3. Determinants for HIV-1 species-tropism

Our early studies on systematic analysis of HIV-1 proviral mutants by site-directed mutagenesis have clearly demonstrated the cell-dependent functionality of some viral proteins (Gag-CA, Vif, Vpu, and Vpx) and the cell-dependent viral replication (Adachi et al., 1999; Kawamura et al., 1994b, 1998; Sakai et al., 1993, 1995; Sakuragi et al., 1995). These results have strongly suggested the presence of specific intracellular factors, other than receptor molecules for viruses, responsible for viral cellular tropism. Importantly, restriction factors against HIV-1 (APOBEC3/Vif, TRIM5α/Gag-CA, and tetherin/Vpu) have been recently identified and molecularly cloned (Neil et al., 2008; Sheehy et al., 2002; Stremlau et al., 2004; Van Damme et al., 2008). Furthermore, a new restriction factor functional in macrophages and antagonized by Vpx has been proposed (Fujita et al., 2008; Fujita et al., 2010; Sharova et al., 2008; Srivastava et al., 2008). Taken altogether, these findings have prompted active researchers to examine whether these cellular proteins are associated with the HIV-1 species-tropism. As results of a series of comparative biological and biochemical studies on the interaction between HIV/SIV and human/monkey restriction factors, it has been revealed that various species-specific cellular proteins in Table 3 determine or modulate the species-tropism of HIV-1. As can be understood in Table 3, viral accessory proteins Vif, Vpu, Vpx (and/or Vpr), and Nef (in the case of some SIVs) play significant roles (Tables 1 and 2) against the restriction factors present in host cells (Malim & Emerman,

Host restriction factors	Viral proteins	Antiviral effects
APOBEC3G/F	Vif	Induction of lethal mutations in the viral genome
CypA and TRIM5α/TRIMCyp	Gag-CA	Block of post-entry replication steps
Tetherin/BST-2	Vpu	Inhibition of virion release
Macrophage factor?	Vpx/Vpr?	Suppression of uncoating / reverse transcription?

Table 3. Restriction factors against HIV-1. Cellular anti-HIV-1 factors identified and one of potential anti-viral factors are listed. As for the details of restriction factors of these two categories, see the text.

2008). It is well-predicted that primate immunodeficiency viruses now have evolved by acquiring the appropriate accessory genes through numerous mutations and recombinations (Kirchhoff, 2009, 2010; Sauter et al., 2009, 2010). Among viral structural proteins, only Gag-CA, which constitutes a major virion component, appears to be deeply involved in the species-tropism of HIV-1. By adapting Gag-CA and accessory proteins to the hostile environment, HIV/SIV could spread, persist, and survive. In this regard, HIV-1 has developed its specific characteristics from the progenitor form, and may be still uniquely altering its virological property through multiple rounds of the infection cycle in human populations.

3.1 Vif and APOBEC3G/F

Accessory protein Vif (Table 1) is essential for HIV/SIV replication in certain cell types such as natural target cells (T-lymphocytes and macrophages) that express APOBEC3G/APOBEC3F. APOBEC3G/F are members of a polynucleotide cytidine deaminase family that displays

diverse functions (Holmes et al., 2007), and are potent inhibitors of viral replication counteracted by Vif. Vif degrades APOBEC3G/F via the ubiquitin-proteasome pathway (Table 1 and Fig. 3). In the absence of Vif, APOBEC3G/F are incorporated into virions, and cause lethal mutations in viral genome during the reverse transcription process in a new infection cycle (Table 3). There are two functional domains in Vif, that is, N-terminal binding region to APOBEC proteins and C-terminal region for degradation (Strebel et al., 2009). Noteworthy, HIV-1 Vif does not degrade APOBEC3Gs of the rhesus macaque and African green monkey probably due to its inability to binding to them. In contrast, SIVmac Vif can inactivate both human and simian APOBEC3Gs. Thus, the interaction of Vif and APOBEC3G/F is critically important for the unique species-tropism of HIV-1. In our experience, APOBEC3G/F is the strongest determinant for this tropism among the restriction factors listed in Table 3. Whether another activity of Vif to induce G2 cell cycle arrest (Izumi, T., 2010) (Table 1) is involved in the species-tropism is presently unknown.

3.2 Gag-CA and its interacting cellular proteins (CypA, TRIM5α and TRIMCyp)

Early studies have already indicated that Gag-CA is responsible for the HIV-1 species-tropism as described above (Shibata et al., 1991; Dorfman & Gottlinger, 1996). Recent works have focused on the interaction of Gag-CA and its counterpart (CypA, TRIM5α and a TRIM5α/CypA fusion protein, TRIMCyp). It is well-established now that CypA, TRIM5α and TRIMCyp act as an inhibitor of HIV-1 replication in a species-specific manner (Lim et al., 2010; Luban, 2007; Nakayama & Shioda, 2010; Price et al., 2009; Towers, 2007; Ylinen, 2010). These cellular proteins exert their anti-viral powers on the incoming virion core in a poorly defined way (Table 3 and Fig. 2). Of note, CypA positively and negatively regulates HIV-1 replication in human and macaque cells, respectively. Importantly, rhesus TRIM5α, cynomolgus TRIM5α and cynomolgus TRIMCyp effectively inhibit HIV-1 replication, but not rhesus TRIMCyp. Therefore, CypA, TRIM5α and TRIMCyp can determine the unique species-tropism of HIV-1. We estimate that Gag-CA is the second strongest determinant for the tropism. It should be stressed here that the polymorphism observed in TRIM5 alleles affects the sensitivity of hosts to virus infection.

3.3 Vpu and tetherin

Accessory protein Vpu (Table 1) is required for optimal replication of HIV-1 in certain cell types that express tetherin. Tetherin specifically inhibits the virion release from cells (Table 3) and is countered by Vpu (Nomaguchi et al. 2008b; Strebel et al., 2009). Vpu degrades cellular tetherin and CD4 effectively. It is generally accepted that Vpu enhances virion release from the cell surface by down-regulation of tetherin (Table1, Table 2 and Fig.3), and thereby promote viral replication. However, Vpu proteins of HIV-1 and some SIVs can not efficiently antagonize simian tetherin molecules relative to those of SIVs with a high ability (Sauter et al, 2009). In fact, HIV-1 NL4-3 scarcely suppressed the anti-viral activity of the rhesus tetherin. Based on this finding, it can be concluded that tetherin is associated with the species-tropism of HIV-1. However, in our experience, the positive effect of Vpu on viral replication is much smaller than those of Vif and Gag-CA. Moreover, another functional activity of Vpu to degrade cellular CD4 is considered to be irrelevant to the HIV-1 species-tropism. Whether Vpu is associated with the HIV-1 pathogenesis is an important question to address. Interestingly and importantly, Env of some HIV-2 isolates and Nef of some SIVs have the Vpu-like ability to enhance virion release (Strebel et al., 2009; Zhang et al., 2009).

3.4 Potential determinants for HIV-1 species-tropism

It has been recently reported that HIV-2/SIVmac Vpx is necessary for the post-entry step of viral replication, such as uncoating/reverse transcription, in monocyte-derived dendritic cells and macrophages (Fujita et al., 2008; Goujon et al., 2007; Srivastava et al., 2008). Vpx is supposed to counter an unidentified anti-retroviral factor(s) present in cells of this lineage (Tables 1-3 and Fig. 2). Because Vpx can also up-regulate the HIV-1 replication, the unidentified macrophage factor appears to be commonly important for HIV/SIV replication. To substantiate the macrophage entity as a restriction factor against HIV/SIV and/or the other retroviruses, its identification is urgently required.

During a systemic characterization of HIV-1mt CA mutants, we have noticed a TRIM5α-independent enhancement of viral infectivity in macaque cells (Nomaguchi et al., manuscript in preparation). This result suggests the presence of unknown anti-viral factor that interact with HIV-1 Gag-CA. We also have found a mutation in the Env-SU region that confers the mutant a significant affinity to macaque CD4, considerably promoting virus replication (Nomaguchi et al., manuscript in preparation). These observations may be relevant to the HIV-1 species-tropism.

4. Generation and characterization of various HIV-1mt clones

To obtain a novel class of HIV-1 that infects, replicates and finally causes AIDS in macaques, we and a research group in USA have independently initiated the work on HIV-1mt and have done macaque model studies (Hatcho et al., 2008; Hatziioannou, 2006, 2009; Igarashi et al., 2007; Kamada et al., 2006, 2009; Kuroishi et al., 2009; Nomaguchi et al., 2008a; Saito et al., 2011; Yamashita et al., 2008). Another group has published a report on HIV-1mt derivatives very recently (Thippeshappa et al., 2011). We now are actively and thoroughly amending the HIV-1mt genome by computer-assisted and structure-guided mutagenesis.

Our prototype HIV-1mt designated NL-DT5R (Kamada et al., 2006) contains a 21-nucleotide SIVmac Gag-CA element (corresponding to the HIV-1 CypA-binding loop) and the entire SIVmac *vif* gene inserted into the genetic background of HIV-1 NL4-3 (Adachi et al., 1986). From this clone, we have systemically generated a series of HIV-1mt clones as shown in Fig. 5. Because CCR5-tropic (R5) viruses of HIV-1 are thought to be clinically more important than CXCR4-tropic (X4) viruses, we have constructed two sets of HIV-1mt clones. Our strategy for generation of HIV-1mt clones pathogenic for macaques are as follows: (i) Adaptation of viruses in macaque cells. Targets for infection are cynomolgus and rhesus macaque lymphocyte cell lines immortalized by Herpesvirus saimiri (HVS) (Table 4).; (ii) In vitro mutagenesis of the clones based on bioinformatics. With the aid of the computational sciences, new viral genome sequences are designed.; (iii) Selection of appropriate clones by their replication kinetics in macaque lymphocyte cell lines in Table 4. Viruses which replicates similarly with or more robustly than SIVmac239 in cynomolgus and rhesus peripheral blood mononuclear cells are then chosen. On the basis of this strategy, we have successfully obtained a number of new generations with increasing ability to replicate from the original prototype NL-DT5R (see below). However, so far, none of the HIV-1mt clones tested are pathogenic for macaques (pig-tailed and cynomolgus) (Igarashi et al., 2007; Nomaguchi et al., manuscript in preparation; Saito et al., 2011), although they all can replicate in the monkeys. The newest clones in Fig. 5 (MN4Rh-3V and MN5Rh-3V), which replicate best in macaque cells among our HIV-1mt clones, have not yet been examined for

their pathogenicity. It should be mentioned here that the replication potentials of the HIV-1mt clones in cell lines parallel with those in individuals.

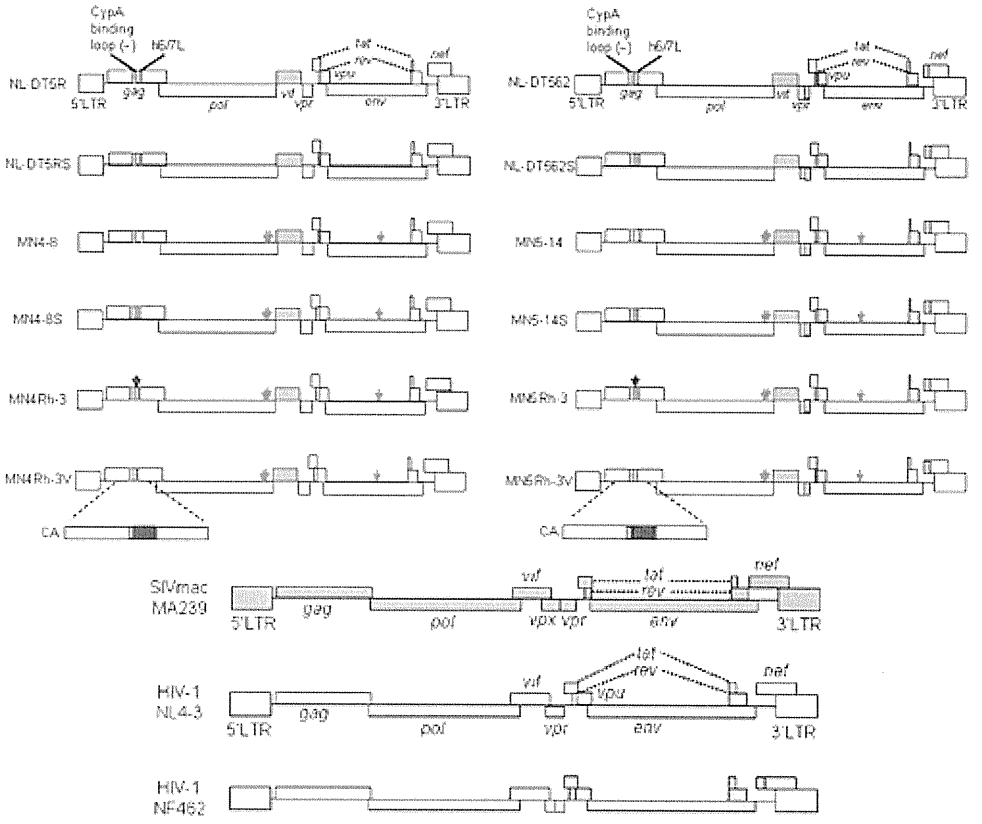

Fig. 5. Genome organization of HIV/SIV and various clones of HIV-1mt. Proviral genome structure is schematically shown. Blue, white and pink areas (boxes) indicate the genes and LTR of SIVmac MA239 (Shibata et al., 1991), X4-tropic HIV-1 NL4-3 (Adachi et al., 1986) and R5-tropic HIV-1 NF462 (Kawamura et al., 1994a), respectively. HIV-1mt clones on the left and right are X4 and R5 viruses, respectively. Arrows indicate the site of each single/double nucleotide-mutation introduced (Nomaguchi et al., manuscripts in preparation). There are several single-nucleotide mutations in the green area of Gag-CA (Nomaguchi et al., unpublished). h6/7L, Loop between helices 6 and 7.

In parallel with the generation and characterization of a series of HIV-1 mt clones, we have searched for and established macaque cell lines suitable for our projects. Table 4 lists the cell lines we routinely use now. Since the lymphocyte cell lines immortalized by HVS do not lose their original characteristics as primary lymphocytes in most cases and are readily maintained for experiments, to biologically characterize viruses like HIV-1, it is quite important for laboratory researchers to have HVS-immortalized cell lines. In our laboratory, cynomolgus HSC-F (Akari et al., 1996; Fujita et al., 2003) and rhesus M1.3S (Doi et al., 2011)

cell lines are chosen as targets for virus infection, and frequently used. HSC-F cells are very sensitive to HIV-1mt and SIVmac clones, and produce a large amount of progeny viruses after infection. M1.3S cells are quite resistant to HIV-1mt and SIVmac clones, and are appropriate for selection of highly replicable and potentially pathogenic viruses. Because we are interested in analyzing the species-tropism of HIV-1, we need to have various target cell lines of human and simian origins with a unique property. Monolayer cell lines of cynomolgus MK.P3 (F) and rhesus LLC-MK2 are easily used for transfection experiments and for monitoring the single-cycle viral infectivity assays. In fact, we have differentially and successfully used the cell lines in Table 4 depending on the purpose of each project.

Macaques	Cell lines	Origins	TRIM5 alleles
Cynomolgus	HSC-F	lymphocyte	TRIM5α and TRIMCyp
	MK.P3 (F)	kidney	TRIM5α and TRIMCyp
Rhesus	HSR1.4	lymphocyte	Mamu-3 and Mamu-4
	HSR5.4	lymphocyte	Mamu-7
	M1.3S	lymphocyte	Mamu-1 and Mamu-3
	LLC-MK2	kidney	Mamu-1 and Mamu-7

Table 4. Cell lines for virological evaluation of HIV-1mt. TRIM5 alleles of the cell lines listed have been determined in our laboratory (Doi et al., 2010; our unpublished results). For the polymorphism of TRIM5 alleles, see the references (Newman et al., 2006; Virgen et al., 2008; Wilson et al., 2008).

We have repeatedly examined the replication kinetics of HIV-1mt clones in various macaque cell lines. Fig. 6 shows the typical kinetics (a schema) based on the results from our numerous infection experiments. In highly sensitive HSC-F cells, all the viruses do replicate to distinct extents. As is clear, MN4Rh-3V and MN5Rh-3V replicate most robustly among HIV-1mt clones. In relatively resistant M1.3S cells, three clones do replicate but the others do not. In both cell lines, SIVmac239 (MA239N) (Doi et al., 2010) displays the best potential to replicate. These results indicate that we still need to improve MN4Rh-3V and MN5Rh-3V to obtain the ideal clone, the pathogenic HIV-1mt. In this situation, there are two directions. These are the selection of host macaques susceptible to the currently available clones and the further efforts to obtain the desired clones. First, pig-tailed and/or the other macaque species sensitive to the viruses can be selected by their TRIM5 alleles (Newman et al., 2006; Virgen et al., 2008; Wilson et al., 2008), and used for infection. Indeed, American research groups have adopted this strategy using the pig-tailed macaques/variants of simian-tropic (st) HIV-1 with a *vif*-substitution only (Hatziioannou et al., 2009; Thippeshappa et al., 2011). However, we very much prefer to take the second possibility. Through this approach, we would be able to better understand the molecular mechanism underlying various events between the pathogen and host. Furthermore, if one is interested in the studies to analyze the mutations, adaptations, and evolution of the pathogen, the pressure-giving environment (Malim & Emerman, 2008), i.e., natural hosts having a wide variety of restriction factors, would be much better. Of a particular note, pig-tailed monkeys infected with various st HIV-1s have not yet develop AIDS (Igarashi et al., 2007; Hatziioannou et al., 2009; Thippeshappa et al., 2011).

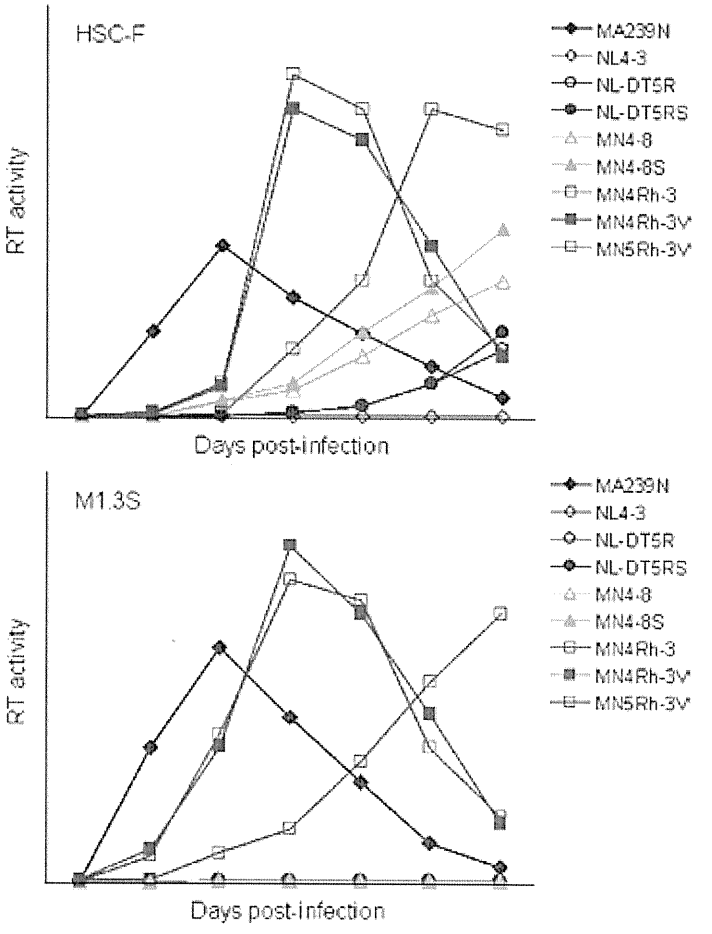

Fig. 6. Schematic representation of replication kinetics of various viral clones. A schema of replication kinetics is illustrated. Molecular proviral clones for study are shown on the right. Routinely, cell-free virus samples are prepared by transfection of proviral clones into 293T cells (Kamada et al., 2006), and viruses produced in cells of equal RT units are inoculated into HSC-F and M1.3S cells (Table 4). After infection, viral replication is monitored at intervals by RT activity in the culture supernatants.

5. Conclusion

We have described the generation of CXCR4-tropic and CCR5-tropic HIV-1 clones with macaque cell-tropism (HIV-1mt) in this chapter. The best X4 and R5 viruses we have now replicate comparably with a standard SIVmac clone in macaque cells, although their pathogenicity for macaques needs to be determined. The genomes of these HIV-1 mt clones contain the entire *vif* gene of SIVmac, some nucleotide substitutions in the *gag* gene to give a small number of mutated amino acids, two adaptive mutations in the *pol* gene, and one adaptive mutation in the *env* gene (Fig. 5).

For the moment, our goal is to have the HIV-1mt clones pathogenic for cynomolgus and/or rhesus macaques with the aid of computational sciences. The clones are expected to have the HIV-1-derived or closely related accessory genes except for the *vif* gene. With these ideal HIV-1mt clones, we would be able to authentically investigate the HIV-1/host interaction including: (i) viral replication in individuals; (ii) viral pathogenesis; (iii) viral mutations/adaptations/evolution. Once these clones are available, a wide variety of basic and clinical studies would be initiated otherwise impossible.

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Many original articles reporting the scientifically new and important findings could not be cited due to the tremendous numbers of publications and the space limitations. We express our sincere regret over these omissions based on rather subjective considerations.

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HIV-1 Vpr and G2 cell cycle arrest

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Priority Paper Evaluation
Future Microbiology

Evaluation of: Belzile J-P, Abrahamyan LG, Gerard FCA *et al.*: Formation of mobile chromatin-associated nuclear foci containing HIV-1 Vpr and VPRBP is critical for the induction of G2 cell cycle arrest. *PLoS Pathog.* 6(9), E1001080 (2010). All primate immunodeficiency viruses encode a unique set of accessory proteins to optimize their replication in hosts. In general, these proteins appear to be multifunctional for virus replication. Viral protein R (Vpr), one of the accessory proteins, has also been reported to exhibit distinct activities, but its exact role in the viral life cycle is still unclear and controversial. However, of particular note, Vpr-mediated G2 cell cycle arrest is conserved among primate immunodeficiency viruses. Belzile *et al.* have characterized and analyzed in detail the punctuate structures on the DNA of host cells formed by HIV-1 Vpr (Vpr nuclear foci). They demonstrate, mainly by confocal immunofluorescence analysis, that highly mobile chromatin-associated Vpr nuclear foci are critical for induction of the G2 cell cycle arrest.

Belzile *et al.* recently reported that HIV-1 Vpr accumulates into unique discrete foci in the nucleus of host cells and that these foci, termed Vpr nuclear foci (VNF), associate with chromatin and rapidly move inside the nucleus [1]. VNF contain the E3 ubiquitin ligase component Vpr-binding protein (DCAF1) and are thus responsible for induction of G2 cell cycle arrest via activation of ataxia telangiectasia-mutated and Rad3-related kinase (ATR) [1]. The overall conclusion of the article is firmly supported by the results obtained from numerous, careful and elaborate experiments.

Primate immunodeficiency viruses including HIV-1 encode unique viral proteins that modulate the virus–host cell relationship [2,3]. Of these accessory proteins, Vpr is least well understood with respect to its functional role in the viral replication cycle [4]. Even results in published studies are sometimes controversial. For example, HIV-1 Vpr has been reported to be essential, dispensable or unnecessary for viral replication in natural target cells, such as macrophage and T lymphocyte cultures. Moreover, whether the observed cytostatic properties of HIV-1 Vpr are directly related to viral pathogenesis remains to be determined. So far, several activities have been attributed to HIV-1 Vpr, including nuclear import of viral DNA, transactivation of viral long terminal repeat, regulation of apoptosis and promotion of a cell cycle arrest at the G2/M phase [4]. Importantly, the ability to induce G2 cell cycle arrest is highly conserved among Vpr of primate immunodeficiency viruses [5,6]. Furthermore, Vpr has been shown to manipulate the Cul4–DDB1–DCAF1 E3 ligase complex to induce the ATR-dependent G2 arrest through proteasomal degradation of an unknown cellular target [7–13]. Similarly, another accessory protein, Vpx, which is a paralog of Vpr but does not induce G2 arrest, is reported to act as a connector in the same Cul4–DDB1–DCAF1 complex to counter an unknown restriction factor in macrophages and dentritic cells by proteasomal degradation [14–16]. In summary, the virological significance of Vpr-mediated G2 arrest is presently unclear. Belzile *et al.* have initiated the work presented in this article based on the assumption that Vpr-mediated G2 cell cycle arrest plays an important role *in vivo* for viral replication and/or pathogenesis. This impairment of cell division by Vpr can be postulated to increase viral replication and to trigger immunomodulation [17–21]. Their hypothesis is not unreasonable because the activity is shared by distinct primate immunodeficiency viruses as described above and abnormal accumulation of cells in G2/M phase is indeed observed in individuals infected with HIV-1 [22].

Belzile *et al.* perform a systemic stepwise study to clarify how Vpr mediates the G2 cell cycle arrest. First, they show that VNFs containing VPRBP are important for Vpr-mediated G2 arrest. Second, the association of VNF with chromatin is demonstrated. Finally, VNF are shown to be highly mobile long-lasting nuclear bodies. In total, the results presented demonstrate that HIV-1 Vpr associates to form chromatin-bound nuclear foci via its C-terminus and that these nuclear foci serve as a mobile scaffold to recruit the DDB1–CUL4A (VPRBP) E3

Keywords
▪ G2 arrest ▪ HIV-1 ▪ primate immunodeficiency virus ▪ VNF ▪ Vpr ▪ Vpx

ubiquitin ligase to induce the ubiquitination and degradation of a chromatin-bound substrate, resulting in DNA replication stress or damage.

Belzile *et al.* have cautiously analyzed the subcellular localization of VNF and VPRBP by confocal microscopy. Vpr formed small circular structures (VNFs) of various relative sizes in the cell nucleus. VPRBP was also found to localize in the nucleus as a punctuate structure. Notably, a considerable proportion of VNF co-localized with VPRBP in primary CD4-positive T-lymphocytes as well as in HeLa, an established permanent cell line. These results strongly suggest that HIV-1 Vpr can develop VNF-containing VPRBP in natural target cells for HIV-1 infection. To exclude the possibility that the observed co-localization of VNF and VPRBP is a coincidence, the *in situ* proximity ligation assay [23], which allows individual interacting proteins in close proximity to be visualized and quantified, was performed. As a result, Vpr was shown to be in close proximity to endogenous VPRBP in dense nuclear foci and thus it was concluded that VNFs contain VPRBP. It is important and interesting to determine whether these VNFs correspond to well-described nuclear bodies. By confocal microscopic examination, VNFs were shown not to co-localize with SC35 (SFRS2) or PML bodies but were revealed to partially co-exist with DNA repair foci components (53BP1, RPA32 and γ-H2AX), consistent with a previously published report [24]. This finding may suggest that formation of VNFs represents an early event in the induction process of G2 arrest that would result in generation of DNA replication stress or DNA damage. In fact, the effects of an inhibitor of ATR and siRNA against VPRBP on G2 arrest, VNF formation, and Vpr-induced formation of DNA repair foci have indicated that Vpr forms VNFs prior to and independently of ATR activation and that Vpr recruits VPRBP to the foci. Moreover, G2 arrest-defective Vpr mutants and G2 arrest-incompetent Vpx were evaluated for the capacity to form nuclear foci. They were defective for foci formation or did not form any foci. Thus, the results obtained indicate that VNF formation is an early event required for induction of G2 arrest. The relationship between VNF formation and Vpr-oligomerization was then analyzed by monitoring affinity (using BRET assays) of pairs of Vpr proteins including C-terminal mutants, which are defective for foci formation. The ability of Vpr to oligomerize did not directly correlate with VNF formation. However, results of trans-complementation of the mutants showed some contribution of the oligomerization of Vpr to the process of VNF formation.

Belzile *et al.* also examined the association of VNF with chromatin by Western blot analysis of fractionated extracts prepared from Vpr-expressing cells. To form functional VNFs, Vpr concentrates at the specific sites in the nucleus by an unknown mechanism. Since VNFs co-localize with chromatin-bound factors at DNA repair sites, some of these chromatin-bound cellular proteins may be responsible for tethering Vpr to the specific regions. Cell extracts containing the chromatin were extracted from Vpr-expressing cells by treatment with Triton X-100 and micrococcal nuclease and were confirmed by Western blot to have chromatin-bound proteins (RPA70 and histone 3) but not cytoplasmic protein (GAPDH). By this fractionation method, Vpr and a portion of VPRBP were shown to specifically associate with chromatin. Results of genetic analysis supported this conclusion. VPRBP did not contribute to the association of Vpr and chromatin as revealed by siRNA-knockdown. To determine whether VNF and VPRBP interact on chromatin, co-immunoprecipitation analysis of chromatin fraction prepared from Vpr-expressing cells was performed. Data obtained showed that VNFs most likely interact with VPRBP on chromatin. Belzile *et al.* have finally studied the dynamic nature of VNF in their article. Nuclear bodies associating with chromatin are generally dynamic structures in mobility and/or in stability. Dynamics of VNF behavior on chromatin was analyzed by time-lapse confocal microscopy in living HeLa cells expressing eYFP–Vpr. Monitoring of eYFP–Vpr and software-assisted tracking of numerous foci have revealed that VNF are highly mobile long-lasting nuclear bodies.

Major conclusions in many of the pre-existing Vpr studies as well as the data presented in this study are consistent with the main claim in this article as described above. Although many of the experiments were carried out in non-HIV-target monolayer cells, such as HeLa and 293T, enough care has been taken to interpret the results obtained. Multiple and diversified experiments have been designed and performed to prove assumptions or hypotheses. Based on this paper, the future directions to understand molecular biology and biology of Vpr have become quite clear. Most importantly, we basic researchers on HIV-1 should know

the virological significance of Vpr-mediated G2 arrest [17–21]. Among four HIV-1 accessory proteins (Vif, Vpr, Vpu and Nef), Vpr is most poorly analyzed for its role in viral replication *in vivo* [2–4]. Vif and Vpu counteract innate restriction factors (a family of APOBEC3 proteins and tetherin/BST-2, respectively) against HIV-1. Nef effectively impairs host immune response. But what does Vpr do for HIV-1 replication? Why does HIV-1 have Vpr? Why does HIV-2 have both Vpr and Vpx? Extensive studies on the interaction of Vpr and its target cellular protein would give a definitive answer to these essential questions.

Conclusion & future perspective

Belzile *et al.* clearly indicate that HIV-1 Vpr forms highly mobile nuclear foci containing VPRBP and that formation of these discrete punctuate structures constitutes a critical event in the induction of DNA damage/stress and G2 arrest by Vpr. Further characterization of these chromatin-bound nuclear foci would better reveal the mechanism by which Vpr activates ATR and induces G2 arrest. Unfortunately, the cellular factor targeted by Vpr is still unidentified and represents a major issue to understand the biology of HIV-1. In this regard, the cellular target protein of Vpx should also be identified. In addition, the activity of HIV-1 Vpr needs to be studied in macaque monkey models [25,26]. Another important point to note is that Vif and Vpr independently induce cell cycle arrest [27,28]. It is shown that Vif-mediated G2 arrest has a considerable positive effect on HIV-1 replication [28]. It would be interesting to examine the biological significance of the G2 arrest caused by HIV-1 for viral infection *in vivo*.

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Executive summary

Objectives

- To determine the subcellular localization where Vpr and Vpr-binding protein (VPRBP; also known as DCAF1) interact.
- To investigate the nature and composition of Vpr nuclear foci (VNF).
- To determine whether the formation of VNF represents an early event in the induction of G2 arrest.
- To determine how VNF forms.
- To correlate the ability of Vpr to form VNF with the ability to associate with chromatin.
- To determine whether Vpr and VPRBP interact on chromatin.
- To investigate the dynamic nature of VNF.

Methods

- Cells processed for fluorescence immunohistochemistry, laser-scanning confocal microscopy, *in situ* proximity ligation assay and flow cytometry.
- Cellular proteins prepared for western blot and immunoprecipitation.
- Transfected cells for bioluminescence reasonance energy transfer assay.
- Cell lysates prepared for chromatin-binding assay.

Results

- Vpr forms VNFs that contain VPRBP.
- VNFs partially co-localize with DNA repair foci components (53BP1, RPA32 and γ-H2AX).
- VNF formation is required for induction of G2 arrest. G2-arrest incompetent Vpx, a paralog of Vpr, does not form any foci.
- Vpr oligomerization does not directly correlate with VNF formation but may contribute to the process to some extent.
- The ability of Vpr to form VNF correlates with its ability to associate with chromatin.
- Vpr interacts with VPRBP on chromatin.
- VNF are highly mobile long-lasting nuclear bodies.

Conclusion

- Vpr forms highly mobile punctuate structures on the DNA of host cells designated VNF.
- Vpr recruits the ubiquitination complex to the VNF and uses them to target a DNA-bound cellular protein for degradation. Vpr thus induces a cell division block.
- Identification of the unknown cellular factor targeted by Vpr is important to understand the role of Vpr for viral replication and AIDS pathogenesis.

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OPINION ARTICLE
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Rhesus M1.3S cells suitable for biological evaluation of macaque-tropic HIV/SIV clones

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Human immunodeficiency virus type 1 (HIV-1) is the causative virus of human acquired immunodeficiency syndrome (AIDS). Due to the lack of appropriate animal models, basic studies on HIV-1 replication, pathogenesis, and evolution, have been limited to cellular and molecular levels (Nomaguchi et al., 2008). Moreover, applied clinical studies *in vivo* also have been forced to use simian immunodeficiency virus isolated from macaques (SIVmac) and/or chimeric virus between SIVmac and HIV-1 (SHIV) based on our pioneering work 20 years ago (Shibata et al., 1991; Sakuragi et al., 1992). This experimental hindrance is mainly originated from the extremely narrow host range of HIV-1. To overcome the difficulty, we and others have made every effort to develop and establish suitable virus/animal systems. Because HIV-1 has adapted itself from the ancestral SIVs in Africa to replicate in humans in a marvelously strict way (Kirchhoff, 2009), the most promising one would be the use of macaque-tropic HIV-1 and macaques (Ambrose et al., 2007; Nomaguchi et al., 2008). As input viruses for infection of macaques, simian-tropic (st)HIV-1, and macaque-tropic HIV-1 (HIV-1mt) were designed, generated and characterized *in vitro* (Hatziioannou et al., 2006; Kamada et al., 2006; Thippeshappa et al., 2011) and *in vivo* (Igarashi et al., 2007; Hatziioannou et al., 2009; Saito et al., 2011; Thippeshappa et al., 2011). However, any of these HIV-1 derivatives neither replicate similarly robustly with the SIVmac standard clone in macaque cells nor are pathogenic for macaques so far. We, therefore, started improving the prototype HIV-1mt clone designated NL-DT5R by viral adaptation in macaque cells and genome manipulation based on bioinformatics (**Figure 1A**). NL-DT5R contains a 21-nucleotide SIVmac Gag-capsid (CA) element that lacks cyclophilin A (CypA) binding activity of the corresponding HIV-1 Gag-CA loop [CypA binding loop (−) in **Figure 1A**] and the entire SIVmac *vif* gene (Kamada et al., 2006). NL-DT5RS contains a loop sequence between helices 6 and 7 (h6/7L in **Figure 1A**) of SIVmac Gag-CA relative to NL-DT5R. MN4–8 carries three adaptive mutations in the *pol*-integrase and *env*-gp120 regions. MN4–8S contains the h6/7L of SIVmac Gag-CA and three adaptive mutations. MN4Rh-3 has an additional mutation in Gag-CA relative to MN4–8S. We now have a newest clone MN4Rh-3V (CXCR4-tropic), which was generated by the introduction of several mutations deduced from structural analysis into Gag-CA. Because CCR5-tropic viruses are thought to be clinically more important than CXCR4-tropic viruses, we similarly have generated a CCR5-tropic clone designated MN5Rh-3V.

To achieve our purpose to obtain HIV-1mt clones potentially pathogenic for macaques, as a prerequisite, it is critical to have macaque cell lines suitable and easily usable to characterize many of the virus clones generated. However, macaque cell lines that are sensitive to various SIV and HIV-1mt clones and maintain the characteristics of natural target cells such as peripheral blood mononuclear cells were not readily available to us. We therefore searched for the appropriate cell lines and finally paid attention to the macaque cell lines immortalized by Herpesvirus saimiri (Akari et al., 1996; Fujita et al., 2003; Doi et al., 2010). Of various cynomolgus and rhesus macaque cell lines in these reports, we were particularly interested in the rhesus MT-IL2I cell line (Doi et al., 2010). It is the most refractory cell line among those examined by us to infection of SIVmac and HIV-1mt clones, but is CD4-positive, CXCR4-positive, and CCR5-positive. Therefore, it was predicted that this cell line expresses potent intracellular restriction factors against viruses. In fact, the TRIM5 allele of this cell line was found to be *Mamu 1/Mamu 3* (Doi et al., 2010), a genotype that phenotypically shows a strong resistance to SIVmac infection (Lim et al., 2010). A subline of the MT-IL2I, after CD4-positive cells had been enriched by sorting, was designated M1.3S and used for infection experiments thereafter. Representative results obtained in M1.3S are shown in **Figure 1B**. Viruses of the early generation (from the prototype NL-DT5R up to MN4–8S) did not replicate at all. However, in cynomolgus HSC-F cells that are very sensitive to virus infection, MN4–8S replicated better than NL-DT5R, NL-DT5RS, and MN4–8. Interestingly, MN4Rh-3 acquired replication ability in M1.3S cells, and MN4Rh-3V exhibited further improved replication potential (**Figure 1B** left). On the other hand, the mutational effects of SIVmac accessory proteins on viral replication are also readily observed in M1.3S cells (**Figure 1B** right). Generally, these effects cannot be easily recognized in the established cell lines. As clearly observed in **Figure 1B**, each mutation to knock out the expression of individual accessory protein has greatly affected SIVmac replication. In particular, the mutations in *vif* and *vpx* genes have demonstrated a drastic negative effect. Therefore, it is quite evident that restriction factors against virus replication (counteracted by viral accessory proteins) are amply present in M1.3S cells.

In conclusion, the M1.3S cell line is exquisitely suitable to characterize macaque-tropic viruses and to investigate anti-viral cellular factors. Although primary cells are indispensable for basic studies on HIV/SIV that have highly adapted themselves to replicate in these cells, M1.3S cells can alternatively be used to address important issues to understand the biology and molecular biology of HIV/SIV.