Merkel Cell Polyomavirus–Positive Merkel Cell Carcinoma Requires Viral Small T-Antigen for Cell Proliferation

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TO THE EDITOR

Angermeyer *et al.* (2013) claim that ''Merkel Cell Polyomavirus–Positive Merkel Cell Carcinoma Cells Do Not Require Expression of the Viral Small T Antigen.'' This controversial conclusion is based on their inability to detect Merkel cell polyomavirus (MCV) small T (sT) protein expression and to inhibit cell growth by putative sT knockdown in MCV-MCC (Merkel cell carcinoma) cells.

These findings contradict existing evidence showing MCV sT protein expression in MCV-MCC cancer tissues (Shuda *et al.*, 2011) and cell lines (see Figure 2, Houben *et al.*, 2010) (Guastafierro *et al.*, 2013). To investigate this discrepancy, we tried replicating the results of Angermeyer *et al.*, (2013) using the same antibodies to detect MCV sT (CM8E6 (Kwun *et al.*, 2009), CM5E1 (Shuda *et al.*, 2011), and 2T2 (Wang *et al.*, 2012), kindly provided by C. Buck) on a panel of MCV-MCC cell lines (Figure 1). MCV sT and large T (LT) are alternatively spliced viral oncoproteins sharing a common N terminus but having different C-termini, thus CM8E6 and 2T2 detect all isoforms of T-antigens, while CM5E1 detects only sT and CM2B4 detects only LT and related isoforms. Differences in protein expression levels between MCV LT and sT are likely dependent on either pre-mRNA or post-transcriptional protein processing. For positive and negative controls, we used UISO cells transiently transfected with the MCV T-antigen locus (JN038578) or with corresponding empty vector. UISO, commonly described as being from MCC origin (Houben *et al.*, 2007), is negative for MCV and miRNA ontology studies show it clusters with cell lines of breast cancer origin (Renwick *et al.*, 2013). In contrast to Angermeyer *et al.*, the 19 kD MCV sT band is readily detected in all MCV-MCC cell lines (open arrows) but not in UISO cells.

IS MCV ST REQUIRED FOR MCC CELL PROLIFERATION?

The knockdown by Angermeyer *et al.*, (2013) used different small hairpin

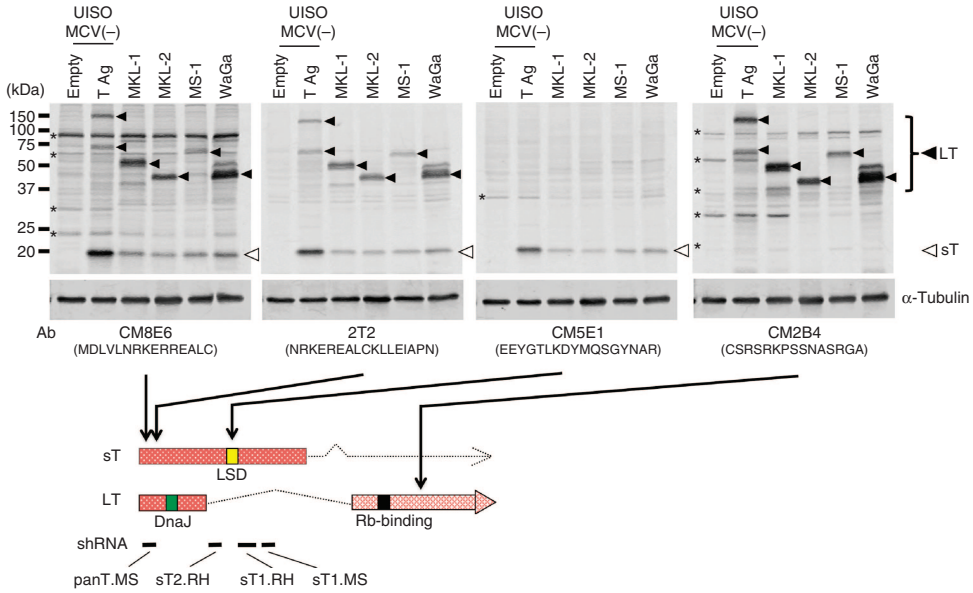

Figure 1. Detection of Merkel cell polyomavirus (MCV) small T (sT) antigen expression by multiple MCV T-antigen antibodies. MCV-positive Merkel cell carcinoma (MCC) cells (MKL-1, MKL-2, MS-1, and WaGa) and MCV-negative UISO cells transfected with MCV genomic T-antigen gene or empty vector as positive and negative controls, were immunoblotted with multiple MCV T-antigen antibodies. α-Tubulin was used as a loading control. Both large T (LT, closed arrows) and small T (sT, open arrows) were detected by CM8E6 and 2T2, sT by CM5E1, and LT by CM2B4. Asterisks indicate non-specific bands. Peptide sequences used for mAb production and shRNA targeting sites are shown in the bottom diagram of T-antigen transcripts with a DnaJ (green box), an Rb-binding (black box) as well as large T stabilization (LSD, yellow box (Kwun *et al.*, 2013)) domains.

Abbreviations: LT, large T; MCC, Merkel cell carcinoma; MCV, Merkel cell polyomavirus; shRNA, small hairpin RNA; sT, small T

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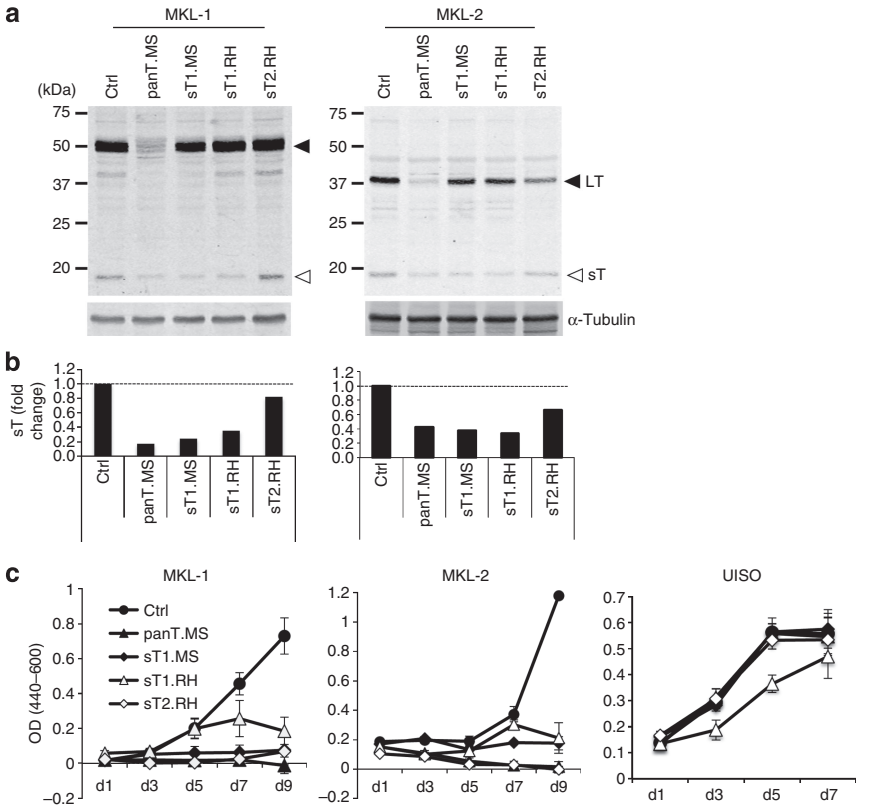

Figure 2. Merkel cell polyomavirus (MCV) small T (sT) antigen knockdown inhibits MCV-positive Merkel cell carcinoma (MCC) cell proliferation. (**a**) MCV-positive MCC cell lines, MKL-1 and MKL-2, were transduced with pLKO.1-based lentiviral shRNAs targeting both LT and sT (panT.MS) or sT alone (sT1.MS, sT1.RH, and sT2.RH) as described (Houben *et al.*, 2010). Both LT (closed arrows) and sT (open arrows) proteins are detected by 2T2. (**b**) Expression of LT and sT was quantitated by LI-COR IR immunoblotting system using α-Tubulin for normalization. Relative sT expression to sh ctrl is shown. (**c**) Small hairpin RNA (shRNA)-transduced MCV-positive (MKL-1 and MKL-2) cells and MCV-negative (UISO) cells were subjected to Wst-1 cell proliferation assay. Error bars indicate SD.

RNAs (shRNAs), and directly contradict our findings that sT knockdown inhibits cell replication in MCV-MCC (Shuda *et al.*, 2011). Since Angermeyer *et al.* were not able to measure sT protein by immunoblotting, efficacy of knockdown could not be determined. To assess cell proliferation, Angermeyer *et al.* (2013) used a competition assay containing mixtures of shRNA-transduced and nontransduced cells that compete with each other for growth. We instead directly measured cell proliferation using standard Wst-1 assays. To resolve this, we generated the same two sT-specific shRNAs cloned in pLKO.1-based lentiviral vector (named here sT1.RH for Roland Houben and sT2.RH) used in their study and compared these two shRNAs to an shRNA previously described to target sT alone (designated here as sh sT1.MS for Masahiro Shuda), an shRNA targeting both LT and sT (sh panT.MS) and a scrambled negative control shRNA (sh ctrl) (Shuda *et al.*, 2011). Both sh sT1.MS and sh panT.MS inhibit sT protein expression measured by quantitative LI-COR immunoblotting (Figure 2a and b) and cell growth (Figure 2c) as previously described (Shuda *et al.*, 2011). One shRNA (sh sT1.RH) of Angermeyer *et al.* also inhibits sT expression and significantly inhibits MCV-MCC cell growth. However, St1.RH also showed reduced proliferation of UISO cells consistent with an off-target effect that precludes its use as a specific targeting agent for MCV sT. The other shRNA (sh sT2.RH) has minimal (MKL-2) or no (MKL-1) sT knockdown activity (Figure 2b). It nonetheless inhibits MCV-MCC cell growth. Given the inability to monitor sT knockdown and off-target effects for the sT.RH shRNAs used in the knockdown studies of Angermeyer *et al.* (2013), attempts to rescue MCC cell proliferation using combinations of LT and sT expression during sT knockdown are not interpretable.

Using the same shRNA constructs described by Angermeyer *et al.* (2013), we show that their conclusion that MCV sT has no role in MCV is not correct. We recommend using sh sT1.MS, which is efficacious in sT knockdown and we are unaware of any off-target activity. Mixed cell competition assays to measure proliferation are fraught with uncertainty since paracrine effects can distort proliferation measurements and more traditional cell counting or Wst-1 measurements are preferred. Finally, as co-equal authors that independently developed T-antigen shRNA knockdowns for the report describing T-antigen knockdown in MCC (Houben *et al.*, 2010), we disagree with these authors' assertion that pan-T knockdown induces apoptosis in MCC. Weak poly ADP-ribose polymerase cleavage (Figure 5B, Houben *et al.*, 2010) can be seen in some cell lines during knockdown, but it is not universally present, and Casp3 or Casp9 cleavage is completely absent. As confirmed by Angermeyer *et al.* (2013), MCV sT is the only known transforming oncoprotein of MCV in rodent cells while MCV LT alone is not sufficient to transform rodent fibroblast cells (Shuda *et al.*, 2011; Angermeyer *et al.*, 2013). In the SV40 T-antigen model of human cell transformation, expression of both LT and sT is required (Hahn *et al.*, 1999). Taking into consideration the higher tumorigenic barrier in human cells as compared to rodent cells and that the spliced sT isoform is expressed together with LT in most MCC (Shuda *et al.*, 2011), it is likely that MCV sT co-contributes with MCV LT to MCC carcinogenesis.

CONFLICT OF INTEREST

YC and PSM are on patents for Merkel cell polyomavirus diagnostic reagents, which have been assigned to the University of Pittsburgh.

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Response to Shuda *et al.*

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TO THE EDITOR

The Merkel cell polyoma virus (MCV), which is associated with Merkel cell carcinoma (MCC), codes for two oncoproteins termed large and small T antigen (LT and sT). Although different MCC cell lines clearly depend on the expression of MCV LT (Houben *et al.*, 2012), our results (complete rescue of growth inhibition induced by an small hairpin RNA (shRNA) targeting both of the differentially spliced T antigens by reexpression of only an shRNA-insensitive LT) recently published in the *Journal of Investigative Dermatology* suggest that MCV-positive MCC cells do not require the expression of sT for proliferation (Angermeyer *et al.*, 2013). Now, Shuda *et al.* (2013) respond to this publication with a letter stating the opposite on the basis of their observation that one shRNA targeting MCV sT (sh sT1.MS) induces reduced sT expression and reduced proliferation in two MCV-positive MCC cell lines (MKL-1 and MKL-2), while having no effect on a control cell line (UISO; Shuda *et al.*, 2013); two other evaluated sT shRNA constructs either lacked significant knockdown capability or affected the growth of a control cell line, thus suggesting off-target effects. Moreover, as LT is essential for MCV-MCC cells, data from the same group showing that sT functions to stabilize LT protein in MCC cells (Kwun *et al.* (2013), Supplementary Figure S2B) would further suggest that sT is also required. Surprisingly, however, such a dependency of LT on sT expression is not supported by the data now presented by Shuda *et al.* (2013) as sT knockdown does not affect LT expression (Shuda *et al.* (2013), Figure 2a). Therefore, the conclusion that MCV-MCC cells require sT is based only on the growth-inhibiting effect of the shRNA sh sT1.MS. Notably, cytotoxic or cytostatic off-target effects frequently affect the interpretation of RNA interference experiments and vary between cell lines (Fedorov *et al.*, 2006). Thus, although the authors demonstrate that the MCV-negative cell line UISO is not affected by sh sT1.MS, such effects cannot be completely ruled out for MKL-1 and MKL-2. Interestingly, within the data set provided by Shuda *et al.* (2013) there is a clear example for such a cell type–specific off-target effect: the construct shRNA sT2.RH, which is described by Shuda *et al.* (2013) to have ''minimal (MKL-2) or no (MKL-1) sT knockdown activity'', completely inhibited the growth of the two MCV-positive MCC cell lines, whereas the proliferation of UISO was not affected at all (Shuda *et al.*, 2013, Figure 2). This observation suggests that UISO may be less sensitive toward shRNA-induced off-target growth inhibition compared with MKL-1 and MKL-2. Consequently, the conflicting results presented by our two groups indicate that further control experiments are essential to remove the question mark behind the title of this letter, or establish the opposite. In this respect, the rescue by reexpression of the targeted protein is considered as the ultimate proof for specificity of shRNA effects (Kittler *et al.*, 2005). We were not able to achieve this result for the sT shRNA constructs used in our study ((Angermeyer *et al.*, 2013), Figure 2). Thus, it would be

Abbreviations: LT, large T antigen; MCC, Merkel cell carcinoma; MCV, Merkel cell polyoma virus; shRNA, small hairpin RNA; sT, small T antigen

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