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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" (Angermeyer *et al.*, 2013). 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 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 Angermeyer et al's results 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 detects 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 premRNA 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.

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Conflict of interests

Yuan Chang and Patrick Moore are on patents for Merkel cell polyomavirus diagnostic reagents, which have been assigned to the University of Pittsburgh.

Is MCV sT required for MCC cell proliferation?

The knockdown by Angermeyer et al. used different shRNAs (Angermeyer *et al.*, 2013), and directly contradict our findings that sT knockdown inhibits cell replication in MCV-MCC (Shuda et al). 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 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 laboratory and sT2.RH) used in their study and compared them to an shRNA previously described to target sT alone (designated here as sh sT1.MS for Masahiro Shuda laboratory), 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 LICOR immunoblotting (Figure 2A and B) and cell growth (Figure 2C) as previously described (Shuda *et al.*, 2011). One of Angermeyer et al.'s shRNA (sh sT1.RH) also inhibits sT expression and significantly inhibits MCV-MCC cell growth. Proliferation of UISO cells, however, was also reduced consistent with an off-target effect that precludes evaluating it as a 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 Angermeyer et al's knockdown studies, 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., we show that their conclusion that MCV sT plays 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 PARP 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., 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 (Angermeyer et al., 2013; Shuda et al., 2011). 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.

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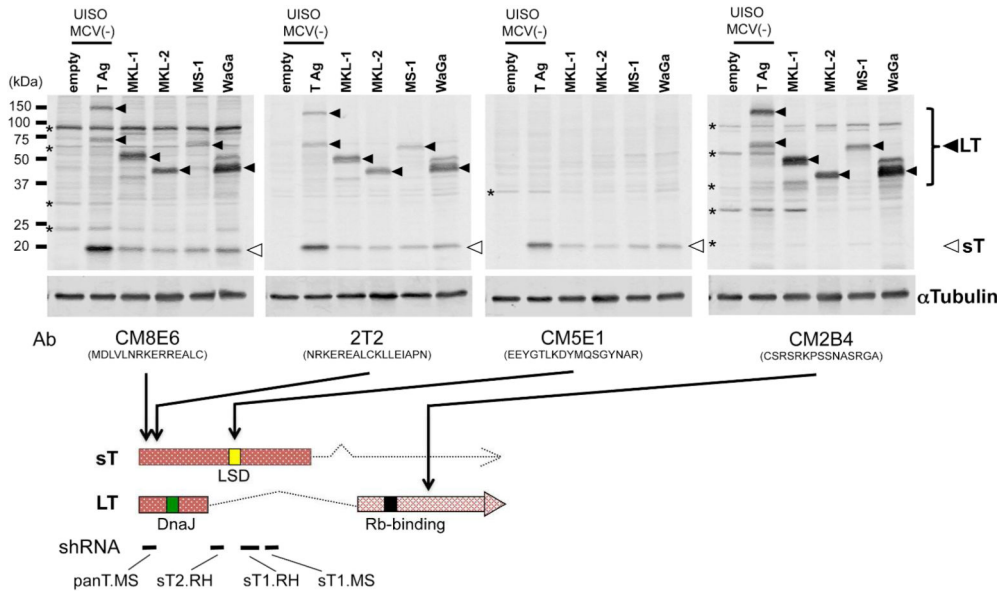

Figure 1.

Detection of Merkel cell polyomavirus (MCV) small T (sT) antigen expression by multiple MCV T antigen antibodies.

MCV-positive 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. αTubulin 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 monoclonal antibody 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.

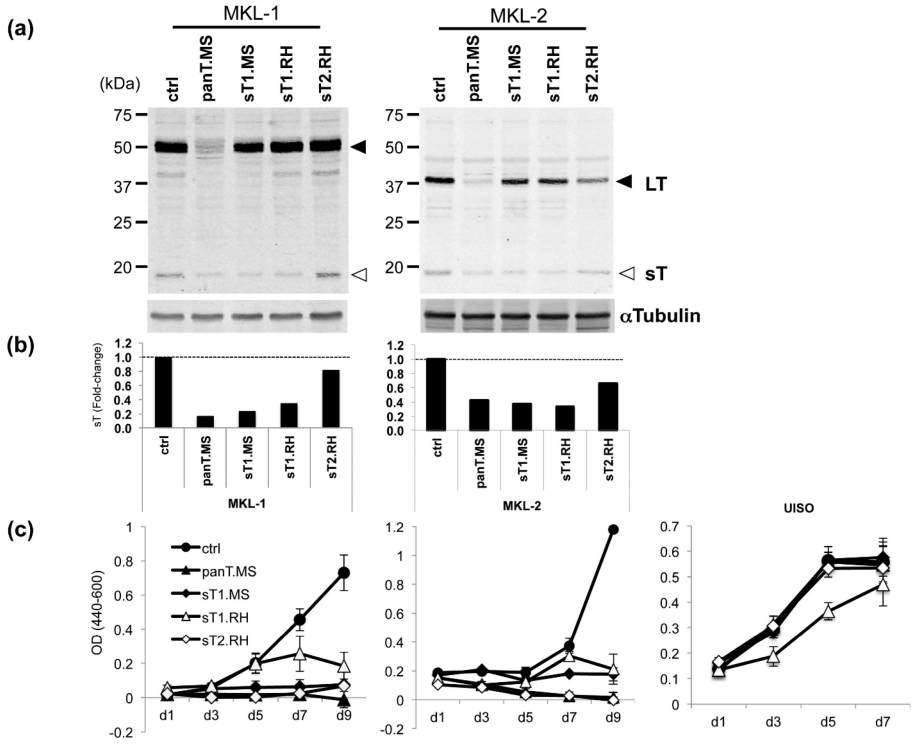

Figure 2.

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 αTubulin for normalization. Relative sT expression to sh ctrl is shown. (**c**) 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 standard deviation.