# Molecular Basis of Pathogenicity of a Rabies Virus Street Strain

（狂犬病ウイルス街上毒の病原性の分子基盤に関する研究）

Rabies is a zoonotic infectious disease caused by the Rabies virus (RABV), a member of the genus *Lyssavirus* in the family *Rhabdoviridae* of the order *Mononegavirales*, characterized by fatal neurological symptoms. Despite being one of the oldest known infectious diseases, the pathogenic mechanisms of RABV have not yet been fully elucidated. While the disease is preventable through vaccination, no effective treatment has been established once symptoms appear, and the mortality rate for infected humans and animals is nearly 100%. Even today, rabies is distributed worldwide except in a limited number of rabies-free countries and regions. It is estimated that approximately 59,000 people die from rabies annually, primarily in developing countries in Asia and Africa. Thus, rabies outbreaks remain a critical public health issue in the international community, making the establishment of new therapeutic strategies and more effective preventive measures essential for its control.

The pathogenicity of RABV is defined by two primary abilities: “neuroinvasiveness,” the capacity of the virus to reach the central nervous system (CNS) from peripheral infected sites, and “neurovirulence,” the capacity to spread within the CNS and induce neurological symptoms. To date, most studies on RABV pathogenicity have focused on neurovirulence, while knowledge regarding neuroinvasiveness—specifically the role of viral replication in muscle tissue—remains scarce. A major reason for this is that conventional RABV research has primarily utilized fixed strains. RABV strains are classified into two groups: street strains, which are field isolates obtained from humans or animals with rabies, and fixed strains, which include laboratory-adapted and vaccine strains. Fixed strains, through serial passage in cells or animal tissues, exhibit a shorter incubation period and uniform pathogenesis compared to street strains; however, they also show distinct characteristics, such as

significantly attenuated neuroinvasiveness. Therefore, findings obtained from research using fixed strains do not necessarily reflect the pathogenicity of street strains. Consequently, research using authentic street strains is extremely important for clarifying the pathogenesis of rabies in natural infections, particularly the mechanism of entry from the periphery into the CNS. To address this issue, this study aimed to elucidate the molecular basis of pathogenicity of a RABV street strain, focusing on infectivity in muscle cells (myotropism) and neuroinvasiveness.

In Chapter I, to analyze the virological characteristics of the Toyohashi strain—a street strain isolated in 2020 from an imported human rabies case in Japan following a dog bite in the Philippines—a reverse genetics (RG) system was established. Infectious virus was successfully rescued from full-length complementary DNA derived from the viral genome. Although there were concerns regarding phenotypic changes due to cell-adaptive mutations during viral recovery in cultured cells, the reconstituted recombinant Toyohashi strain (rToyo) exhibited growth kinetics comparable to the parental strain. Furthermore, in infection experiments using mice, rToyo maintained characteristics typical of street strains, showing higher neuroinvasiveness and an inconsistent incubation period approximately seven days longer than the fixed Challenge Virus Standard (CVS) strain. Next, to analyze the intracellular dynamics of Negri body-like structures, which function as sites for RABV transcription and replication, a recombinant virus expressing a fluorescent mCherry protein fused to the *P* gene (rToyo-P-mCherry) was generated. Live-cell imaging using time-lapse microscopy successfully visualized the formation and chronological accumulation of mCherry-positive Negri body-like structures within the cytoplasm of infected cells. These results demonstrate that the RG system established in this study is a valuable tool for clarifying the molecular basis of RABV street strain replication and pathogenesis.

In Chapter II, the RG system established in Chapter I was utilized to identify viral factors and analyze the mechanisms regulating the myotropism and neuroinvasiveness of the Toyohashi strain. First, myotropism and neuroinvasiveness were compared between the street Toyohashi strain and the fixed CVS strain. Evaluation of viral growth using myoblast cell lines and mouse hindlimb muscle tissue, along with survival analysis of mice following intramuscular inoculation, showed that the Toyohashi strain exhibited more efficient replication in myoblasts, higher infectivity in mouse hindlimb muscle, and a higher mortality rate than the CVS strain following intramuscular inoculation. These results confirmed that the street Toyohashi strain possesses higher myotropism and neuroinvasiveness than the fixed CVS strain. To identify the specific RABV genes contributing to this phenotype, the RG system was used to generate recombinant chimeric viruses by swapping each structural gene (*N*, *P*, *M*, *G*, and *L*) between the Toyohashi and CVS strains. *In vitro* and *in vivo* evaluations revealed that introducing the CVS-derived *G* or *L* gene into the Toyohashi backbone reduced myotropism and significantly attenuated pathogenicity after peripheral infection. Conversely, introducing the Toyohashi-derived *G* and *L* genes into the CVS backbone enhanced myotropism and increased mortality rate following intramuscular inoculation. Thus, the *G* and *L* genes were identified as critical factors regulating myotropism and neuroinvasiveness in the Toyohashi strain. Furthermore, functional analysis using pseudotyped viruses showed that the Toyohashi G protein facilitates viral entry into muscle cells more effectively than the CVS G protein. Additionally, minigenome assays revealed that the Toyohashi L protein promoted higher viral genome replication and transcription efficiency in infected muscle cells than the CVS L protein. These findings suggest that the Toyohashi strain achieves efficient proliferation in muscle cells through the coordinated action of G protein-mediated entry and L protein-mediated replication/transcription, potentially resulting in high neuroinvasiveness.

This study established an RG system that enables detailed virological analysis of the clinically isolated street Toyohashi strain and provided new insights into the molecular basis of myotropism and neuroinvasiveness in street strains. The results strongly suggest the importance of using street strains to accurately understand the RABV infection process under natural conditions. These findings are expected to lead to the elucidation of universal molecular mechanisms among street RABV strains and the development of new therapeutic and preventive methods targeting viral entry and replication processes.