

# Reverse genetics of rabies virus: new strategies to attenuate virus virulence for vaccine development

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**Abstract** Rabies is an ancient neurological disease that is almost invariably fatal once the clinical symptoms develop. Currently, prompt wound cleansing after exposing to a potentially rabid animal and vaccination using rabies vaccine combined with administration of rabies immune globulin are the only effective methods for post-exposure prophylaxis against rabies. Reverse genetic technique is a novel approach to investigate the function of a specific gene by analyzing the phenotypic effects through directly manipulating the gene sequences. It has revolutionized and provided a powerful tool to study the molecular biology of RNA viruses and has been widely used in rabies virus research. The attenuation of rabies virus virulence is the prerequisite for rabies vaccine development. Given the current challenge that sufficient and affordable high-quality vaccines are limited and lacking for global rabies prevention and control, highly cell-adapted, stable, and attenuated rabies viruses with broad cross-reactivity against different viral variants are ideal candidates for consideration to meet the need for human rabies control in the future. A number of approaches have been pursued to reduce the virulence of the virus and improve the safety of rabies vaccines.

The application of reverse genetic technique has greatly advanced the engineering of rabies virus and paves the avenue for utilizing rabies virus for vaccine against rabies, viral vectors for exogenous antigen expression, and gene therapy in the future.

**Keywords** Rabies virus · Reverse genetic technique · Attenuation · Vaccine

## Introduction

Rabies is an acute ancient fatal neurological disease that affects almost all kinds of mammals, including humans (Dietzschold et al. 2005). The mortality of rabies is almost 100 %, and it has been estimated by the WHO that more than 55,000 human deaths are caused by rabies through bites from rabid animals annually worldwide with the majority of cases occurring in developing world such as Asia and Africa where canine rabies is endemic and resources are limited (Briggs et al. 2013). Following India, China has the second highest incidence of rabies in the world, and rabies is still posing a great threat to the public health in China (Zhang et al. 2005).

The causative agents of rabies are viruses belonging to the *Lyssavirus* genus in the family *Rhabdoviridae* of which the prototypic rabies virus (RABV) is responsible for the vast majority of cases (Rupprecht and Plotkin 2013). The RABV genome is a single-stranded, negative-sense RNA of approximately 12 kb encoding five structural proteins in the order (3' to 5') nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent RNA polymerase (L) (Albertini et al. 2011). The RABV genome is tightly encapsidated by N, P, and L protein to form a ribonucleoprotein (RNP) complex which acts as the entity responsible for virus gene transcription and genome replication within the

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cytoplasm of host cell (Albertini et al. 2011). The RNP complex is packaged into a bullet-shaped structure and wrapped by an envelope comprising an inner layer of the M protein and the transmembrane spike G trimers, both of which play pivotal roles in viral assembly and budding (Mebatsion et al. 1996; Mebatsion et al. 1999).

Despite the tremendous progress that has been made in understanding of the RABV pathogenesis and the development of rabies vaccines, rabies is still almost invariably fatal once clinical symptoms occur. For this reason, prompt and thorough cleansing of the wound following a bite from a potentially rabid animal are critically important to reduce the risk of infection. Subsequently, post-exposure prophylaxis with rabies vaccine supplemented by the administration of rabies immune globulins infiltrated around the wounds has been proven to be highly effective to prevent rabies (Briggs et al. 2013; Rupprecht and Plotkin 2013). However, although rabies is largely brought under control in developed countries, it still causes great threats to developing countries where most human cases occur because of canine rabies and sufficient and affordable vaccines needed for effective post-exposure prophylaxis are insufficient and limited (Briggs et al. 2013). Therefore, considerable efforts are needed to develop safe, effective, and inexpensive vaccines to globally control rabies in the future. Since the first development of rabies vaccine by Pasteur in the late nineteenth century, rabies vaccine has improved greatly and vaccination has been widely used in both domestic animals as well as reservoir species (Briggs et al. 2013; Rupprecht and Plotkin 2013). The development of human rabies vaccines follows a trend from brain passage to cell adaptation primarily because of safety considerations (Wu et al. 2011). Human rabies vaccines can be produced either from animal tissues or cultured cells; however, severe adverse reactions and significant poor antigenic responses have been reported using the tissue-derived rabies vaccines either from the nerve tissues or whole avian embryos, thus falling short of modern standards of vaccines that are pure, potent, safe, effective, and cost-beneficial (Wu et al. 2011). The advent of modern industrial cell cultivation and fermentation techniques has greatly promoted the capacity of producing vaccines with high quantity and quality, and vaccines using cultured cells have quickly outdated, if not completely eliminated, the use of tissue-derived rabies vaccines and will undoubtedly act as the primary substrates for rabies vaccine production in the future (Wu et al. 2011).

Contrary to the classical genetics which is based on visible results of reproductive attributes to explore the genetic basis for a phenotype or trait, reverse genetics takes advantage of the availability of gene sequence to investigate the specific role of a gene in phenotype determination. The advent of reverse genetic technique has greatly facilitated the molecular biology analysis of RNA viruses, as it avoids the difficulty to manipulate the RNA molecule directly (PeKosz et al. 1999).

After *in vitro* transcription, the RNA genome of a virus is converted to the corresponding DNA and the subsequent manipulation of the DNA sequence, such as site-specific mutation, gene deletion/insertion, and substitution, could easily be performed on the DNA level to study the structure and function of a specific gene and its role in virus replication cycle. After the pioneering work by Schnell et al. (1994) to rescue an attenuated, fixed RABV strain SAD B19 through reverse genetic technique (Schnell et al. 1994), numerous studies have utilized this powerful tool to investigate the molecular biology of RABV as well as other negative-strand RNA viruses, and reverse genetic technique has shown promising prospect in developing novel rabies vaccines which are safer and more desirable than transitional vaccines.

In this review, we briefly present the general approaches to recover infectious RABVs from cloned complementary DNA (cDNA) and discuss recent advances in engineering RABV as viral vectors for vaccine development. While a number of excellent reviews have been written focusing on reverse genetics of negative-strand RNA viruses and the history and development of rabies vaccines (Briggs et al. 2013; Conzelmann 2013; Finke and Conzelmann 2005; Hicks et al. 2012; Nel 2005; Rupprecht et al. 2002; Wu et al. 2011), this review mainly summarizes the current strategies using reverse genetics to reduce the virulence and improve the safety of RABV for potential use in rabies vaccine production and the development of multivalent vaccines capable of stimulating host immune responses against other pathogens. Finally, some of the issues concerning the application of reverse genetics in negative-strand RNA viruses that need to be addressed are discussed.

### Recovery of infectious recombinant RABV from cloned DNA

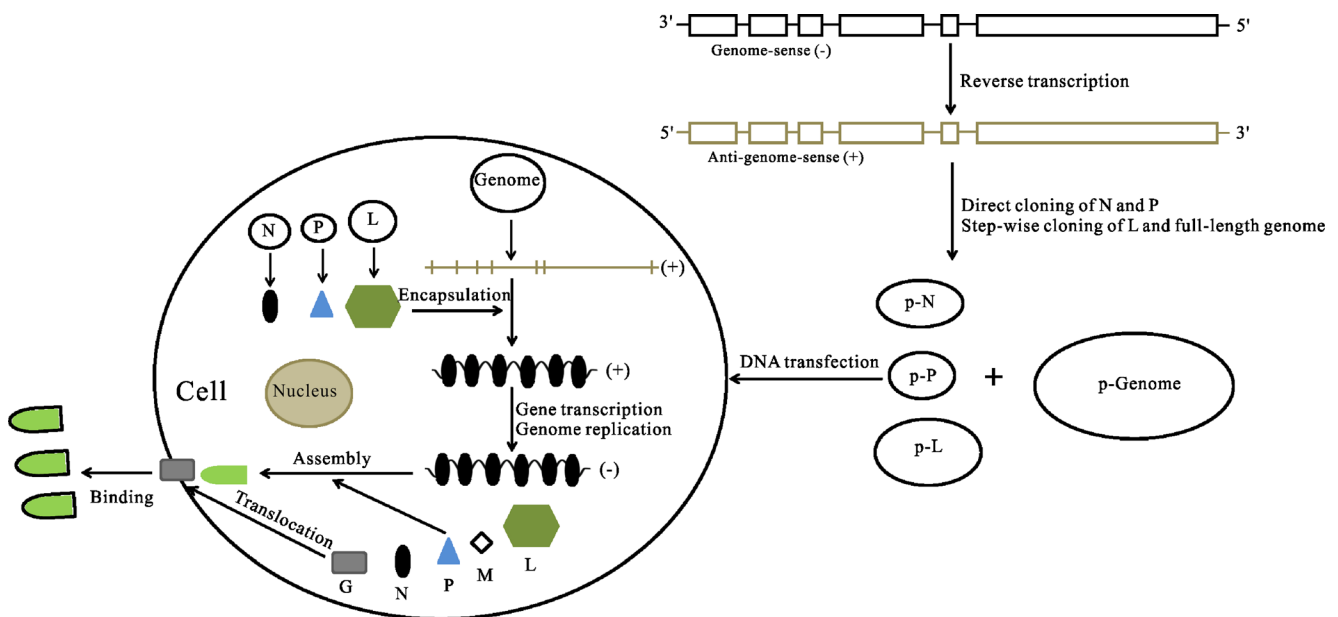
Since the first recovery of phage Q $\beta$ , a positive-stranded RNA virus in 1978, a number of positive-stranded RNA viruses have been successfully rescued using reverse genetics (Taniguchi et al. 1978). As the genomes of positive-stranded RNA viruses can function as messenger RNA (mRNA) for translation of the viral proteins, including viral polymerase, therefore an infectious cycle could be easily initiated by introduction of the genome analogs, such as transcripts generated by standard DNA-dependent RNA polymerases from viral genomes, into a cell. However, in contrast to positive-stranded RNA viruses, the template of the polymerases of negative-stranded RNA viruses is exclusively the RNP complex and the genomes of negative-strand RNA viruses could not be used as mRNAs to provide viral proteins involved in virus replication and transcription, meaning that all those essential proteins have to be provided *in trans* (Schnell et al. 1994). These characteristics of negative-strand RNA viruses

hindered the generation of infectious virus from cloned cDNA.

A pioneering breakthrough in the generation of RABV from cloned cDNA was accomplished by Schnell et al. 1994, which subsequently has revolutionized the study of RABV as well as other negative-strand RNA viruses (Schnell et al. 1994). Briefly, to rescue RABV, cells are transfected with four plasmids, with three helper plasmids expressing the N, P, and L proteins (which together with the RNA genome form the RNP complex), respectively, and one plasmid transcribing the full length of antigenome-sense RNA transcripts (Fig. 1). The full length of RABV cDNA is stepwisely assembled into a plasmid through restriction enzymes to provide template for virus replication and for transcription. A key point for successful recovery of RABV is the use of complementary antigenome-sense rather than the genome-sense transcripts to initiate a productive infection. Although either the genome-sense or the complementary antigenome-sense transcripts might be used as the template, only the antigenome-sense transcript is feasible as it effectively avoids hybridization between minus-sense genomic RNA with those plus-sense mRNAs from the protein-encoding

plasmids which could probably interfere with genomic RNA encapsidation and protein translation.

A number of improvements have been made to facilitate the manipulation and increase the recovery efficiency of RABV. For the supply of T7 RNA polymerase, instead of infecting cells with recombinant vaccinia virus, a cell line constitutively expressing T7 RNA polymerase BSR-T7/5 was developed (Buchholz et al. 1999). A further improvement has been developed first in a segmented negative-strand RNA virus, the influenza A virus, which was based on cellular polymerase I or II for gene transcription (Fodor et al. 1999; Hoffmann et al. 2000; Neumann et al. 1999), and was subsequently applied in RABV recovery to achieve high recovery efficiency (Inoue et al. 2003). Furthermore, as it has been demonstrated that extra nucleotides at the viral terminal end might affect rescue efficiency (Le Mercier et al. 2002), the coding sequences of two ribozymes, hammerhead ribozyme (HamRz) and hepatitis delta ribozyme (HdvRz), were anchored to the 5' and 3' end of the full-length cDNA, respectively, to generate full length of RABV transcripts with precise termini (Inoue et al. 2003). These innovations significantly expand the feasibility of reverse genetics of RABV to various



**Fig. 1** Schematic diagram of recovery of infectious RABV from cloned cDNA. The standard protocol for recovery of infectious RABV from cloned cDNA involves at least simultaneous transfection of four plasmids, encoding N, P, L, and the full-length cDNA of the virus, respectively, into a cell. The negative-sense viral RNA genome is first reverse transcribed into the corresponding positive-sense cDNA (antigenomic). Three helper plasmids expressing N (p-N), P (p-P), and L (p-L) are constructed through amplification of the specific gene from full-length cDNA. To avoid mutation, the L gene is generally assembled from stepwisely cloned fragments. Similarly, the viral cDNA is stepwisely assembled into a plasmid (p-Genome) and is oriented to give rise to a positive strand antigenomic-sense RNA. Transcription of these four plasmids is directed by either T7 RNA polymerase or suitable

polymerases such as endogenous cellular RNA polymerase I or II. To generate precise 5' and 3' ends of viral RNA, the cloned viral cDNA is generally flanked by autocatalytic ribozyme sequences. N, P, and L expressed from helper plasmids encapsulate the transcribed full-length antigenomic-sense RNA to form the RNP complex, which then acts as the template for further expression of the five viral proteins (N, P, M, G, and L) and amplification of negative-strand genome-sense RNAs (also exist as RNP), thus initiating an autonomous infection cycle. The replicated genome-sense RNP complex is condensed into the bullet-shaped form by M protein and bud from plasma membrane where the glycosylated trimeric transmembrane G is accumulated. Finally infectious virions are produced and the next infection cycle ensues

cell lines and allow rapid and efficient generation of recombinant RABV.

A predominant characteristic of RABV and other members of the *Lyssavirus* genus is the presence of the pseudogene region ( $\psi$ ) located between the G and L open reading frames and is transcribed as the nontranslated 3' terminal part of the G cistron mRNA (Walker et al. 2011). Previous studies have identified that a RABV lacking the  $\psi$  region displayed normal growth characteristics in cultured cells and no difference in axonal spread or pathogenicity in mice (Ceccaldi et al. 1998; Schnell et al. 1994). Therefore, the  $\psi$  region is an ideal target to accept insertions of additional genes and lays a foundation for various genetic manipulation of the RABV genome as described below.

### Construction of replication-deficient virus

Due to the almost 100 % mortality of rabies, currently only inactivated vaccines have been approved for human use. However, inactivated viral vaccines can only induce CD<sup>4+</sup> T cell and humoral responses but are unable to elicit CD<sup>8+</sup> T cell responses which play pivotal roles in cellular immune response against viral infection (Dudek and Knipe 2006). Therefore, live, highly attenuated, or even totally avirulent RABV strains which could trigger CD<sup>8+</sup> and CD<sup>4+</sup>, as well as humoral immune responses without causing disease, are more potent than those traditional rabies vaccine strains and thus are ideal candidates for future vaccine development.

Reverse genetics has been used to elucidate functions of viral genes and develop new types of vaccines against specific pathogens. Replication-deficient viruses are deleted for specific viral genes that are essential for viral replication, and therefore, they can only replicate in cells that express the deleted viral genes while fail to establish infection in normal cells, thus allowing stringent control of viral replication and high level of safety feasible (Dudek and Knipe 2006). A prerequisite for reasonable use of such gene deletion strategies is an appropriate complementation system to provide sufficient viral proteins not at the expense of causing host cell cytopathy.

Of the five genes encoded by RABV, the P and M genes have been deleted to generate replication-deficient viruses to test the potential for vaccine development. P or M gene deletion abolished progeny virus production and was a pathogenic in adult and suckling mice even when inoculated intracranially (Ito et al. 2005; Morimoto et al. 2005; Shoji et al. 2004). Furthermore, inoculation of the P or M gene deleted virus into mice induced high virus-neutralizing antibody (VNA) titer and protected mice from lethal virus challenge (Ito et al. 2005; Morimoto et al. 2005; Shoji et al. 2004). As for the rest, N, G, and L genes, their deletions either affected viral immunogenicity or could not be rescued readily and are thus not appropriate for vaccine production (Morimoto et al. 2005).

Therefore, the P or M gene deleted RABV would be a potential candidate for safe live-attenuated rabies vaccine development.

### Targeting specific viral gene

#### Overexpression of the G protein

The RABV G protein is the only viral protein exposed on the surface of the virus. It is not only the major determinant of viral pathogenicity but is also the major protective antigen responsible for the induction of protective immunity against rabies (Cox et al. 1977; Faber et al. 2002; Wiktor et al. 1973; Zhu et al. 1996). The G protein facilitates the interaction of virus particles with appropriate cell surface receptors to mediate virus entry and is also involved in efficient virus budding as well as the neurotropism of RABV (Dietzschold et al. 1983; Langevin et al. 2002; Lentz et al. 1984; Morimoto et al. 2000; Morimoto et al. 1999; Seif et al. 1985; Thoulouze et al. 1998; Tuffreau et al. 1998). Typically, attenuated RABV strains express higher levels of G in infected neurons than highly pathogenic street strains (Morimoto et al. 1998; Yan et al. 2001), and attenuated RABV causes significantly apoptotic cell death in neurons while few signs are observed upon pathogenic street strain infection (Rupprecht and Plotkin 2013). In addition, it has been shown that attenuated RABV actively activates, while pathogenic RABV evades, the host innate immune and antiviral responses (Wang et al. 2005). Therefore, the G protein expression level is an important determinant in virus pathogenicity and is a potential target to attenuate virus virulence.

Indeed, it has been shown that RABV containing two copies of G gene increased the expression of G and at the same time significantly improved the efficacy of the vaccine by enhancing its immunogenicity, reducing its pathogenicity, and resulting in higher levels of VNA production (Faber et al. 2009; Tao et al. 2011; Zhang et al. 2013). Furthermore, the G protein expression level was shown to be inversely correlated with the pathogenesis of RABV virus, and elevated G expression was associated with enhanced apoptosis which contributes to the induction of strong upregulation of genes related to host immune responses observed in neurons infected with attenuated RABV strains (Faber et al. 2009; Faber et al. 2002). Most importantly, increasing G expression level in the viral seed strain has the advantages that not only significantly decrease the probability of reversion to pathogenicity but also greatly improve the manufacturing capacity and biosafety and substantially reduce the production costs, all of which are critical for modern production of pure, potent, safe, and affordable rabies vaccines that are urgently needed especially in the developing countries. Therefore, recombinant



RABV overexpressing the G protein might be a useful candidate for development of new rabies vaccines.

### Amino acid mutation in the G protein

Previous studies have demonstrated that a single amino acid substitution at position 333 of the G protein from a positively charged arginine (R) or lysine (K) residue to glutamine (Q) or isoleucine (I) rendered a virulent RABV strain to be apathogenic in adult mice by intracerebral inoculation (Faber et al. 2005b). However, other studies have found that amino acid 333 of the G protein is not entirely responsible for the pathogenicity of an RABV and that some RABV strains having a Q333 substitution retained neuroinvasive capacity and pathogenicity. Furthermore, RC-HL, a nonpathogenic RABV fixed strain, has an R at this position (Ito et al. 1994; Morimoto et al. 2001; Sato et al. 2009; Yang and Jackson 1992). Subsequently, other amino acids, such as those between amino acids 164 and 303 in the G protein of the RABV strain Nishigahara, especially amino acids 242, 255, and 268, also played a role in determining the apathogenic phenotype of RC-HL strain (Ito et al. 2010; Takayama-Ito et al. 2006). Interestingly, it was found that an amino acid substitution at 194 on the G protein from asparagine (N) to K was solely responsible for the recovery of pathogenic phenotype in the nonpathogenic, highly attenuated RABV strain SPBNGA (Faber et al. 2005b). Therefore, multiple sites in the G protein should be considered to be mutated to further attenuate the RABV virulence. In general practice, researchers usually constructed recombinant RABV strains which carry two or more G genes encoding mutated G protein at important amino acids such as amino acid 149, 194, and 333 to ensure high attenuation and abate the risk that an attenuated virus might revert to a pathogenic phenotype due to low fidelity of virus RNA polymerase and high mutation rate during viral replication ensues.

### Deletion of the cytoplasmic domain of G protein

A previous study has shown that the RV G cytoplasmic domain (CD) promotes efficient viral budding and spread within infected cells, possibly through ensuring efficient incorporation of the G protein into the envelope (Mebatsion et al. 1996). In addition, a recombinant *vesicular stomatitis virus* (VSV) with the G protein CD-deleted caused no weight loss in immunized mice, implying that the G CD might have a role in virus pathogenesis (Roberts et al. 1999). To investigate the role of the G CD in RV pathogenicity, the G CD was deleted in the RV vaccine vector SPBN (SPBN- $\Delta$ CD). Mice pathogenicity study indicated that SPBN- $\Delta$ CD showed attenuated pathogenicity in mice and, notably, deletion of the G CD did not result in attenuation of the humoral response against the RV G (McGettigan et al. 2003), demonstrating that RABV

with the G CD deleted could be utilized as strategy to develop efficacious antiviral vaccines.

### Amino acid mutation in the M protein

The RV M protein is a multifunctional protein. It has been shown to regulate the balance between transcription and replication of RV (Finke and Conzelmann 2003; Finke et al. 2003). In addition, M also interacts with the RNP complex, which it condenses into a dense bullet-shaped form and recruits for assembly and budding (Mebatsion et al. 1999). A common feature of M protein is the presence of “late” or L-domains, which mediate efficient virus budding through interacting with host cell proteins. To date, four L-domain core motifs, PPxY, PT/SAP, YxxL, and FPIV where x can be any amino acid, have been described, and a PPxY motif (PPEY) closing to the amino terminus of M was identified in RABV M protein (Wirblich et al. 2008).

Point mutation analysis identified that the PPEY motif in M is required for efficient virion release from cell membrane with the first proline being the most important amino acid (Wirblich et al. 2008). Importantly, mutation of the PPEY motif resulted in reduced spread of virus infection, and the recombinant virus was highly attenuated in mice without compromising the ability to elicit potent antibody responses against RABV G protein (Wirblich et al. 2008). Therefore, the PPEY motif of M protein can be targeted to increase the safety of rabies vaccine vectors.

### Mutation of the P protein

The RABV P protein is a noncatalytic cofactor of the viral polymerase complex where it associates with the major catalytic component L protein and functions to regulate the viral polymerase complex activity. Furthermore, it has been shown that the P protein forms a complex with N, thus keeping the N protein in a suitable form and conferring the specificity of genomic RNA encapsidation by N (Yang et al. 1998). Besides the full-length P protein, four other amino-terminally truncated P products (P2, P3, P4, and P5), translated from P mRNA by a leaky scanning mechanism from internal in-frame AUG initiation codons, have been identified (Chenik et al. 1995). These different forms of P proteins have distinct cellular localizations and play important roles in counteracting host immune responses on multiple levels (Schnell et al. 2010).

Interestingly, it has been found that the cytoplasmic dynein light chain (LC8), which is a subunit of cytoplasmic dynein motor complex and is involved in the intracellular retrograde transport of organelles, interacts with the RABV P protein (Jacob et al. 2000; Raux et al. 2000). It was initially speculated that the P-LC8 interaction might account for the axonal transport of the RNP complex via a microtubule-mediated process.

This hypothesis was disputed as the deletion of the dynein LC8 binding domain within the P did not inhibit RABV entry into the central nervous system (CNS) (Mebatsion 2001; Tan et al. 2007). Instead, deletion of the dynein LC8 binding domain significantly suppressed viral transcription and replication in the CNS (Tan et al. 2007). However, deletion of the dynein LC8 only specifically decreased virus growth on neuronal cells whereas the growth pattern on nonneuronal cells remained unchanged (Tan et al. 2007). In vivo studies demonstrated that deletion of the dynein LC8 binding domain retained pathogenicity in suckling mice (Mebatsion 2001) but was nonpathogenic in adult mice (Tan et al. 2007). In another study, the internal ribosome entry site (IRES) from picornaviruses was introduced to control the expression of P, and an in vivo study showed reduction of replication in cell culture, increased activation of the beta interferon (IFN- $\beta$ ) promoter, and attenuation in mice infected intracerebrally (Marschalek et al. 2009). Taken together, targeting the P protein through either mutating the dynein LC8 binding domain or translational control of expression is a promising strategy to attenuate virus virulence of live RABV.

## Exogenous expression of genes with antiviral activities

### Antiviral genes

Tumor necrosis factors (or the TNF family) are a class of proteins known as cytokines that can cause apoptosis. As the best known member of the TNF family, TNF- $\alpha$  has a wide variety of functions in host adaptive and innate immunity (Blum and Miller 2000). TNF- $\alpha$  is produced by T cells and cells of the monocyte/macrophage lineage (Bette et al. 2003; Bette et al. 1993) and plays important roles in countering viral and bacterial infections, such as inducing the expression of IFNs (Jacobsen et al. 1989; Mestan et al. 1986) and exhibiting direct antiviral activity against avian, swine, and human influenza viruses with a potency greater than that of IFNs (Faber et al. 2005a; Seo and Webster 2002).

To explore the role of TNF- $\alpha$  in RABV infections, a copy of TNF- $\alpha$  was inserted into the recombinant RABV vector SPBN to express TNF- $\alpha$  (SPBN-TNF- $\alpha$ +) (Faber et al. 2005a). It was found that compared to SPBN expressing inactivated TNF- $\alpha$  (SPBN-TNF- $\alpha$ -), the spread and production of SPBN-TNF- $\alpha$  were significantly reduced. However, surprisingly, the expression of TNF- $\alpha$  was not accompanied with increased cell death or induction of IFNs. Furthermore, mice infected intranasally with SPBN-TNF- $\alpha$  showed significantly less virus spread in the brain than those infected with SPBN-TNF- $\alpha$ -, and SPBN-TNF- $\alpha$ + caused no death in infected mice whereas 80 % of mice infected with SPBN-TNF- $\alpha$ - succumbed to death (Faber et al. 2005a). In brains of mice infected with SPBN-TNF- $\alpha$ +,

enhanced CNS inflammation, including T cell infiltration and microglial activation, was observed (Faber et al. 2005a).

IFNs are a class of proteins known as cytokines produced by host cells as protective defenses in response to challenges such as virus infection and the presence of tumor cells (Fensterl and Sen 2009). IFNs, especially type I IFNs (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$ , and IFN- $\omega$ ), not only are known for their anti-viral immunity activities but also have recently gained recognition for their roles in coordinating the innate and adaptive immune responses (Kotenko 2011). In addition, IFNs induce the production of hundreds of other proteins collectively known as interferon-stimulated genes (ISGs) that have important roles in combating virus infections (Platanias 2005). Many viruses have evolved mechanisms to resist host IFN activity, such as circumventing the IFN response by blocking downstream signaling events and inhibiting the functions of proteins that are induced by IFN, to establish infection (Lin et al. 2004; Navratil et al. 2010).

It has been shown that RABV vector SPBN expressing IFN- $\beta$  (SPBN-IFN- $\beta$ +) was highly attenuated and that virus replication in cultured cells was significantly decreased (Faul et al. 2008). However, mice immunized with SPBN-IFN- $\beta$ + had a significantly greater number of activated CD $^{8+}$  T cells through IFN- $\beta$  signaling dependent way. This attributes to activate CD $^{8+}$  T cell production by IFN- $\beta$  expression might be beneficial for certain vaccine approaches in that RABV vectors carrying both IFN- $\beta$  and another viral antigens such as HIV-1 Gag have advantages as a molecular adjuvant to increase immune response against HIV-1 infection (Faul et al. 2008). Besides the type I IFN, the type II IFN, IFN- $\gamma$ , also has many important functions in both innate and adaptive immunity (Kotenko 2011). Previous studies have shown that control of RABV replication in CNS begins at the onset of T cell entry and IFN- $\gamma$  production in the CNS prior to the production of VNAs (Barkhouse et al. 2014). It was shown that SPBN expressing IFN- $\gamma$  highly attenuated the pathogenicity of the virus predominantly via enhancing the production of type I IFNs, and the pathogenicity of the virus was reduced in both normal and immunocompromised mice (Barkhouse et al. 2014). Recent studies have confirmed the importance of IFN- $\gamma$  during RABV clearance from the CNS and demonstrated the concept that I IFN- $\gamma$  is located in the center of the signaling pathway in the brains of mice infected with attenuated CVS-B2c strain, compared to the central role of TNF- $\alpha$  in the network generated from mice infected with street DRV-Mexico (Chai et al. 2014).

Taken together, the above results demonstrate that expression of antiviral genes such as TNFs and IFNs using RABV vector has the potential to control and treat rabies in the future.

### Pro-apoptosis genes

Laboratory-attenuated RABV strain infection induces extensive host innate immune, antiviral responses, and apoptosis

while street viruses cause little neuronal pathology or damage in the CNS and apoptosis was barely observed upon street virus infection (Morimoto et al. 1999). Apoptosis has been considered to be beneficial for host to counteract viral infection as several studies have shown that the pathogenicity of an RABV was inversely correlated with their capacity to induce apoptosis (Faber et al. 2002; Morimoto et al. 1999). Furthermore, the extent of apoptosis also appeared inversely correlated with the expression level of the G protein as high level of G protein induced widespread apoptosis and strong antiviral responses (Faber et al. 2002). These observations raise the possibility that enhanced virus-induced cell death was associated with increased antiviral immune responses thereby functioning in protecting CNS from infection by RABV.

The effect of inducing apoptosis on RABV pathogenicity was investigated through exogenous expression of the proapoptotic protein cytochrome *c* in SPBN vector (SPBN-Cyto *c+*). As expected, cytochrome *c* expression triggered extensive apoptosis in primary neuron cultures in vitro (Pulmanusahakul et al. 2001). On the other hand, enhanced immunogenicity and attenuated pathogenicity were achieved in mice infected with SPBN-Cyto *c+* in vivo (Pulmanusahakul et al. 2001). Furthermore, cytochrome *c* significantly elevated the VNA titers in immunized mice, and the mortality in mice infected intranasally with SPBN-Cyto *c+* was substantially lower than in mice infected with control virus (Pulmanusahakul et al. 2001). Therefore, RABV vectors expressing cytochrome *c* promise as a candidate for a live-attenuated rabies vaccine in the future.

### Cytokine/chemokine genes

Cytokines are a broad group of small proteins with molecular masses of 8–14 kDa that are important in cell signaling. They are produced by hemopoietic system, immune system, or activated cells in inflammatory responses and play pivotal roles in cellular proliferation and differentiation through autocrine or paracrine (Zlotnik and Yoshie 2000). Based on the structure and function, cytokines have been classified into chemokines, IFNs, interleukins, lymphokines, TNFs, growth factors, and colony-stimulating factors (CSFs).

It was found that although the street silver-haired bat RABV (SHBRV) strain was more pathogenic than the laboratory-attenuated CVS-B2c strain, SHBRV induced fewer inflammatory and host gene expression changes than the attenuated CVS-B2c strain (Yan et al. 2001). Microarray technique was used to investigate the host responses to RABV infection through comparing gene expression profiles in the brains of mice infected with CVS-B2c or SHBRV. Of the genes altered by CVS-B2c infection, genes involved in host immunity/antiviral responses and apoptosis were significantly upregulated in mice infected with CVS-B2c than those in

mice infected with SHBRV, implying that the host innate immune responses triggered by CVS-B2c infection might be responsible for the attenuated phenotype of CVS-B2c strain (Wang et al. 2005). Given that cytokine/chemokine expression has been well documented to be involved in CNS inflammation, host immune responses, and direct antiviral activities (Hosking and Lane 2010; Niu et al. 2011; Ubogu et al. 2006), cytokine/chemokine has been recognized as critical mediators of a variety of biological processes during viral infection (Hosking and Lane 2010).

Several studies have demonstrated that recombinant RABV expressing cytokine/chemokine genes, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), showed further attenuation of viral virulence and enhanced host innate and adaptive immunity through inducing the expression of innate immune molecules and VNAs, the infiltration of inflammatory cells into the CNS, and the enhancement of blood–brain barrier (BBB) permeability (Chai et al. 2014; Kuang et al. 2009; Wang et al. 2011; Zhao et al. 2009). The enhanced BBB permeability is critical for the entry of immune effectors into the CNS to clear the virus and prevent the development of rabies by allowing immune effectors to enter the CNS and clear RABV (Kuang et al. 2009; Niu et al. 2011). Recently, it was demonstrated that the ability of attenuated RABV strain to enhance BBB permeability was associated with the downregulation of the tight junction proteins in the CNS largely caused by virus-induced inflammatory cytokines/chemokines (Chai et al. 2014). Most importantly, expressing cytokines/chemokines did not cause apparent signs in adult mice inoculated intracerebrally with up to 10<sup>7</sup> fluorescent focus unit of viruses and could protect mice from infection as late as 5 days post-infection (Wang et al. 2011). Therefore, these recombinant RABV expressing cytokines/chemokines have obvious advantages over traditional RABV vaccines in its potential to be used in delayed rabies treatment.

It should be noted that although overexpression of cytokines/chemokines has important roles in controlling virus infection and RABV attenuation, certain cytokines/chemokines, such as RANTES or IP-10, when overexpressed, are detrimental (Zhao et al. 2009). It has been shown that when RANTES or IP-10 was overexpressed, the RABV pathogenicity in mice was increased owing to persistent and high-level expression of chemokines, extensive infiltration of inflammatory cells in the CNS, and severe enhancement of BBB permeability (Zhao et al. 2009). Therefore, much work should be performed in the future to comprehensively investigate the physiological effect of cytokines/chemokine expression before RABVs overexpressing cytokines/chemokines are to be developed to treat clinical rabies or other CNS diseases due to the complex responses triggered by cytokines/chemokines.

## Gene rearrangement

Gene expression of the nonsegmented negative-strand RNA viruses is primarily controlled at the transcriptional level by the order of genes relative to the single transcriptional promoter located at the 3' end of the genome (Abraham and Banerjee; Ball and White 1976), thus resulting in obligatorily sequential expression of the individual genes with gradual decrease in transcriptional abundance (Iverson and Rose 1981; Villarreal et al. 1976). This reduction in transcript yield is called transcription attenuation and is caused by the conserved intergenic junction separating adjacent genes. It has been estimated that approximately 25 to 30 % of the polymerase complex dissociates from the RNP as they cross each gene junction, leading to reduction of the downstream product accordingly (Iverson and Rose 1981). This is what is called stop/start model of transcription and is widely accepted for nonsegmented negative-strand RNA viruses (Whelan et al. 2004). This type of transcriptional regulation is a very simple and efficient method to achieve differential expression levels of gene products, which is suggested to directly reflect the relative protein amounts needed for completion of the viral life cycle (Whelan et al. 2004).

Therefore, rearranging the position of a specific gene relative to the single transcriptional promoter is expected to change the expression level of that gene accordingly, thus resulting in altered virus replication and immune responses triggered in the host. Consistently, it has been shown that translocation of the most promoter-proximal N gene to successive positions down the genome reduced N expression and genomic replication in a stepwise manner (Wertz et al. 1998). Besides, translocation of the N gene also decreased virus replication in cultured cells and virus lethality in mice without compromising their ability to elicit protective immunity, protect against virus challenge, and produce high levels of VNAs (Wertz et al. 1998). In another study, translocation of the G gene closer to the 3' promoter increased the G expression and elicited earlier and enhanced immune response in inoculated mice, and this phenomenon was even more pronounced when the N gene was rearranged to reduce N gene expression simultaneously (Flanagan et al. 2000). Taken together, these studies demonstrated that gene rearrangement can be an effective strategy to develop vaccines for nonsegmented negative-sense RNA viruses, such as RABV, and was especially advantageous given that gene rearrangement excluded the risk of introducing heterologous genes into the RABV genome.

## Outlook

Since the first successful recovery of infectious RABV from cDNA clones in 1994, the past two decades have witnessed tremendous advantages of reverse genetics on expanding our

understanding of the molecular biology of RABV as well as other negative-strand RNA viruses. At the same time, researchers have been continuously refining this powerful tool to improve the recovery efficiency and simplify the operation processes. Currently, a number of RABV strains have been reported to be rescued and reverse genetics has been widely used in studying the pathogenesis, genome organization, and gene function of RABV. Most importantly, reverse genetics has opened up a new window for utilizing recombinant RABV as novel viral vectors and vaccine delivery agents. A number of intrinsic features of RABV and other negative strand RNA viruses are ideal for gene delivery vectors for many applications. The simple and conserved makeup of RABV genomes allow high degree of amenability of these viruses to genetic engineering and express foreign genes. Moreover, the helical structure of RNP complex of RABV has rather flexible plasticity and could tolerate considerable genome size changes, thus allowing inserting relatively large fragments or multiple foreign genes. Importantly, the encapsidation of viral genome RNA into RNP complex possesses the advantage to significantly reduce the chance of recombination between viruses and maintain the genome stability. Indeed, it has been shown that the frequency of homologous recombination is rare, if any, in negative-strand RNA viruses, and therefore, those strategies using reverse genetics to manipulate RABV genome to alter its biological characteristics are theoretically highly stable and irreversible, meaning that the rearranged viruses were stable following their introduction into and replication in their natural hosts, and the risks of inter-genomic exchange with other RABV strains to restore virulence were effectively avoided.

One should highlight, though, that there remains many aspects on reverse genetics that should be improved before it would be widely used in rabies vaccine development. According to the transcription pattern of RABV, it seems that the best position for foreign gene expression would be the closest to the 3' end of the RABV genome for maximal expression, that is, in the junction of N and P genes. However, insertion of a foreign gene proximal to the promoter might interfere with virus replication more profoundly than in a promoter-distal position. Furthermore, it has been shown in VSV that not all positions of the VSV genome were appropriate for foreign gene insertion and that expression of a foreign gene inserted between N and P genes would be quickly suppressed within two passages (Wertz et al. 2002). In other study using the Newcastle disease virus, it was shown that although the mRNA level of a green fluorescence protein gene decreased gradually as the gene was inserted gradually distal from the 3' end of the virus genome, the green fluorescence protein fluorescence intensity in infected cells was most abundant when the gene was inserted between the noncoding region of the P and M gene, suggesting that the noncoding region between the P and M genes was the optimal insertion site for foreign gene



expression compared to other positions, including the promoter–proximal position (Zhao et al. 2014). Furthermore, cautions should be taken about the high frequency of reversion during virus passage in infected cell due to low fidelity of viral RNA polymerase, arguing the need for multiple alterations to be introduced simultaneously. Last but not the least, although the frequency of homologous recombination was extremely rare in RNA viruses, recent studies have clearly shown that it indeed happens in negative-sense RNA viruses (Chare et al. 2003; Han and Worobey 2011; Liu et al. 2011), raising the risk that recombination might occur between attenuated viruses with wild-type street virus. Therefore, considerable efforts should be exerted to better manipulate RABV genome through reverse genetics for vaccine production and expression of interested gene products, and the balance between satisfactory levels of bio-safety and appropriate immunogenicity would also require systemic investigation, strategic planning, and careful designing to orchestrate the development of future rabies vaccines.

Live-attenuated viruses have proven to be effective vaccines against many diseases, such as smallpox, yellow fever, and measles, and live-attenuated viruses are at some circumstances more effective than inactivated viruses in triggering host immune response. Due to high mortality of rabies, live-attenuated RABV strain has been only approved for animal use. However, after systematically manipulating the RABV genome using reverse genetic technique, it would be desirable to obtain highly attenuated live RABV strains with elevated immunogenicity which promise to provide a novel approach for development of vaccines against rabies as well as other numerous problematic viral pathogens.

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