

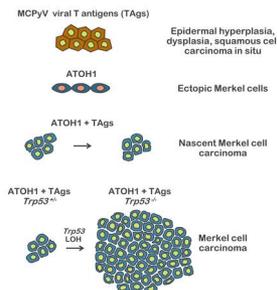
Direct cellular reprogramming enables development of viral T antigen-driven Merkel cell carcinoma in mice

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**Direct cellular reprogramming enables development of viral T antigen-driven
Merkel cell carcinoma in mice**

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Conflict of Interests

The authors have declared that no conflict of interests exist.

Abstract

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer that frequently carries an integrated Merkel cell polyomavirus (MCPyV) genome and expresses viral transforming antigens (TAGs). MCC tumor cells also express signature genes detected in skin-resident, post-mitotic Merkel cells, including *ATOH1*, which is required for Merkel cell development from epidermal progenitors. We now report the use of in vivo cellular reprogramming, using *ATOH1*, to drive MCC development from murine epidermis. We generated mice that conditionally expressed MCPyV TAGs and *ATOH1* in epidermal cells, yielding microscopic collections of proliferating MCC-like cells arising from hair follicles. Immunostaining of these nascent tumors revealed p53 accumulation and apoptosis, and targeted deletion of *Trp53* led to development of gross skin tumors with classic MCC histology and marker expression. Global transcriptome analysis confirmed the close similarity of mouse and human MCCs, and hierarchical clustering showed conserved upregulation of signature genes. Our data establish that expression of MCPyV TAGs, in *ATOH1*-reprogrammed epidermal cells and their neuroendocrine progeny, initiates hair follicle-derived MCC tumorigenesis in adult mice. Moreover, progression to full-blown MCC in this model requires loss of p53, mimicking the functional inhibition of p53 reported in human MCPyV-positive MCCs.

Introduction

MCC is an aggressive neuroendocrine skin tumor with a poor prognosis (1), although up to 50% of patients with advanced disease respond to immunotherapy (2). In 2008, a clonally-integrated polyomavirus was discovered in eight of ten MCCs (3). Subsequent studies confirmed that most MCCs contain integrated Merkel cell polyomavirus (MCPyV) genome and express two viral TAgS, small T (sTAg) and truncated large T (tLTAg) (4, 5). MCPyV-negative MCCs have a high mutation burden with a predominance of UV signature mutations whereas relatively few mutations are detected in MCPyV-positive MCCs (6-9), arguing that viral TAgS play a central role in virus-positive MCC tumorigenesis. In keeping with this notion, MCPyV TAgS transform cultured cells (10, 11) and are tumorigenic when expressed in vivo (12-16), but a bona fide mouse model of MCC has not been reported despite over a decade of effort by several laboratories.

MCC tumor cells express multiple transcription factors and lineage markers in common with Merkel cells, which are rare, non-proliferative neuroendocrine cells that reside beneath specialized compartments of epidermal cells and transduce light touch and itch sensation to adjacent sensory nerves (17, 18). The cell of origin of MCC is not known (11, 16), hindering efforts to develop a viable mouse model testing the role of MCPyV TAgS in MCC development. However, normal Merkel cells are specified from KRT5⁺ epidermal progenitors through the action of the bHLH transcription factor ATOH1 (19, 20), and some human MCCs are closely associated with epidermal tumors (21-24), raising the possibility of a common cellular origin. Moreover, ectopic ATOH1 expression can reprogram epidermal cells to form post-mitotic Merkel cells in adult mice (25), and we have previously shown that expression of ATOH1

together with MCPyV sTAg yields MCC-like cells in mouse embryos (15). Given the failure of conventional approaches to generate a mouse model of MCC, we set out to ascertain whether ATOH1 could be utilized as a novel tool to reprogram TAg-expressing epidermal cells into the Merkel cell lineage in adult mice, enabling the development of murine tumors resembling human MCC.

Results and Discussion

We generated and validated transgenic mouse strains with doxycycline-inducible co-expression of MCPyV sTAg and tLTA_g carrying IRES-driven RFP and GFP reporters, respectively (Fig. 1A and Supplementary Fig. 1). We next performed crosses with *K5-CreERT2* (26), *R26-LSL-rtTA* (27), and *tetO-Atoh1* mice (28) to generate *K5-CreERT2;R26-LSL-rtTA;tetO-sTAg/tetO-tLTA_g;tetO-Atoh1* mice, which we designated *SLA* (Fig. 1A), as well as *SL* mice, which were missing the *tetO-Atoh1* allele (see Methods). Mice were treated with tamoxifen to activate Cre function and rtTA expression, and with doxycycline to induce expression of sTAg and tLTA_g, +/- ATOH1, in *Krt5*-expressing epidermal cells and their progeny.

Although the *K5-CreERT2* strain drives recombination broadly in the basal layer of hair follicles as well as interfollicular epidermis (26), examination of tissue sections from TAg-expressing *SL* mice 2.5 weeks or more after transgene induction revealed that LTA_g expression became largely restricted to hair follicle epithelium (Supplementary Fig. 2). Moreover, histological analysis of sections from *SLA* mice collected two weeks after transgene induction revealed spatially restricted, atypical-appearing cellular aggregates near the normally quiescent hair follicle stem cell compartment called the bulge (29, 30) (Fig. 1). The cells in these aggregates contained scant

cytoplasm, condensed chromosomes, and pyknotic nuclei (Fig. 1B), and they expressed ATOH1, the Merkel cell/MCC markers Keratin 8 (KRT8) and SOX2, proliferation marker Ki67, apoptosis marker cleaved caspase 3 (CC3), MCPyV tLTA_g, and p53 (Fig. 1C), none of which were detectable at appreciable levels in control hair follicles (Supplementary Fig. 3). While a reliable sTA_g antibody is not available, the sTA_g target RCOR2 (31) was also detected in the atypical cellular aggregates (Supplementary Fig. 4A). Although microscopic cellular aggregates with KRT8⁺ cells could be detected at all time points examined between 2 weeks and 12 months after transgene induction, progression to gross tumors resembling MCCs was not detected in *SLA* mice (N=15). These findings suggest that expression of MCPyV TAGs together with exogenous ATOH1, in epidermal cells located specifically near the hair follicle stem cell niche, is sufficient to initiate tumorigenesis but fails to drive progressive growth and formation of grossly evident MCCs.

The accumulation of p53 in nascent MCCs (Fig. 1C) was unexpected since MCPyV sTA_g functionally inactivates p53 in human cells by increasing the levels of both MDM2 and CK1 α , which activates MDM4 (32). Given the presence of apoptotic cells in the microscopic tumor-like aggregates in *SLA* mice (Fig. 1C), we considered that the failed progression to full-blown MCC might be due at least in part to p53-mediated cell death. To explore this possibility, we next generated mice designated *SLAP* which also carried one floxed p53 allele (*Trp53^{wt/fl}*) (33) (Fig. 2A), yielding cells hemizygous for *Trp53* following recombination. Six out of 14 *SLAP* mice were euthanized for humane reasons, reflecting unanticipated morbidity also reported in other mouse models expressing MCPyV TAGs and deficient in p53 (12, 13). Each of the remaining eight mice developed one or more grossly visible tumors resembling human MCCs between 11

and 22 weeks after transgene induction (Fig. 2B,C). Notably, the wild-type *Trp53* allele was lost in all MCCs where DNA was available for analysis (N=5) (Supplementary Methods and Supplementary Fig. 5, showing absence of sequence from amplicons covering *Trp53* exons 2-10), pointing to a requirement for complete loss of p53 for tumor expansion.

Ten of the eleven skin tumors arising in *SLAP* mice exhibited histologic features highly characteristic of human MCCs, including a monomorphous small blue cell phenotype, finely-stippled chromatin, prominent mitoses, and nuclear molding (Fig. 2C) (see Methods). Variable numbers of tumor cells also expressed tLTA_g and ATOH1 (Fig. 2D) as well as multiple protein markers detected in human MCCs, including ISL1, INSM1, SOX2, POU3F2, and KRT8, the latter in a dot-like pattern highly characteristic of MCC (Fig. 2E). In addition, multiple sTA_g target proteins (31) were detected by immunoblotting or immunostaining (Supplementary Fig. 4). Despite tumor initiation from hair follicle epithelium (Fig. 1B-C), the mouse MCCs, like the great majority of human MCCs, were largely localized within the dermal compartment of skin without obvious connections to either the epidermis or hair follicles.

To further investigate the similarity between human MCCs and MCC-like skin tumors arising in *SLAP* mice we performed RNA sequencing (RNA-seq) on tumor specimens. We also compared the MCC tumor transcriptomes to those of normal mouse skin, normal human skin, and mouse basal cell carcinoma (34), a common epithelial skin tumor. Principal component analysis revealed a high degree of similarity between mouse and human MCC tumor samples which clustered together; additionally, these samples clustered separately from normal mouse and human skin as well as mouse BCCs (Fig. 3A). To better define the similarity among MCCs, we

also generated a heatmap of pairwise Spearman correlations across tumor and skin transcriptomes (Supplementary Fig. 6). Hierarchical clustering again grouped the mouse and human MCCs together, with high overall similarity among MCCs from both species based on an average Spearman correlation of 0.74. In addition, the scatter plot in Supplementary Fig. 7 shows concordance of the most highly upregulated genes in MCCs of both species when compared to normal skin, and Supplementary Figure 8 shows pathway enrichment analysis comparing mouse MCCs to normal skin. Finally, examination of transcripts highly expressed in mouse Merkel cells (35) (Fig. 3B), or neuroendocrine variants of lung, prostate, and bladder cancers (36) (Supplementary Fig. 9), highlighted the molecular similarities of mouse and human MCCs to both normal Merkel cells as well as neuroendocrine cancers arising in other organs, respectively.

Although multiple polyomaviruses infect humans (37), only MCPyV has been convincingly linked to a human cancer. Using direct in vivo cellular reprogramming with ATOH1, we have generated the first adult murine model of MCC. Several of our findings are noteworthy. Despite the broad expression pattern of the *Krt5* promoter in skin epithelia, initiation of TAg-driven mouse MCC in our model appears to occur in or near a restricted domain of the hair follicle that harbors several stem cell populations (30). This is of interest since MCPyV-positive human MCCs have a low burden of UV mutations, in keeping with a cell of origin that resides in deeper compartments of skin including the hair follicle rather than more superficial regions such as the interfollicular epidermis (see Fig. 1B). In addition, the hair follicle is a site of relative immune privilege (38), perhaps allowing for survival and expansion of viral antigen-expressing cells that may be eliminated if recognized as foreign in other regions of skin. Finally, stem or progenitor

cells may have greater plasticity and thus be preferentially susceptible to ATOH1-mediated postnatal reprogramming into the Merkel cell lineage.

The requirement for *Trp53* deletion in our model is also of interest since *TP53* mutations are uncommon in MCPyV-positive human MCCs, perhaps because p53 is depleted due to sTAg-mediated upregulation of MDM2 and the MDM4 activator CK1 α (32). However, efficient disruption of p53 function seems unlikely in our murine *SLA* model given the accumulation of p53 in nascent tumors (Fig. 1C). The differential requirement for loss of *Trp53* in viral TAg-driven mouse MCC, but not human MCC, may also be due to the striking divergence of p53-regulated target genes in mouse versus human cells (39). Our data argue that either functional inactivation of p53 in human MCCs, or genetic deletion of *Trp53* in our mouse model, is required for MCPyV-driven MCC tumorigenesis. Importantly, our immunostaining studies and transcriptomic data highlight the strong similarity between human and mouse MCCs mice despite the different mechanisms leading to inhibition of p53.

In summary, our findings underscore the utility of modulating cell fate to generate a neoplasm without a defined cell of origin; establish a pivotal role for MCPyV T antigens in the pathogenesis of virus-positive MCCs; demonstrate how tumors that appear to reside entirely within the dermis may originate from follicle epithelia; and set the stage for future studies centered on gaining deeper insight into MCC biology, mechanisms underlying viral TAg-driven tumorigenesis, and preclinical testing of novel therapeutics.

Methods

Mouse Models

Transgenic mouse production. Transgenic mice carrying doxycycline-inducible MCPyV T antigens and fluorescent reporters, designated *tetO-sT/tLT*, were generated by co-injection of *tetO-sTAg-IRES-RFP (tetO-sT)* and *tetO-tLTAg-IRES-GFP (tetO-tLT)* cassettes (Supplementary Fig. 1A) into fertilized (C57BL/6 x SJL) F2 mouse oocytes by the University of Michigan Transgenic Animal Model Core. Details and characterization of *tetO-sT/tLT* transgenic mice are provided in Supplementary Methods and Supplementary Fig. 1.

Generating mice with inducible transgene expression. To generate a model that would allow tight control of transgene expression both spatially and temporally, we employed a triple-transgenic model that included i) a hormone inducible Cre allele, ii) a Cre-inducible rtTA allele, and iii) *tetO*-driven effector alleles. We employed the *K5-CreERT2* strain (26) to drive tamoxifen-inducible Cre activity in K5-expressing epidermal cells, including Merkel cell progenitors; B6.Cg-*Gt(ROSA)26Sor^{tm1(rtTA,EGFP)Nagy}/J* mice (Jackson Laboratory Stock No. 005670) (27), designated *R26-LSL-rtTA*, to drive expression of rtTA in recombined cells and all of their progeny; and *tetO-sT/tLT* strains for expression of MCPyV sT and tLT, in mice with a target genotype of *K5-CreERT2;R26-LSL-rtTA;tetO-sT/tLT (SL)*. A *tetO-Atoh1* allele (28) was added to drive cells into the neuroendocrine lineage in *K5-CreERT2;R26-LSL-rtTA;tetO-sT/tLT;tetO-Atoh1 (SLA)* mice. In mice treated with tamoxifen to activate Cre function, recombination at the ROSA locus leads to rtTA expression, and transgene expression is induced by doxycycline. To also alter *Trp53* gene dosage, mice were crossed with conditional B6.129P2-*Trp53^{tm1Brn}/J (Trp53^{fl/fl})* mutant mice (The Jackson Laboratory, Stock No. 008462) (33) to

generate *K5-CreERT2;R26-LSL-rtTA;tetO-sT/tLT;tetO-Atoh1;Trp53^{fl/wt} (SLAP)* mice, enabling deletion of one copy of the *Trp53* gene after recombination. Additional details are provided in Supplementary Methods and Supplementary Table 1.

Transgene induction and tumor monitoring. Transgene expression +/- *Trp53* deletion were induced in *SLA* (N=15; 11F/4M) and *SLAP* (N=14; 10F/4M) mice starting at P21-24 by continuous administration of tamoxifen chow at 400mg/kg in Teklad Global rodent diet (Envigo) and doxycycline (200µg/mL, Fisher Scientific) in drinking water containing 5% sucrose. Mice were monitored bi-weekly for skin phenotypes and tumor development. *SLA* mice were monitored for up to 12 months with no apparent skin tumors developing during this time. *SLAP* mice were monitored until skin tumors developed or until euthanasia was required for humane reasons (N=6/14), which in two mice included growth of grossly evident internal tumors that were not MCCs.

Tumor Development. Gross skin tumors (N=11) arising between 11 and 22 weeks after transgene induction in *SLAP* mice (N=8) were GFP+ and RFP+ and expressed ATOH1 and SOX2 as well as other markers including ISL1, INSM1, POU3F2 and KRT8, in at least focal areas consistent with human MCC. All tumors except for one were scored as histologically consistent with human MCC by a board-certified dermatopathologist, PWH. The outlier arose on an ear at the site of an ear tag, was classified histologically as undifferentiated, and did not express appreciable levels of most MCC markers. Internal tumors with an undifferentiated phenotype were identified in 3/8 *SLAP* mice with cutaneous tumors. Basal cell carcinomas arising in *K5-Gli2* mice were harvested at 7-9 months of age.

Immunostaining, acquisition of human tissue, RNA isolation and sequencing, and processing and analysis of human and mouse RNA-seq datasets are described in Supplementary Methods.

Study Approval

Animal Studies. All mice were housed and maintained, and procedures performed, according to University of Michigan Institutional Animal Care and Use Committee guidelines under animal protocol #PRO00008710.

Human studies. Human MCC tumor specimens were collected from patients according to protocol approved by the University of Michigan Institutional Review Board (IRB Study IDs: HUM00050085 and HUM00046018). Normal skin punch biopsies (5mm) from healthy volunteers were collected after informed written consent under a protocol approved by the local ethics board at the University Hospital Schleswig-Holstein, Campus Kiel, Germany (Reference: A100/12).

Author Contributions

AAD and MEV conceived, designed, and supervised the study. MEV, JJVG, JA, DW, HZ, MG, and TLS performed animal modeling experiments including generation of transgenic mice. AAD, MEV, PWH, SYW, and IB analyzed the mouse data. KH, CJL, and AMU performed experiments for targeted DNA sequencing, including processing data and statistical analysis. PWH, MTP, SW, and LCT performed transcriptomic analysis of human and mouse data sets. MG, MCK, and PC contributed mice or mouse tumors. PWH, SW, JEG, AMU, AMC, and LCT contributed human MCC tumor or human skin data sets or analysis tools for cross-species transcriptome analysis or NGS analysis. AAD and MEV wrote the manuscript, and PWH, SW, LJS, JSR, SYW, IB, MC, AMU, and LCT provided edits or made significant conceptual contributions, with all authors reviewing and approving the final version.

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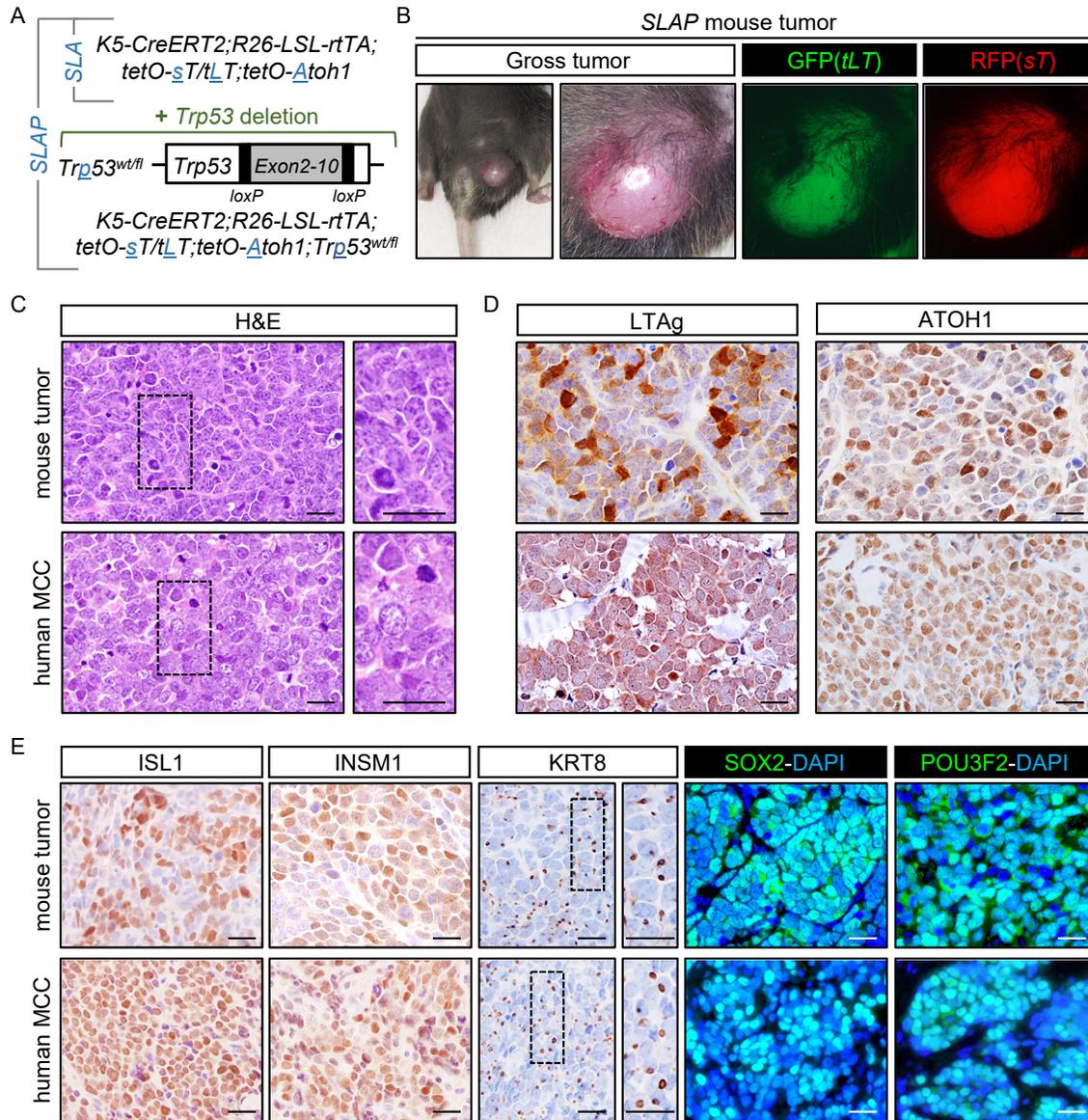


Figure 2. In vivo reprogramming using ATOH1 in p53-deficient cells enables development of full-blown murine MCC. (A) Addition of conditional *Trp53* allele to generate SLAP mice expressing MCPyV sTag, tLTA_g, and ATOH1, which are also deficient in p53, in Krt5-expressing cells and their progeny. (B) Gross tumor arising in SLAP mouse 4 months after transgene induction. (C) Similar histopathology of SLAP mouse tumor and human MCC. Immunostaining for (D) transgene expression and (E) MCC marker expression. Scale bars: 25 μ m.

Fig. 3

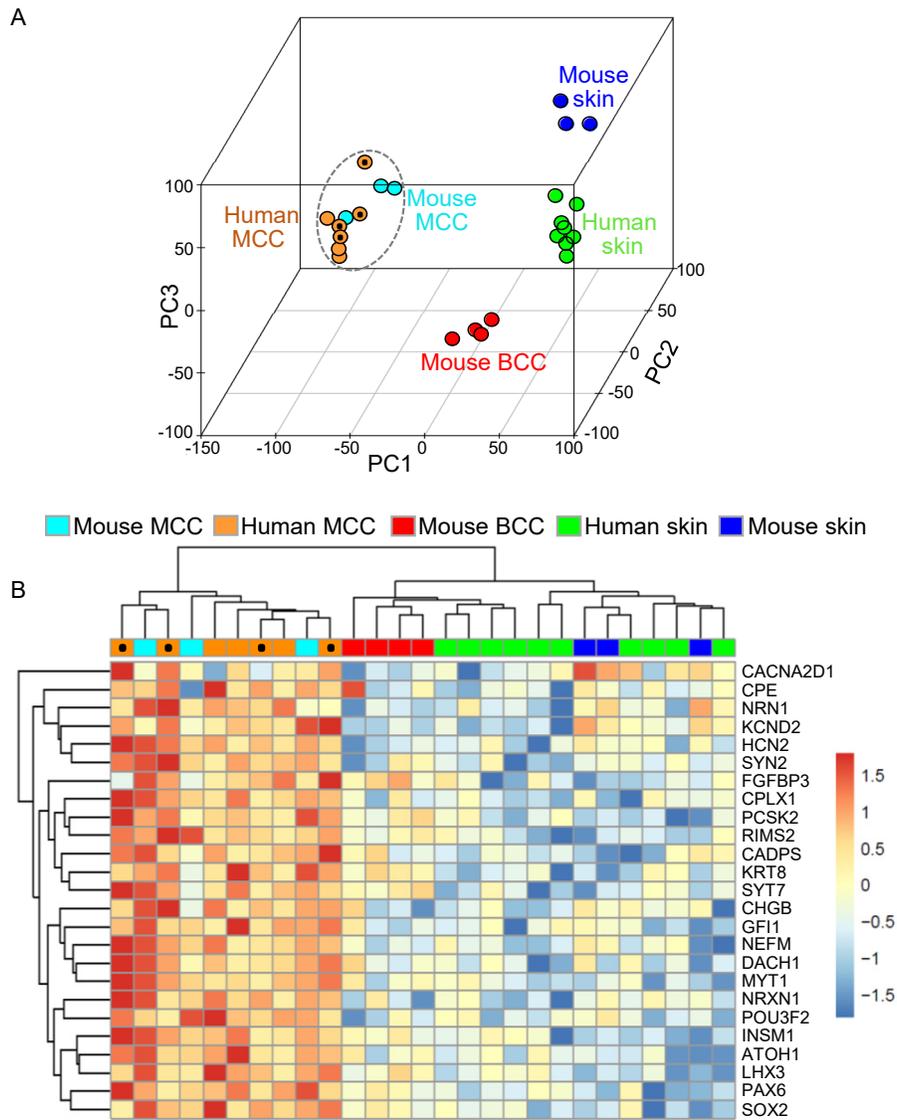


Figure 3. Cross-species transcriptome analysis of MCC. (A) Principal component analysis plot of global transcriptomes showing similarity of mouse (N=3) and human (N=7) MCCs, with a well-defined separation from normal mouse (N=3) and human (N=10) skin as well as mouse BCCs (N=4). (B) Hierarchical clustering of transcripts enriched in normal mouse Merkel cells show similar expression patterns in mouse and human MCCs. Data from MCPyV-positive human MCCs are marked (•).