cells and those expressing HPV E7, upregulation of CDT1 is associated with re-replication, leading to increased genome instability (Ballabeni *et al.*, 2013; Fan *et al.*, 2013). It will be of interest to investigate the gene expression patterns of CDT1 and GMNN in MCC and whether transcript levels correlate with increased DNA re-replication and genome instability.

In addition to regulating the cell cycle/DNA replication, a second set of enriched gene sets corresponding to immune signalling pathways are upregulated in the presence of tumour-derived LT in an LXCXE-dependent manner. In line with SV40 gene expression studies, we found that tumour-derived MCV T antigen upregulated IFN-induced genes and chemokine/cytokine transcripts. Tumour-derived LT highly upregulated CXCL1 and IL-8, which are CXC chemokine family members that, when activated, induce cellular proliferation and migration (Dhawan & Richmond, 2002; Luca *et al.*, 1997; Mackay, 2001; Schadendorf *et al.*, 1994). The proliferation- and migration-promoting effects of IL-8 are associated with epidermal growth factor receptor transactivation, as well as increased expression and activity of metalloproteinases (MMPs), several of which are also over-represented in tumour-derived LT-expressing cells (Li *et al.*, 2003; Schraufstatter *et al.*, 2003). The effects on CXC chemokines and MMP-related genes by tumour-derived T antigens may allow increased invasion and metastasis following tumour development. Interestingly, a study reported that HPV16 E6 induced IL-8 and IL-6 production and subsequent upregulation of MMPs, indicating that this pathway may be commonly targeted by DNA tumour virus oncoproteins (Cheng *et al.*, 2008; Shiao *et al.*, 2013; Toussaint-Smith *et al.*, 2004). In addition to confirming gene expression alterations, we measured IL-8 protein levels and found

increased secreted IL-8 levels in the supernatant of cells expressing tumour-derived LT plus sT. While there was a trend towards increased secretion of IL-8 in tumour-derived LT-expressing cells, this increase did not reach significance. These data suggest that IL-8 secretion is not directly correlated with mRNA abundance or that a threshold of increased gene expression is required to obtain significant IL-8 secretion in BJ-hTERT fibroblasts. Further investigation into the ability of sT to stimulate IL-8 secretion will be needed to determine whether the observed increase is due to the ability of sT to stabilize LT or if sT facilitates secretion independent of LT. Future functional analyses are needed to elucidate whether or not our observations regarding overall increased immune response regulation in the presence of MCV T antigens can be validated and proven significant in MCV biology.

METHODS

Cell lines and generation of stable cell lines. BJ-hTERT fibroblasts and HEK293FT were grown in Dulbecco's Modified Eagle Medium (DMEM) (Corning Cellgro) with 10 % FBS (Sigma). All cells were grown at 37 °C in humidified air containing 5 % CO₂. Stable cells were established using lentiviral transduction of cDNA constructs that encoded full-length LT, tumour-derived LT₃₃₉, tumour-derived LT₃₃₉ with a mutated LFCDE interaction domain (LT₃₃₉-LFCDK), tumour-derived LT₃₅₀, tumour-derived LT_{MKL-1}, LTΔLFCDE or tumour-derived LT₃₃₉ plus sT protein (Arora *et al.*, 2012; Shuda *et al.*, 2011). Primers for construction of tumour-derived LT₃₅₀, tumour-derived LT_{MKL-1} and LTΔLFCDE from the codon-optimized full-length LT are included in Fig. S3. As described previously (Arora *et al.*, 2012; Shuda *et al.*, 2011), lentiviruses for pLVX and pSMPUW overexpression constructs of MCV T antigens were produced in HEK293FT cells (Invitrogen) using psPax2 and pMD2.G helper constructs (Addgene). At 3 days post-transfection, virus-containing supernatants were harvested and filtered through 0.45 μm filters. Lentiviral transduction was performed in the presence of 6 μg polybrene ml⁻¹. The medium was changed after 24 h, and antibiotic selection was added (puromycin at 1 μg ml⁻¹; G418 at 400 μg ml⁻¹; Sigma) on day 3 after infection. Cells were grown under selection medium until all mock-infected cells had died. Stable cells were then grown in the absence of antibiotic selection prior to harvesting for microarray or immunoblot analysis.

Illumina BeadChip array. RNA was extracted from biological triplicate stable cell lines of BJ-hTERT empty, tumour-derived LT₃₃₉, tumour-derived LT₃₃₉-LFCDK and tumour-derived LT₃₃₉ + sT cells using TRIzol reagent (Invitrogen). The RNA was treated with DNase I (Ambion), and RNA integrity was assessed. For microarray analysis, the integrity of RNA samples was confirmed with an RNA integrity number of 6 or above, which correlates to the amount of degradation observed following electrophoresis (RNA nanochip; Agilent). Human HT-12 v4 Expression BeadChips (Illumina) were processed in the Genomics Research Core at the University of Pittsburgh. Microarray data were normalized to total fluorescence intensity using cubic spline normalization. For multiple probes per gene, means of all probes were used for individual gene analyses.

Microarray data analysis. Cubic spline-normalized data were imported into Excel and analysed using BRB (Biometric Research Branch)-ArrayTools developed by Dr Richard Simon and the BRB-ArrayTools Development Team (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). Hierarchical clustering of genes was performed using the one minus correlation with mean linkage on centred and

scaled genes, and was visualized using the dynamic heatmap viewer. The class comparison tool was used to identify genes that were differentially expressed among sample classes using a random-variance *t*-test (Wright & Simon, 2003). Genes were considered statistically significant if their calculated *P* value was less than 0.01. A global test was performed to determine whether the expression profiles differed between the classes by permuting the labels of which arrays corresponded to which classes. For each permutation, the *P* values were recomputed and the number of genes significant at the *P* < 0.01 level was noted. The proportion of the permutations that gave at least as many significant genes as with the actual data were the significance level of the global test permuting the labels of which arrays corresponded to which classes.

Lists of regulated biological functions were generated through the use of Qiagen's Ingenuity Pathway Analysis (IPA; Qiagen, <http://www.ingenuity.com>). DAVID analysis Gene Ontology results based on significantly regulated gene lists were ranked based on an EASE score, which is a modified Fisher's exact *P* value that is adapted to measure gene-enrichment in annotation terms (Huang *et al.*, 2009a, b). Venn diagrams were designed based on the generation of area-proportional Venn diagrams by BioVenn (Hulsen *et al.*, 2008). NextBio from Illumina (<http://www.nextbio.com>) was used to compare the microarray results of this study with relevant publicly available datasets (Kupersmidt *et al.*, 2010).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002).

Immunoblotting. Cells were lysed in radioimmunoprecipitation assay buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1 % SDS, 0.5 % sodium deoxycholate, 1 % NP-40 and protease inhibitors (Complete Cocktail; Roche). Lysates were kept on ice for 15 min, sonicated and then centrifuged at 20 000 g for 10 min at 4 °C. Supernatants were mixed with 5 × SDS loading dye and denatured at 100 °C for 5 min. Proteins were separated by SDS-PAGE and transferred onto a Hybond-C nitrocellulose membrane (GE Healthcare). Viral proteins were detected using mAbs CM2B4 (LT) and CM8E6 (sT) as described previously (Kwun *et al.*, 2009; Shuda *et al.*, 2009). Cyclin E was detected using the mouse mAb HE12 (Cell Signalling) at 1 : 1000. CDK2 was detected using the mouse mAb clone D-12 (Santa Cruz) at 1 : 500. Cyclin D1 was detected using the rabbit polyclonal antibody M20 (Santa Cruz) at 1 : 1000. α-Tubulin was detected with the rabbit mAb clone 11H10 (Cell Signalling) at 1 : 2000 or the mouse mAb clone 12G10 developed by J. Frankel and E. M. Nelsen (obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, USA). For secondary antibody detection, an Odyssey Infrared Imaging system (Li-Cor) was used with IRDye 680- or 800-conjugated secondary antibodies (1 : 8000 dilution; Rockland Immunochemicals).

Quantitative RT-PCR. RNA was extracted from BJ-hTERT stable cell lines using TRIzol reagent (Invitrogen) following the protocol. The RNA was treated with DNase I (Ambion) and cDNA was synthesized using a SuperScript III First Strand Synthesis kit (Invitrogen). The primer sequences are provided in Fig. S3. qRT-PCR was performed on a CFX96 cyclor (Bio-Rad) using SsoFast Evagreen (Bio-Rad) according to the manufacturer's protocol. Results were normalized to glyceraldehyde 3-phosphate dehydrogenase and standardized relative to the empty vector BJ-hTERT qRT-PCR results. Two independent RNA isolations were performed. Each of the RNA isolations was tested twice with technical duplicates.

Cellular proliferation assay. Cells were seeded into 96-well plates at 5000 cells per well. Proliferation was monitored using a Cell Counting Kit-8 (Dojindo). Absorbance at 440 nm was obtained using a Synergy plate reader (BioTek). Background absorbance from wells containing

medium alone, as well as absorbance at 650 nm, was subtracted from all samples. Absorbance values were divided by the absorbance value of day 1 for normalization, and fold increase was used to evaluate cell proliferation. The proliferation assay was performed twice with technical triplicates.

ELISA. Two millilitres containing 4×10^6 cells was added to each well of a six-well plate. After 48 h, supernatants were collected and analysed using a sandwich ELISA. Cells were harvested to determine total protein concentration. A Human CXCL8/IL-8 DuoSet ELISA development system and ancillary reagent kit were purchased from RnD Systems. Absorbance at 450 nm was obtained and compared with background absorbance at 570 nm. Absorbance values were normalized to the total protein content. ELISA data were obtained from three independent supernatant harvests; each assay was performed with technical duplicates. Significance was determined by Student's *t*-test.

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