The trichodysplasia spinulosa-associated polyomavirus : infection, pathogenesis, evolution and adaptation

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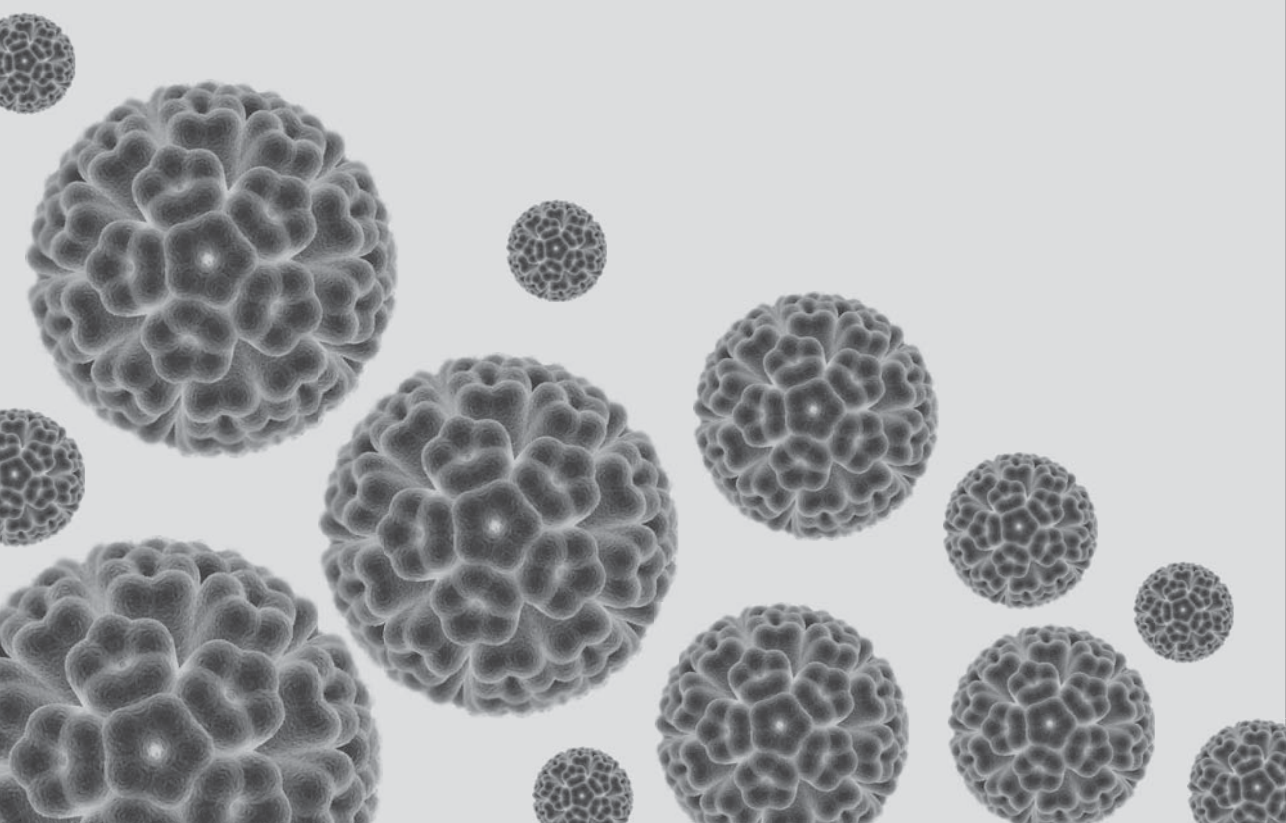
VePyV1 CaPyV JCPyV MPyV SA12 RacPyV GHPyV BKPyV
BatPyV CPyV OraPyV1 FPyV MptV APPyV1 SqPyV LPyV CSLPyV
EPyV PRPyV1 APPyV2 KIPyV MiPyV PtvPyV2c OtPyV1 STLPyV
MFPyV1 HPyV9 SV40 TSPyV CoPyV1 PPPyV CPPyV HPyV12
MXPyV PtvPyV1a PDPyV EiPyV1 AtPPyV1 HaPyV GggPyV1
CdPyV DRPyV MWPyV APyV CAPyV1 HPyV7 ChPyV MasPyV
WUPyV HPyV6 BPyV MCPyV OraPyV2 MMPyV SLPyV HPyV10

Part IV

Discussion and Summary



Chapter 7

Summarizing discussion

General aspects of TSPyV infection

Discovery of the trichodysplasia spinulosa-associated polyomavirus (TSPyV) in 2010 paved the way for research focusing on the role of this virus in the pathogenesis of trichodysplasia spinulosa (TS) [1, 2]. When one considers a causal relationship between a pathogen and a certain (proliferative) disease, as proposed by Fredericks and Relman for instance [3], the prevalence of the putative pathogen is expected to be higher in the specified patient group compared to a healthy population (**Chapters 2** and **3**) [2, 4], and the pathogen (viral)-load of symptomatic tissue is expected to significantly exceed that of healthy tissue (**Chapter 3**) [4]. Equally important, by action of its encoded proteins, the pathogen should possess specific pathogenic (transforming) properties that render the infected cell, tissue or organ in a state reminiscent of the indicated disease (**Chapter 4**) [5]. In the process, these interacting viral proteins responsible for adaptation in the host may mutate (**Chapter 6**). Some of these mutations may be recurrent in the virus family arguing for a conserved mechanism of virus adaptation (**Chapters 5**).

In this summarizing chapter, most of these aspects of TSPyV infection, including its prevalence, localization, latency, and persistence, as well as viral pathogenesis and TSPyV host adaptation and evolution will be discussed in the light of available literature to establish a causative role for TSPyV in the development of TS.

Virus reservoir for latency/persistence

TSPyV is a ubiquitous virus similarly to most other human polyomaviruses (HPyVs) as can be concluded from its high seroprevalence (**Table 1**). In addition, the intensity of measured TSPyV antibody responses is high [8 - 10]. Apparently, TSPyV infection creates sufficient viral antigen to promote strong, possibly lifelong, antibody responses, suggesting that efficient TSPyV replication takes place at compartment(s) in the body where virus progeny is produced. Whether replication takes place only in the skin, or elsewhere in the body as well, is unclear at the moment. The intensity of the measured seroresponses, to some extent comparable with BKPyV-seroresponses, might indicate circulation of TSPyV in blood (viremia).

In contrast to its high seroprevalence (~75%), the TSPyV DNA prevalence analyzed with PCR in skin-derived samples of groups of asymptomatic (healthy and immunocompromized) individuals is much lower (<5%) (**Table 1** and **Chapter 2**) [2, 11 - 14]. When TSPyV is detected on the skin, its load is usually very low (**Chapters 2** and **3**). The discrepancy between DNA-prevalence and seroprevalence is evident for many polyomaviruses, for instance KIPyV and WUPyV, and HPyV9 (**Table 1**).

Despite explanations of a technical nature, such as differences in sensitivity to detect TSPyV infection, this discrepancy might be explained by persistent infection of body site(s) other than the skin, for instance of internal organs after dissemination. In this regard, it is worthwhile to note that for instance JCPyV persistently infects lymphocytes and urothelial cells, whereas upon reactivation it invades central nerve cells and causes encephalitis

in immunocompromized hosts [16, 41]. A recent finnish study suggested that TSPyV might persist in lymphoid tissues, specifically the tonsils, of healthy individuals [11], and an american study showed the presence of TSPyV in a kidney biopsy [42]. Nevertheless, the detected percentage in these tissues did not exceed the prevalence found on the skin. Previous studies that looked into lymphoid systems or mucosa (e.g., tonsils) as a residence or reservoir for HPyV infections suggested that these body compartments might play a role in polyomavirus latency/persistence [43 - 46], which might be the case for TSPyV as well. Alternatively, these findings may simply imply a respiratory transmission route for these viruses, which results in transient detection in these lymphoid systems or mucosal tissues (e.g., tonsils).

A possible mechanism of polyomavirus latency/persistence

In general, it is believed that particular virus infections may reside in two distinct non-pathological or asymptomatic states, namely the (non-replicative) latency and low-level persistent replication state, together designated latency/persistence. For this state of latency/persistence, viruses have evolved mechanisms that focus on evading detection and clearance by the host immune system. For instance, viruses encode microRNAs that interfere with host cellular processes [47, 48]. These small non-coding RNAs [49] are normally utilized by polyomaviruses to regulate their own transcriptome, but can also target the host transcriptome, for example involved in cellular innate and adaptive immune responses (**Figure 1**) [50]. Thus, next to virus transcriptome auto-regulation – shown for MCPyV [51, 52], BKPyV

Table 1. Prevalence of human polyomavirus infection in immunocompetent individuals measured by PCR and serology

	Prevalence of polyomavirus infection	
	Viral DNA	Viral serum antibodies
Polyomavirus*	Range in % (major detection site) [Ref]	Range in % [Ref]
BKPyV	30 - 50 (Kidney) [15]	80 - 100 [16 - 19]
JCPyV	10 - 40 (Kidney) [20]	40 - 70 [16 - 19]
KIPyV	1 - 12 (Respiratory tract) [21 - 23]	55 - 90 [17, 18, 24, 25]
WUPyV	1 - 16 (Respiratory tract) [22, 26]	70 - 100 [17, 18, 24, 25]
MCPyV	60 - 80 (Skin) [27 - 29]	40 - 80 [9, 17, 18, 30 - 32]
HPyV6	14 - 50 (Skin) [30, 33]	50 - 80 [9, 30]
HPyV7	11 - 17 (Skin) [30, 33]	35 - 60 [9, 30]
TSPyV	2 - 4 (Skin/Tonsil) [2, 11 - 14]	70 - 80 [8 - 10]
HPyV9	1 - 17 (Skin) [33, 34]	30 - 50 [9, 34]
HPyV10**	1 - 5 (Stool) [35 - 37]	N/A
STLPyV	0 - 1 (Stool) [35]	60 - 70 [38]
HPyV12	1 - 11 (GI tract) [39]	17 - 23 [39]
NJPyV	N/A (Endothelial cells) [40]	N/A

*, For polyomavirus name abbreviation see **Chapter 1**, **Table 1**

**, HPyV10 includes MWPyV and MXPyV

GI-tract, Gastro-intestinal tract; N/A, No data available so far

[53] and JCPyV [50, 54] – microRNAs play most probably key roles in controlling specific host factors to evade the immune system, which could promote latency/persistence (**Figure 1**) [50, 52, 55]. In this context, preliminary genome analysis of TSPyV suggests that it might express a microRNA compatible with regulation of its early transcripts (**Chapter 2, Figure 2; Chapter 5, Supplementary Figure S2**). Whether this microRNA also targets host cell factors, specifically those involved in immune recognition, is unknown.

TSPyV active infection and trichodysplasia spinulosa

From immunosuppression to disease development; a causal relationship

It seems apparent that all other established pathogenic HPyVs described so far, i.e., JCPyV, BKPyV and MCPyV, follow similar rules; i) an asymptomatic primary initial infection at an early age, ii) widespread prevalence in human populations, and iii) upon reactivation pathogenic consequences observed only in the elderly and/or immunocompromized individuals. TSPyV, with its described (sero)prevalence and its pathogenic consequences in severely immunocompromized hosts, seems to fit these rules.

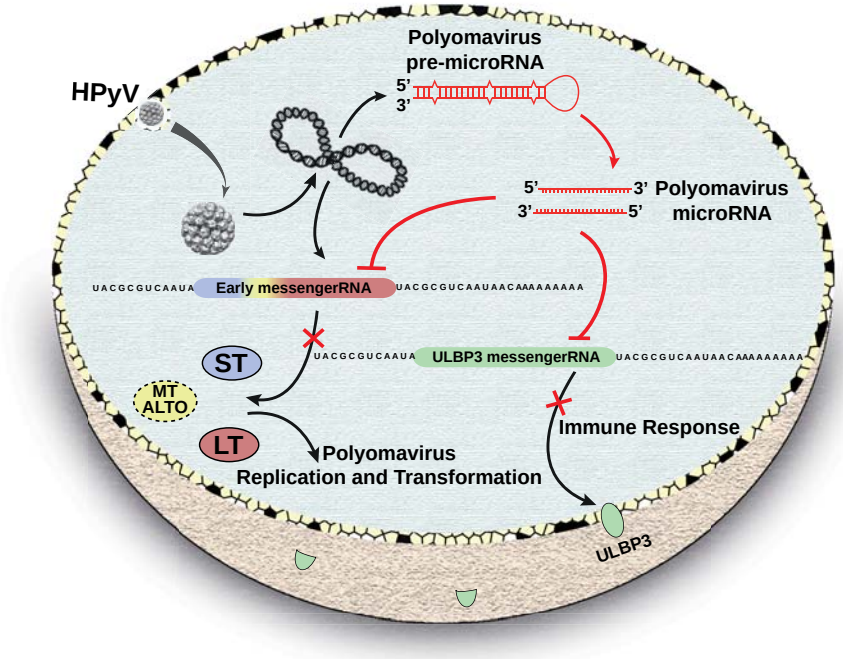


Figure 1. Schematic overview of putative function of polyomavirus microRNA related to infection. Polyomavirus-encoded microRNAs exert an auto-regulatory role by targeting their own early messengerRNAs. Upon viral infection, the innate immune response produces factors, such as the stress-induced ligand ULBP3, which are essential for recognition of infected cells by the immune system (e.g., NK cells). This viral microRNA plays also a putative role in regulation of this host immune response by targeting the ULBP3 messengerRNA. Figure adapted from [48].

One of the aims described in this dissertation was to study and describe the pathogenic consequences of TSPyV infection. In **Chapter 3** it was reported that high viral loads were exclusively measured in lesional samples originating from TS patients, which indicates that TSPyV is actively replicating in these lesions [4]. In healthy subjects and tissues, TSPyV was almost undetectable (**Chapters 2** and **3**). Another indication that active TSPyV infection is involved in the development of TS was the detection of viral proteins VP1 solely in the affected hair follicles (**Chapter 3**). Furthermore, TSPyV LT-antigen was expressed in all lesional TS samples analyzed, lesions which are known to be hyperproliferative (**Chapter 4**) (discussed in next paragraph) [5, 56].

For BKPyV and JCPyV it is well established that when higher viral loads are reached in affected organs, this could have pathogenic consequences [57 - 59]. According to Rothman *et al*., this could fit the complete causal effect, which states that "an agent that causes disease should be both necessary and sufficient for the disease to occur and should be reproducible and consistent in different settings, and in studies performed by different investigators" [60]. In this regard, hypothetically speaking, it could be stated that, when i) the correlation is strong – all TS cases tested are positive for TSPyV, compared to healthy subjects, ii) reproducible – other research groups reporting TSPyV presence in TS lesions, and iii) predictive – when detection of high TSPyV loads in TS lesions is evident, then a causative conclusion is highly probable [4, 42, 61, 62]. Equally important iv) would be the molecular evidence of active infection in this regard (discussed next). Furthermore, other criteria's could be considered for testing to establish a causal relationship between a microbe and a disease, which in this case would also be in line with the Koch's postulates, by investigating the TSPyV infection and pathogenesis in animal models.

Altogether, despite the limitations in number of TS cases described and available worldwide, and the number of tissue samples analyzed, the studies described in **Chapter 2** and particularly in **Chapter 3** provide strong evidence that active TSPyV infection is associated with TS, and TSPyV is most probably the causative agent of this disease. Obviously, more studies are needed from independent laboratories that further confirm the epidemiological and experimental studies to underscore causality.

Molecular pathology of trichodysplasia spinulosa

Possible pathogenic events in polyomavirus-associated disease development

During polyomavirus-associated human disease development, a complex interplay takes place between multiple factors, like host immunity, ageing and genetics, and environmental exposure. In addition, virus-host molecular interactions occur, which can induce changes in viral and cellular expression profiles and potentially disrupt physiological cellular pathways. This issue is best illustrated by MCPyV, the HPyV associated with Merkel cell carcinoma (MCC) [63, 64], a skin tumor already introduced in **Chapter 1** [65]. While MCPyV infects

humans early in life in a fashion comparable to many others, including non-pathogenic polyomaviruses, several successive events (although very rare) can result in neoplasm development. At first, an exogenous mutagenic exposure such as UV-radiation causes DNA damage, for instance DNA double-strand breaks. The subsequent repair of this damage could facilitate virus genome integration into the host DNA [66] (**Figure 2A**). Additional successive mutations, for instance also as a result of UV-radiation, that abrogate the LT-antigen function involved in viral DNA replication without disturbing the pathogenic (transforming) domains (e.g., the LXCXE pRB binding site), is an important event in MCC cell survival and proliferation (**Figure 2A**).

Comparable to MCPyV, TSPyV induces robust proliferation of its host cells located in the hair follicles (see **Chapter 4** and discussion next) (**Figure 2B**) [5]. Whether TSPyV can integrate into the host genome is unknown so far. In symptomatic TS disease, however, the measured high TSPyV loads argue against integration in the productive stage of infection (**Chapter 3**).

Viral-host molecular interplay

Historically, polyomavirus research has repeatedly revealed identification of specific viral-protein and host-protein interactions that lead to important insights into mammalian cell biology [70, 71]. Polyomaviruses have evolved mechanisms that enable them to hijack host regulatory cell factors for the sake of fulfilling their life cycle (**Figure 3**). The ability of a polyomavirus to oncogenically transform its host cells was first demonstrated for SV40. Inoculation of SV40 DNA into rat and mouse cells resulted in uncontrolled cell growth and proliferation [72]. Ever since, large tumor (LT), middle tumor (MT) and small tumor (ST) antigen of several polyomaviruses have been investigated and were postulated as prototype virus oncoproteins that can abrogate several host cell regulatory factors (**Figure 3**). Until recently, MT-antigen expression was demonstrated only for rodent polyomaviruses [71]. New data shows that MT-antigen and/or its derivative ALTO are expressed also by MCPyV [73] and TSPyV (Van der Meijden and Feltkamp *et al.*, unpublished observations), as briefly mentioned in **Chapters 5** and **6**.

Figure 2. Molecular events in the (putative) pathogenesis of MCC and TS. (**A**) (**1**) In general, MCPyV infection (red particles) is acquired at early childhood. (**2**) In a state of decreased immunity (either iatrogenic or age-related), reactivation and replication of MCPyV may occur. (**3**) At some stage, such a burst of infective virus production could facilitate viral integration in susceptible cells. (**4**) Mutagenic events could result into replication-competent virus infection. (**5**) Expression of MCPyV mutation-truncated LT-antigen that provides pro-proliferative signals (e.g., phosphorylation of pRB) leading to clonal expansion and MCC development [67]. Figure adapted from [68]. (**B**) (**1**) Also TSPyV infection (blue particles) is acquired at early childhood. (**2**) During decreased immunity, TSPyV reactivation and replication may occur. (**3**) Alongside high viral replication, tumor antigens are expressed that provide pro-proliferative signals (e.g., phosphorylation of pRB) [5]. (**4**) Hair follicles accumulate with viral particles – which are also shed from the skin – and trichohyalin-protein aggregates resulting into keratinized, spiny structures protruding the skin [4, 69].

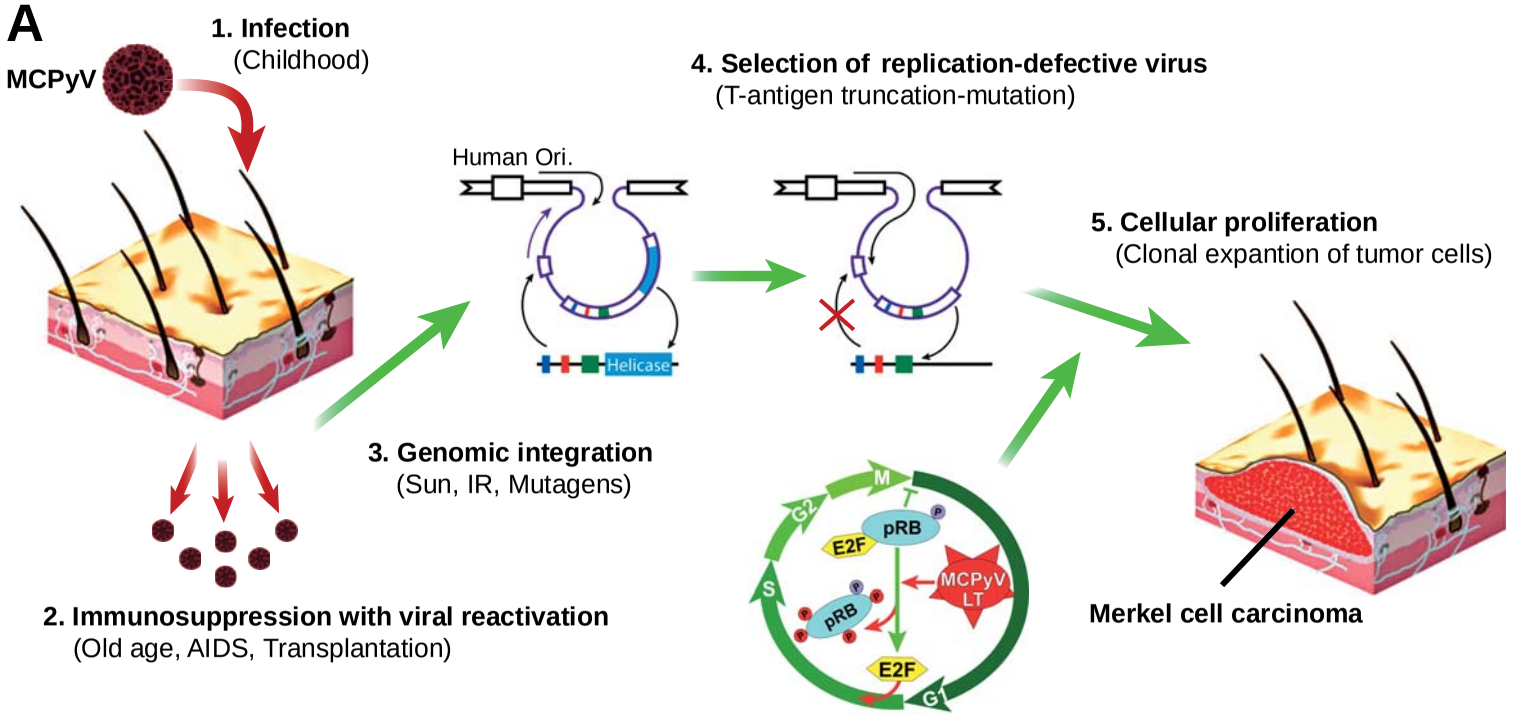


Figure 2. For legend text panel **A** see previous page. For panel **B** see next page.

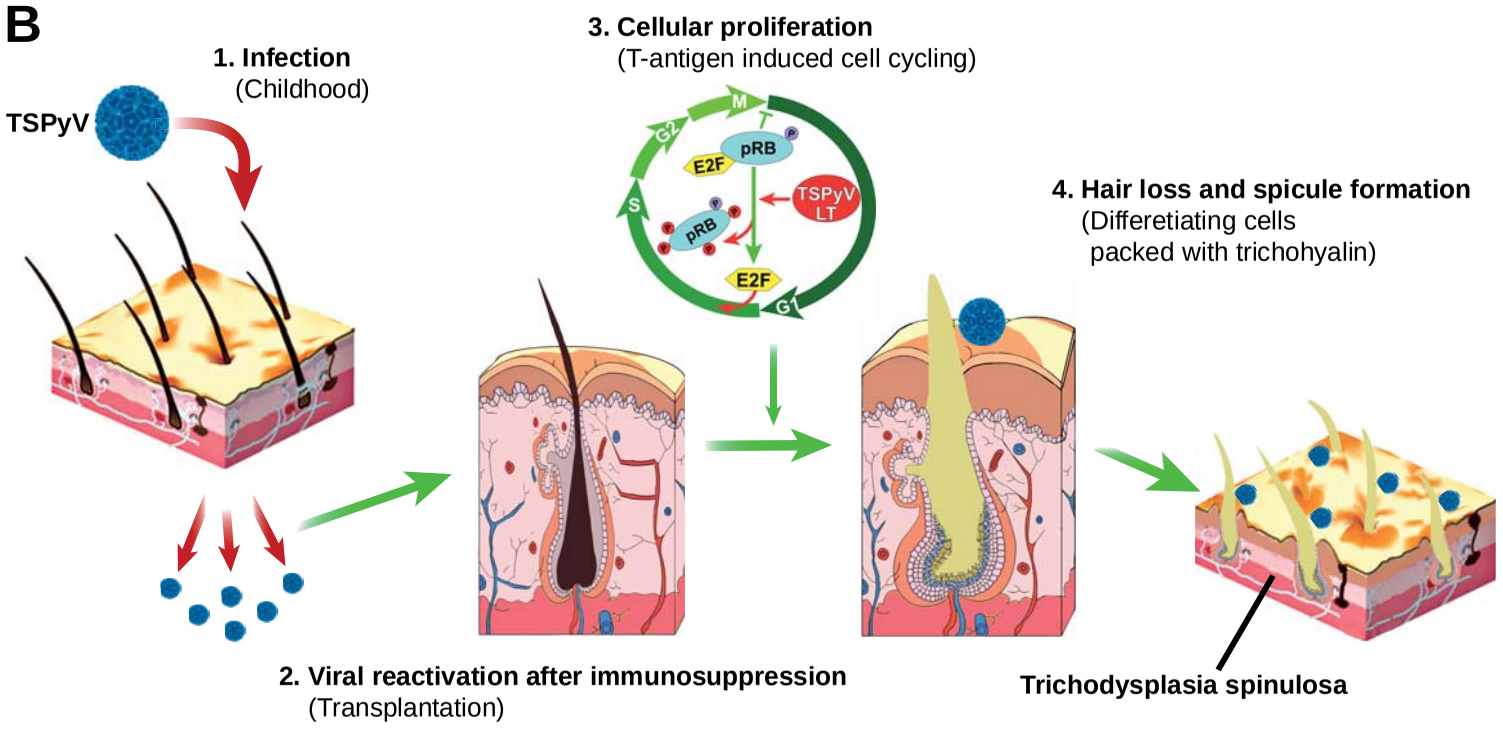


Figure 2. For legend text panel **B** see page 176.

The patho/oncogenicity of the polyomavirus-encoded transforming proteins, most notably the LT-antigen, in cell culture and in animal models has been known for decades. Cellular interacting proteins in this regard are the retinoblastoma tumor suppressor protein (pRB) and its family members (p107 and p130) (discussed next) [74]. The poly-functional LT-antigen is expressed at early stages of the viral life cycle and is involved in both viral replication (ori-binding and helicase activity [75]) and virus-induced cellular transformation and growth [74]. In addition to the full-length LT-antigen, alternative splicing of the LT-antigen messengerRNA can occur, as well as premature termination of translation because of mutations (discussed before). Truncation of LT-antigen usually causes removal of the helicase and putative p53-binding domains, but preserves most of its other domains, for instance the LXCXE domain important for interaction with the RB family members [76]. Preliminary findings suggest that the LT-antigen of TSPyV, comparable to other *in vitro* studied polyomaviruses, demonstrates comparable versatility which expression is regulated through the alternative splicing of TSPyV pre-T-antigen messengerRNA (Van der Meijden and Feltkamp, personal communication). The regulation of (L)T-antigen expression emphasizes that TSPyV, just like other polyomaviruses, has developed an evolutionary feature that enables switching between roles in both viral replication and cellular transformation using different versions of the same protein (other polyomavirus evolutionary aspects are discussed next).

Probably one of the most described interactions and a key mechanism in cellular transformation caused by polyomaviruses is the LT-antigen interaction with the RB family proteins (**Figure 3**). RB family proteins (i.e., pRB, p107 and p130) are important regulators of the G1- (rest) to S-phase (DNA synthesis) transition of cells during cell cycling initiation [99]. Hypophosphorylated pRB, for instance, inhibits function of the E2F transcription factor that regulates gene expression required for host DNA synthesis, whereas hyperphosphorylated pRB induces pRB–E2F complex dissociation and cell-cycle entry. The latter can be reverted by several inhibitory proteins such as $p16^{ink4a}$ and $p21^{waf}$ [5, 99]. For MCPyV it was shown that ST- and LT-antigen, including its derivative 57kT that bears also the pRB-binding LXCXE motif, are required for the proliferation of MCC cells [91, 96, 100 - 102]. The TSPyV LT-antigen also contains the conserved LXCXE motif identified *in silico* (**Chapter 5**) [1, 6]. The colocalization of phosphorylated pRB with proliferation marker Ki-67 observed in TS lesions could indicate that pRB is inactivated, which explains the hyperproliferative nature of these lesions (**Chapter 4**). Thus, through its LT-antigen or one of its truncated derivatives, TSPyV might induce cell cycle progression through disruption of the RB-regulatory pathway, and create a reservoir of proliferating cells that permit viral DNA replication. This putative mechanism could be assessed in future *in vitro* studies – aside other factors indicated in **Figure 3** – that may be involved in the pathogenic process of cellular transformation.

The characteristic TS phenotype observed repeatedly and most notable on the face, remains unexplained so far. However, terminal differentiation of the large proliferating pool of cells into inner root sheath (IRS) might explain the accumulation of trichohyalin-positive cells that facilitate formation of the characteristic spicules (**Figure 2B, Chapters 3** and **4**). IRS

cells are known to produce keratins and trichohyalin that serve as an intracellular “cement”, which gives strength to the IRS layer of a hair follicle to support and mold the growing and protruding hair shaft [103]. Hypothetically, the excess amounts of keratins and trichohyalin produced could shape the spicule on the skin when the cells have undergone differentiation. To investigate spicule formation and involvement of TSPyV T-antigens in more detail, TSPyV T-antigen-expressing organotypic raft culture systems could be exploited, which mimic skin growth in a sophisticated 3D-Skin culture system [104].

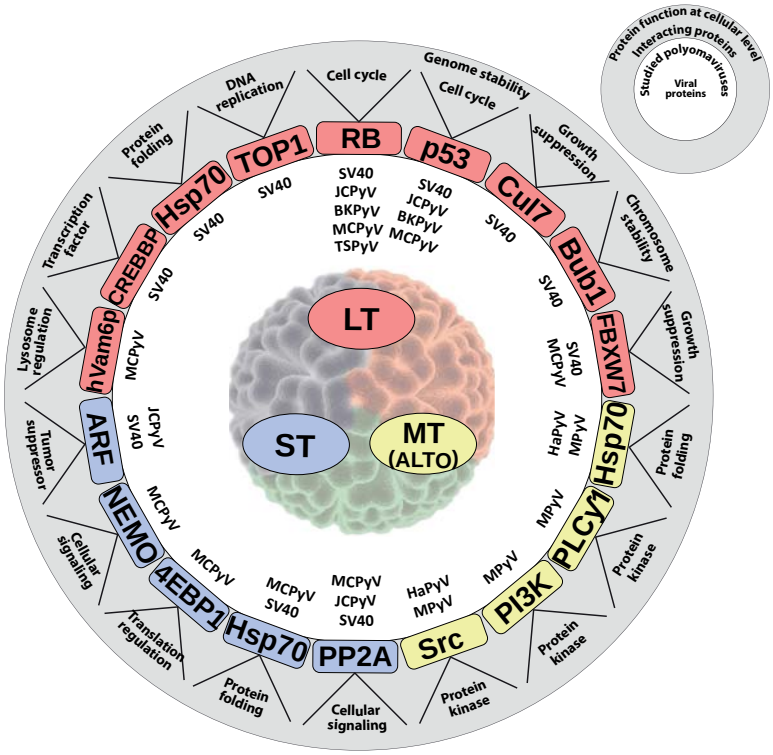


Figure 3. A selection of host cellular factors targeted by polyomavirus early proteins. So far known, polyomavirus LT- (red), ST- (blue) and MT-antigen (yellow), as shown in the inner circle for each virus protein, interact with many cellular proteins (colored according viral proteins), as depicted in the outer circle with their function at cellular level on top (see also inset, top-right). For LT- and ST-antigen, most experiments that have identified these interactions have been shown around SV40 polyomavirus [74, 77]. The J-domain, shared by all early T-antigens, recruits Hsp70 protein homologues that aids in protein folding [78 - 80]. Other regions inside LT-antigen have been shown to bind other important factors, like CREB-binding protein (CREBBP) [81], TOP1 [82], p53 [74, 76, 83], Cul7 [84], Bub1 [85] and FBXW7 [86]. MCPyV LT-antigen, in addition, can probably alter hVam6p protein function that is important in lysosome regulation [87]. LT-antigen of the human pathogenic polyomaviruses (JCPyV, BKPyV and MCPyV) has been shown to abrogate functions of RB proteins (i.e., pRB, p107 and p130), which probably includes TSPyV LT-antigen (**Chapter 4**) [5, 88 - 91]. A major function of ST-antigen is ascribed to its ability to modulate PP2A cellular signaling protein [92 - 94]. Aside that, recently NEMO [95] and 4EBP1 [96] modulation was identified for MCPyV ST-antigen. MT/ALTO-antigen, expressed by MPyV, HaPyV, MCPyV, TSPyV and likely other members of the *Orthopolyomavirus-I* (**Chapter 5**), can regulate cellular signal transduction though modulation of protein kinases Src, PI3K and PLCγ1 [71, 97, 98].

TSPyV evolution and host adaptation

Molecular signatures/footprints of polyomavirus host-adaptation

Virus host adaptation facilitates virus dissemination, which could also promote virus speciation. Virus adaptation to the host involves fixation of beneficial mutations under the selection pressure imposed by the host. Successful adaptations may eventually lead to establishment of a new virus species that is genetically fit to occupy a separate niche in the host or multiple hosts. Virus evolution is constrained by host environment and many structural and genetic factors [105], including overlapping genes [106, 107]. Overlap regards to the expressible nucleotide sequence of one gene that can be part of expressible nucleotide sequence of another gene in an alternate reading frame, which may contribute to the expression and function of one or more gene products. The overlapping gene is always *de novo*, in case of polyomaviruses the ALTO/MT-antigen, which overlaps an ancestral gene, i.e., the LT-antigen, and the evolutionary forces active on these genes may differ [108]. When the ancestral gene product regions are evolutionary conserved - called the short linear motifs (SLiMs) - because of their critical role in regulatory function, protein-protein interaction or signal transduction [109, 110], selection pressure may act more meticulous on that genomic region.

In **Chapter 5**, we studied an overlapping open reading frame (ORF), called ORF5, that is conserved in a large monophyletic lineage of polyomaviruses designated the Ortho-I genogroup of the *Polyomaviridae* family (**Chapter 1, Figure 8**) [111], which includes four human viruses, i.e., MCPyV, TSPyV, HPyV12 and NJPyV. We have discovered this conservation independently from Carter *et al.* [73], who designed and called this overlapping ORF ALTO in their study on MCPyV. Besides the above viruses, Ortho-I genogroup includes two rodent polyomaviruses whose ORF5 encodes the second exon of MT-antigen (**Figure 3**) [71]. Sequence region of ORF2 that encodes the N-terminal part of LT-antigen, and the overlapping ORF5 encoding the ALTO or second exon of MT-antigen, encompasses the LXCXE domain and the ORF5m2 motif, respectively, both of which are considered SLiMs. Accelerated switching between Valine and Alanine was shown in this SLiM of ORF5 in ORF5-plus viruses as a result of the Cysteine residue conservation in LXCXE domain (**Chapter 5**). This was also the predominant site of amino-acid-changing mutations in TSPyV (**Chapter 6**). This switching between residues – called the COCO-VA toggling – implied a positive (purifying) selection on this site of ORF5m2, indicating the importance of this SLiM to preserve the overall function of the ALTO/MT-antigen. This observation lends support to ORF5 expression – either "directly" through the synthesis of MCPyV ALTO protein [73] or upon alternative splicing events directing the synthesis of TSPyV MT-antigen [71] (Van der Meijden and Feltkamp, personal communication).

The MPyV and HaPyV MT-antigens have been investigated for decades now. It is generally accepted that MT-antigen is involved in cellular transformation through modulation of several cellular protein kinases and signal transduction mediators (**Figure 3**) [71].

The observed toggling in ALTO/MT-antigen could emphasize a site-reversion mutation that involves, for instance, escape from immunological pressure facilitating adaptation of the protein to its niche [112]. Still, many questions remain unanswered. How common is CO-CO-VA toggling in other (overlapping) SLiMs? Is it one of many only in polyomaviruses or also evident even beyond this virus family? These are just few questions for future research. Finally, as an extreme case of adaptive evolution, COCO-VA toggling may enlighten evolutionary thinking and stimulate development of applications to extend the research on other (polyoma)virus (overlapping) SLiMs.

TSPyV evolution as an example of polyomavirus host-adaptation

Polyomaviruses do not encode viral DNA polymerase enzymes, but rather rely on host-cell enzymes for their replication [75, 82]. Only during cellular DNA synthesis-phase, an optimal condition is created that facilitates viral replication because of excess supply of host polymerases and deoxyribonucleotides [113]. Human DNA polymerase enzymes are of high fidelity and therefore HPyV DNA mutation rates are expected similar to the host mutation rates. For TSPyV was calculated that it indeed has low intra-host viral DNA substitutions per site per year [114], comparable to those established for BKPyV and JCPyV [115, 116]. Taken into account the host DNA substitution rate and the time-span over which these viral substitutions could have taken place, it was calculated that BKPyV and JCPyV may have diverged recently from a common ancestor – less than 1000 and 350 years, respectively, which on the evolutionary scale is comparable to what we found for TSPyV – less than 4300 years. Still, these data on TSPyV estimation of divergence (**Chapter 6**), and notably also on BKPyV and JCPyV [115, 116], should be sought with caution because of the large uncertainty as a result of extremely large confidence intervals of the calculated evolutionary rates.

The dominant evolutionary model of polyomavirus divergence until recently was virus-host co-evolution [117 - 119]. On the contrary, recent studies showed that polyomavirus evolution could have taken place at least two orders of magnitude faster making the assumptions of a zoonotic transmission of primate polyomaviruses more probable. The calculated time of divergence of the most recent common ancestor (tMRCA) of TSPyV and its closest polyomavirus relative OraPyV1, provided that it may be valid to assume that these two viruses emerged recently from a common ancestor [114]. Similar assumptions exist for JCPyV [116] and BKPyV [115, 120], viruses that most probably have emerged from a common ancestor closely related to SV40 and SA12 – both baboon viruses – with a phyletic position in the Ortho-II polyomavirus genogroup (**Chapter 1, Figure 8**) [111]. Although similar estimates are lacking for most of the recently identified HPyVs, when analysing the polyomavirus phylogenetic tree and looking at the Ortho-I genogroup, one can note that many HPyVs appear to be closely related to viruses isolated from non-human primates (**Chapter 1, Figure 8**) [111]. In this regard, HPyV9 is very similar to the B-lymphotropic polyomavirus (LPyV, also known as the African green monkey polyomavirus, AGMPyV). Furthermore, MCPyV resembles the Gorilla gorilla gorilla polyomavirus 1 (GggPyV1), as well as the Pan

troglodytes verus polyomaviruses (PtvPyV). As indicated above and assessed in **Chapter 6**, TSPyV resembles Bornean orangutan polyomavirus 1 (OraPyV1). Finally, the recently identified NJPyV shows close relationship to the Chimpanzee polyomavirus (ChPyV) [40]. Thus, both virus-host co-evolution as well as a zoonotic non-human to human jump of most (if not all) primate polyomaviruses should be considered. Which of these hypothetical scenarios may be true for TSPyV and new HPyVs, awaits future estimations with the help of larger cohorts of virus genome isolates and sophisticated polyomavirus evolutionary estimation-models.

As discussed in the previous paragraph, genome and proteome adaptation of viruses to the host involve mutations because of the selection pleasure imposed by the host. In **Chapter 5**, we described COCO-VA toggling as a probable evolutionary selection mechanism. In **Chapter 6**, we provided genetic evidence for this mechanism with ORF5 being the predominant place of non-synonymous substitutions during TSPyV evolution. The four identified motifs in this ORF5 are under selection pressure because they overlap with the conserved ORF2 motifs. The motif involved in COCO-VA mechanism was located in the ORF5m2 motif. Out of three TSPyV lineages (TSI, TSII and TSIII), the TSI and TSII viruses used the Alanine residue most frequently at the COCO-VA site, implying that the Alanine residue provided a fitness-gain for the virus to adapt to its human host and may have facilitated additional TSPyV speciation (see **Chapter 6** and previous discussion). Experimental studies on TSPyV ALTO/MT-antigen could reveal molecular forces that drive toggling in this protein, in particular in the concerning SLiMs that facilitate in protein function by playing critical roles in protein regulation, protein-protein interaction or signal transduction. Taken together, without restraint one could hypothesis that the putative ALTO/MT-antigen of TSPyV is acting as a cellular protein kinase and signal transduction protein to stimulate cell growth and cellular transformation and future *in vitro* studies could prove this proposition.

Concluding remarks and future perspectives

We are only beginning to comprehend the basics regarding TSPyV and many knowledge gaps remain. One of the unknowns in TSPyV research is the mechanism(s) underlying latency/persistence and reactivation. In this regard, it is still unknown whether TS disease is the result of a primary TSPyV infection in the midst of immunosuppression or whether it concerns a reactivation from a latent site, be it skin or another not yet identified body location. Especially, limitations in the number of TS patients and availability of follow-up samples obtained after primary infection from different organs and sites have prevented further insights in this matter. *In vitro* study of (putative) TSPyV microRNA regulation, which is known to play a key role in latency/persistence and reactivation in other (polyoma)viruses, might offer additional opportunities in this regard.

As indicated in **Figure 3**, the knowledge about (putative) molecular interactions between T-antigens of polyomaviruses and cellular pathways is fragmented. Many factors have

been analyzed only for SV40 and needs to be validated for other (human) polyomaviruses as well. The lack of an experimental model for TSPyV makes answering these questions difficult. Skin-mimicking organotypic culture models could provide means for investigating the TSPyV and other cutaneous polyomaviruses. Knowledge about the cell type(s) that serve as the viral reservoir(s) could help to study specific mechanisms and pathways locally in monolayers, for instance IRS cells for TSPyV. With these specific and tailored *in vitro* culture systems at hand for research, one could identify interacting host molecular partners of TSPyV T-antigens *in vivo*.

Bioinformatics analysis of the polyomavirus evolution is an area of investigation that is gradually evolving and recent discoveries highlight that a broader taxonomic range of hosts may exist. The *Polyomaviridae* family tree may grow beyond what is known. The close relationship of primate polyomaviruses may provide interesting leads for further studies into inter- and intra-specific evolution of this virus family, as well as virus adaptation and speciation. First, with regard to virus-host evolution, TSPyV is probably a good example because of its possible recent zoonotic ancestry. Together with OraPyV1 they could be used as a model to elucidate intra-host evolution and adaptation of other HPyVs that also closely resemble non-human primate polyomaviruses. Second, with regard to host adaptation and speciation, the novel evolutionary mechanism called COCO-VA toggling described in this dissertation, could be an excellent starting point for studies investigating the potential effect of protein-residue toggling on mechanisms that circumvent host defenses. *In silico*, one could try to identify how common this COCO-VA toggling mechanism is in other (viral) proteins that show overlapping ORFs, whether it involves SLiMs and what it means for virus adaptation and speciation. These are just few experimental and bioinformatics questions for future research that have surfaced from research described in this dissertation.

Altogether, the research described in this dissertation provided many new insights regarding the prevalence, pathogenesis, evolution and host adaptation of TSPyV, which is one of the newer human polyomaviruses. These insights have substantially increased our understanding of TSPyV and the etiology of trichodysplasia spinulosa. Furthermore, they will allow us to better comprehend the clinical impact of polyomavirus infections in general, and provide new leads for future studies devoted to the identification of (antiviral) options to prevent or treat polyomavirus-associated diseases.

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