Journal of
Antivirals & Antiretrovirals
ISSN: 1948-5964

Silver et al., J Antivir Antiretrovir 2013, 5:3
DOI: 10.4172/jaa.1000065

Review Article Open Access

Newly Developed Approaches for Studying Pathogenesis of Varicella Zoster Virus

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Abstract

Varicella-zoster virus (VZV) is a herpesvirus that is the causative agent of chickenpox and herpes zoster. The virus manifests as chickenpox upon primary infection, establishes latency in the sensory neurons, and can later reactivate as herpes zoster when the immune system is compromised. Although the v-Oka vaccine has been introduced to most children worldwide, the virus remains neurovirulent and is thus still able to reactivate and cause herpes zoster. Knowledge about VZV pathogenesis has been limited due to its highly cell-associated nature in culture, difficulty in generating recombinant viruses, and obstacles in *in vivo* viral pathogenesis studies. However, a combination of new approaches has made it possible to functionally profile the entire VZV genome, screen for tissue tropic factors, and analyze recombinant virus replication and pathogenesis *in vivo*. These approaches include the bacterial artificial chromosome technology for generating recombinant VZV, the humanized mouse model for studying viral tropism and pathogenesis *in vivo*, and a bioluminescence marker for monitoring viral growth in all models. From these recombinant virus studies, open reading frame 7 of the VZV genome was revealed to be a novel skin- and neurotropic factor. In addition, studies of the closely related Simian Varicella Virus and other VZV pathogenic studies in conjunction with genetic analyses of the entire VZV genome will not only lead to a better understanding of VZV pathogenesis, but also contribute to the development of a safer, more effective neuroattenuated vaccine candidate.

Keywords: Varicella-zoster virus; Herpesvirus; Bacterial Artificial chromosome; Bioluminescent imaging; Mutagenesis

Introduction

Varicella-zoster virus (VZV) is a member of the human herpesvirus family, a virus to which the majority of the world's population has been exposed [1]. There are eight viruses within the human herpesvirus family divided into three subfamilies: α, β and γ human herpesvirus. The α-human herpesvirus subfamily is comprised of herpes simplex virus-1 (HSV-1) and -2 (HSV-2), which are responsible for oral and genital herpes, and VZV, the causal agent of chickenpox upon primary infection [2,3]. Chickenpox is typically a mild and self-resolving disease that manifests most notably as itchy, red pockmarks covering the entire body [4]. During the active infection, VZV will enter the bloodstream, reaching the sensory neurons among other sites, and remain latent for life unless reactivation occurs due to a weakened immune system. Reactivation of this virus results in herpes zoster (HZ), also known as shingles, where VZV erupts through the sensory nerves and causes belt-like rashes on the thoracic, cranial or lumbosacral dermatomes. Herpes zoster can lead to severe nerve damage that lasts long after the herpetic rash resolves, a condition called post-herpetic neuralgia (PHN) [2,5,6]. PHN is a serious sequela that not only greatly compromises quality of life, but also requires a costly amount of health care treatments which can be detrimental to patient health [7,8]. For the elderly and sick, the effects of herpes zoster are existing and troublesome realities.

Over 90% of the US population has been exposed to VZV [9]. However, the number of hospitalizations and deaths caused by chickenpox has decreased dramatically since 1995 after the US mandated the childhood anti-VZV vaccination. The current vaccine strain for VZV is known as v-Oka, and was developed by passaging its parental strain p-Oka, a Japanese clinical isolate, through human embryonic cells and semi-permissive guinea pig fibroblasts where the virus underwent numerous spontaneous mutations to reduce virulence [10]. Therefore, v-Oka is a mixture of numerous genotypically distinct viruses [11]. The v-Oka strain is very effective in preventing chickenpox, providing 90% protection from the disease. Nevertheless, the skin attenuated virus remains neurovirulent and is thus able to establish latency in the vaccinated population [9,12,13]. The vaccine currently used for protection against shingles is comprised of this same v-Oka strain, except at a titer 14 times that of the chickenpox vaccine [9]. Due to VZV's cell-associated nature, whole infected cell lysate must be used for vaccine production.

However, the production yield of the shingles vaccine is relatively low and requires numerous resources to produce this high titer virus. Therefore, the current standard for vaccinating against shingles is inadequate to supply the population with immunity to the debilitating disease [9]. The v-Oka vaccine may also indirectly increase the incidence of shingles. This is because the decreasing rate of natural VZV infections, a direct result of the varicella vaccine, limits the number of subsequent exposures that would have naturally boosted one's immunity [4,9,14-16]. Moreover, debates persist over the protective attributes of the shingles vaccine, as the vaccine has been reported to only reduce the risk of incidence by 51% [17]. Most importantly, the v-Oka vaccine establishes latency in the sensory nerves, which raises concerns for its potential for reactivation [9,11-13]. For these reasons, it is imperative that studies focus on VZV gene function and viral pathogenesis in order to produce a safer, neuroattenuated vaccine strain that can be ubiquitously supplied without constraints as seen with the v-Oka vaccine.

VZV contains a relatively large genome, consisting of 125-kb

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Received March 20, 2013; **Accepted** May 16, 2013; **Published** May 18, 2013

Citation: Silver B, Lin V, Zhu H (2013) Newly Developed Approaches for Studying Pathogenesis of Varicella Zoster Virus. J Antivir Antiretrovir 5: 062-071. doi:10.4172/jaa.1000065

double-stranded DNA that encodes 70 unique ORFs (Figure 5). The genome is divided into a 100-kb unique long (U_L) that is bounded by terminal long (TRL) and internal long (IRL) repeats, and a 5.4-kb unique short (U_S) regions that is bounded by internal short (IRS) and terminal short (TRS) regions (Figure 1A) [18]. Obstacles in studying VZV include its large genome size, high cell-associativity in culture, extremely narrow host range, and marked differences between *in vivo* and *in vitro* cell replication [18]. Until recently, there were no viable genetic methods to efficiently generate mutant strains and no animal models for *in vivo* testing. Advancements in virological research, including the development of the bacterial artificial chromosome, have circumvented these problems and allowed for more efficient generation of VZV ORF deletion mutants that allow for the study of factors required for viral invasion of and egress from specific tissues during the course of natural infection [2,10]. In addition, the Simian Varicella Virus (SVV) and VZV are closely related alphaherpesviruses with more than 70% DNA homology [19]. Based on the similarities of SVV and VZV pathologies, studying SVV in monkeys serves as a suitable model for investigating how VZV behaves in humans. Continuing studies of individual ORFs in VZV, and also in Simian Varicella Virus (SVV) homologs, will further elucidate VZV pathogenesis and allow for the development of a novel, efficacious vaccine against both disease manifestations of VZV.

Bacterial Artificial Chromosomes

Previous methods of creating recombinant VZV deletion mutants utilized the four-cosmid system, in which VZV DNA was split into four overlapping segments called cosmids. In this system, one cosmid would be made to contain the desired mutation, and all four segments are then co-transfected into human cells to produce the recombinant VZV variant upon multiple homologous recombination events [20,21]. This advancement has also been utilized in the creation of SVV mutants by the cosmid-system approach within Vero cells [22]. This cosmid based method, however, was deemed inefficient as it required co-transfection of four large cosmids into human cells and multiple successful homologous recombination events within a single cell to create the full-length viral genome [18]. Fortunately, building upon the overlapping cosmid system, the bacterial artificial chromosome (BAC) technology yielded a new and more efficient method for generating recombinant viral mutants. Most vectors are adopted from plasmids, small circular DNA separate from their genomes. However, the genomes of the herpesviruses are far larger than the carrying capacity of a plasmid. The BAC, however, is a vector used to carry a large piece of DNA as a bacterial chromosome within bacteria. BACs were developed to hold larger pieces of DNA up to 350 kb, and are therefore suitable for studying herpesviruses [23], which range from 100 kb to 250 kb. In addition, BACs are very useful due to their low copy number and the ease of producing mutants with the advantage of harboring them in modified *E. coli* with λ prophage homologous recombination machinery [24]. Insertion of a BAC vector into one of the genomic cosmids via homologous recombination is the key step that allows for the rapid creation of recombinant viruses that can be genetically manipulated in *E. coli*. After insertion of the BAC vector into one of the cosmids, the overlapping cosmids are cotransfected into mammalian cells, as previously mentioned, to produce the full-length viral genome.

The viral BAC must contain an antibiotic resistance marker for selection and maintenance of bacterial colonies containing the BAC, as well as a selectable marker, such as green fluorescent protein (GFP), for visualization and purification of recombinant viruses in infected human cells. The BAC vector must also be flanked on both sides by 500-1000 bp sequences homologous to the viral genome where the BAC will be inserted in order to facilitate homologous recombination. Lastly, two loxP sites are usually at the ends of the BAC sequence to excise out the BAC vector, thereby removing almost all foreign DNA once the recombinant viruses are generated [23,25]. The BAC system holds many advantages over the cosmid system, allowing for simple manipulation of the viral genome and efficient isolation of recombinant viruses [26-29]. The genetic manipulation of large viral genomes without the requirement for *in vitro* replication in eukaryotic cells is

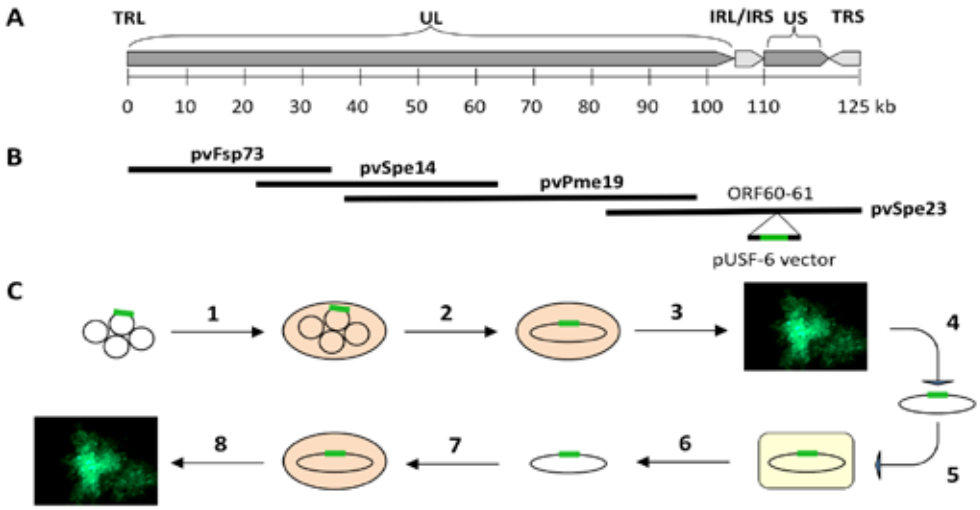

Figure 1: Construction of VZV BAC. **A.** Schematic diagram of the VZV p-Oka genome. Image adapted from Zhang et al. (2007). **B.** Four cosmids containing overlapping VZV genomic segments are shown. A BAC vector (pUSF-6, shown in green) was inserted between ORF60 and ORF61 in VZV cosmid pvSpe23 by homologous recombination. Image adapted from Zhang et al. (2007). **C.** All four VZV cosmids were transfected into MeWo cells (1), and upon multiple successful homologous recombination events (2), the resulting recombinant virus was able to replicate and produced a green fluorescent plaque (3). The VZV BAC DNA was extracted from the cells (4) and transformed into electrocompetent DY380 *E. coli* in the presence of chloramphenicol (5). The infectivity of the recombinant BAC is tested by extraction from the *E. coli* (6) and transfecting it into human MeWo cells (7). The VZV BAC produced GFP-expressing virus that formed plaques (8).

a certain advancement realized by the increasing number of individual ORF deletion mutant studies in a number of herpesviruses that have been cloned into viral BACs.

Generation of the VZV BAC

A commonly used method for creating herpesvirus BACs involves co-transfection of the BAC vector and viral genomic DNA into permissive human cells and then insertion of the BAC vector into the viral genome via homologous recombination. This method, however, is hardly used with VZV due to its highly cell-associated nature. The virus is confined to within a living host cell and lacks cell-free viral particles, making isolation of the viral genome difficult. Therefore, alternative strategies had to be adopted, and the BAC technology was utilized to generate recombinant VZV. The BAC vector containing the origin of replication and selectable markers is inserted in one of the four cosmids (Figure 1B) [23]. The overlaps between cosmids allow for homologous recombination between the segments (Figure 1C) and formation of a single, circular DNA sequence that encompasses a full-length infectious VZV genome. Viral replication and plaque formation can be visualized due to the GFP marker in the BAC vector. The BAC DNA containing the VZV genome in the infected cells is then purified and transformed into *E. coli* by electroporation. Antibiotic-resistant colonies are then selected and the desired VZV BAC DNA is isolated. The integrity of the VZV BAC is verified by restriction enzyme digestion and gel electrophoresis for parallel comparison.

Similar approaches have been used in creation of other herpesvirus BACs, notably the creation of the SVV and the Human Cytomegalovirus (HCMV) BACs, and have exhibited the utility of the BAC for a range of herpesviruses with differing genome sizes [19,30]. Furthermore, the ability to generate viral deletion mutants via VZV and SVV BAC methodologies has facilitated the investigation of the role of specific genes during acute infection, latency, and viral reactivation. These advances will provide new opportunities for studying viral pathology in a focused and purposeful manner with the capability of producing new antiviral and vaccine strategies for VZV.

Bioluminescent imaging

A principle method for studying viral pathogenesis has been to observe viral infection by monitoring cellular pathological effects (CPE) in infected cells under a light microscope. However, with new advents in the virology, it is now possible to exclusively focus on cells that contain the replicated virus by inserting certain features into the viral genome. For instance, when the GFP gene is inserted into the viral genome via the BAC vector, visualization of *in vitro* infections under a fluorescence microscope becomes clearer [31,32]. Without GFP visualization, it is markedly more difficult to note CPE where cells fuse together as infectious centers. These are often crowded areas where the demarcated borders of individual cells are difficult to distinguish. Therefore, GFP is readily preferred over traditional CPE observation in early VZV infection when plaques have not clearly formed and when determining the presence of polykaryons (syncytium). However, it is near impossible to quantify the amount of GFP expressed to accurately analyze the growth kinetics of viruses, especially when an animal model is used. For this reason, bioluminescent imaging (BLI) has become a powerful tool for studying herpesviruses *in vivo* and *in vitro* [33,34]. BLI is a novel method that enables the monitoring and measurement of viral replication in live cells, tissues and animals. The method involves the use of reporter genes, such as luciferase, that are inserted into the viral genome and expressed only as viral proteins are expressed during replication [35,36]. When exposed to the D-luciferin substrate, luciferase will catalyze the substrate's bioluminescent oxidation, thus producing light whose intensity is dependent on the amount of luciferase present [35,37].

BLI has several advantages over other imaging systems such as fluorescent-based imaging. Firstly, BLI is especially effective for *in vivo* applications because D-luciferin can rapidly permeate the tissues of a living animal, allowing the substrate to reach any site of infection in the body, and therefore poses no limits for *in vivo* applications. Additionally, in comparison to GFP, the shorter wavelength of the photons emitted from the luciferase-luciferin reaction allows the light to penetrate tissues and return to the detection instrument for accurate quantification. D-luciferin also has a low toxicity that allows the substrate to be used repeatedly in the same animal [33,34]. Above all else, the main advantage of BLI over other methods is that it can monitor viral growth and provide real-time detection of spatial and temporal growth of viruses *in vivo*. This makes quantification of viral growth *in vivo* possible, an attribute necessary for analyzing recombinant viral growth kinetics and tissue tropism [38-41].

Use of bioluminescence in VZV studies

As previously discussed, the VZV BAC is a step forward in cloning and genetic manipulation of the VZV genome. Coupling VZV with BLI by adding a luciferase gene into viral DNA offers even more advantages for understanding VZV pathogenesis. A luciferase expressing virus can be used to detect areas of viral infection and, more importantly, study the growth kinetics of recombinant mutated viruses and interpret the resulting data to identify tissue tropic genes. Furthermore, using a bioluminescent-capable VZV facilitates studies of anti-VZV compounds, VZV pathogenesis and mutational analyses.

Generation of a VZV_{LUC} BAC

A firefly luciferase expression cassette was inserted between ORF 65 and 66 of VZV BAC to generate VZV_{LUC} BAC (Figure 2A) [10]. This DNA was transfected into human cells to create the VZV_{LUC} virus upon replication. Growth curve analysis shows that VZV_{LUC} grows like its parent VZV p-Oka. After two days, the cell culture media is replaced by media containing D-luciferin, and bioluminescent signals are visualized using the *In Vivo* Imaging System (IVIS) (Perkin Elmer) [42]. Bioluminescence is only detected in VZV_{LUC}-infected cells when the D-luciferin substrate is added to the cell culture media (Figure 2B). The IVIS instrument's ability to detect bioluminescence activity *in vivo* is shown in Figure 2C, with an example panel of a viral growth analysis in a VZV mouse model. For the bioluminescence assay to be used as an accurate estimate of viral titer in growth kinetic analyses, it was imperative to confirm a tight correlation between bioluminescence activity and viral growth kinetics. Using the traditional plaque assay as the standard in determining viral titer, the bioluminescence assay proved to be a valid method for studying viral growth kinetics (Figure 2D). Overall, the VZV_{LUC} BAC has made it possible to generate VZV ORF deletion mutants within bacteria, produce the virus in human cells and carry out growth kinetic studies using bioluminescence assay.

BAC Mutagenesis

Many aspects of the VZV genome are not well understood. Initially, very little was known about the role of various genes in VZV pathogenesis, and still less than 50% of the genome has been functionally profiled [10]. New technological advances and combinational approaches, as discussed earlier, have allowed for analysis of genes

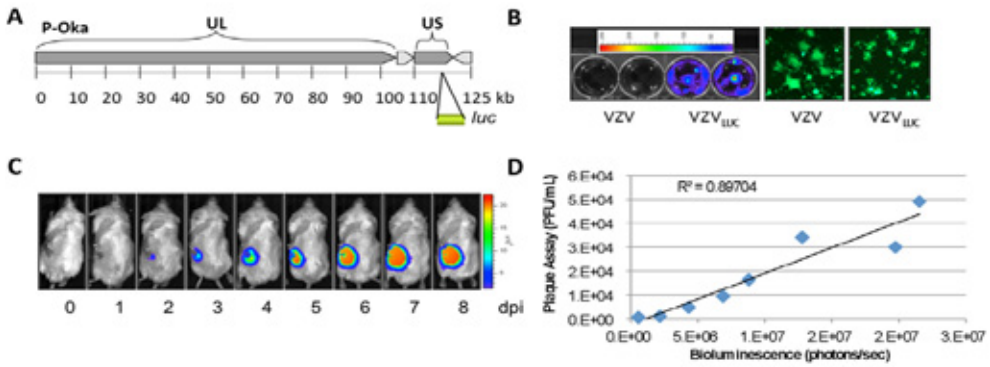

Figure 2: Construction of recombinant VZV containing a luciferase gene (VZV$_{LUC}$) **A.** To generate a VZV$_{LUC}$ strain, a luciferase expression cassette was inserted into the intergenic region between ORF65 and ORF66 of the VZV$_{LUC}$ genome. This clone was then transfected into MeWo cells to produce the VZV$_{LUC}$ strain. **B.** MeWo cells were grown in six-well culture dishes and infected with VZV BAC or VZV$_{LUC}$ BAC. Bioluminescence from VZV$_{LUC}$ infected cells was then visualized and measured using IVIS (Xenogen) imaging. Strong bioluminescence was detected only from VZV$_{LUC}$ -infected cells. In addition, green fluorescent plaques were observed for all wells using fluorescence microscopy, indicating that both viruses were infectious. **C.** Example of *in vivo* IVIS imaging for growth curve analysis in a mouse VZV model. Image adapted from Zhang et al. (2007). **D.** Correlation between traditional viral plaque assay and luciferase-based IVIS bioluminescence growth analyses for accurate estimation of viral titer.

essential for VZV replication on a global scale and provide necessary insight into VZV pathogenesis. By characterizing individual ORF functions, the genes responsible for VZV tissue tropism and other imperative pathogenic factors can be discovered.

Homologous recombination-mediated BAC mutation in *E. coli*

As previously mentioned, BAC technology has allowed for easy manipulation of VZV since the BAC is harbored within bacteria that possess efficient homologous recombination machinery. The DY380 *E. coli* strain is used for recombinant BAC production because it possesses a highly regulated λ-prophage homologous recombination system that allows for recombination between homologies at an efficient length of 40-bp [43,44]. The system is regulated by a temperature-sensitive cI repressor, in which the recombinase can be induced when incubated at 42°C for 15 minutes [44] and will not sporadically cause recombinatory events without first being activated.

Generation of VZV deletion mutants using drug-based selection system

In order to generate a deletion mutant clone (ORFX Δ), DY380 *E. coli* carrying the VZV$_{LUC}$ BAC must undergo E.C. cell preparation and recombination system activation. A PCR generated antibiotic resistance marker cassette will replace the ORF of interest upon homologous recombination, generating the VZV$_{LUC}$ ORFX Δ mutant BAC clone. To verify the successful generation of a VZV ORF deletion mutant clone [44], the clone is checked for antibiotic sensitivity, PCR of the region containing the deletion, and restriction endonuclease digestion. The mutant BAC DNAs is extracted and chemically transfected into human cells until the infectious virus is observed. The growth kinetics of the mutant virus can be measured using the IVIS system for growth curve analysis in comparison to WT VZV$_{LUC}$ to study characteristic functions of specific ORFs (Figure 3A).

Generation of VZV rescue/mutant clones in a drug-based selection system

VZV ORF deletion rescue clones are generated in order to demonstrate that any mutations in the mutant clones are caused solely by the removed DNA and not by other factors. Thus, the viral rescue clones are fully restored forms of the WT virus. On the contrary, further mutational studies can also be performed by replacing the drug selection marker with a mutated form of the ORF. To generate either a rescue clone or mutant clone for the drug-based mutagenesis, a plasmid containing a different antibiotic resistant gene is flanked by loxP sites and used to clone either the ORFX (for rescue) or ORFY (for introduction of mutated ORFX or a new gene). Upon electroporation and homologous recombination, a VZV$_{LUC}$ ORFX/Y rescue BAC is generated. Furthermore, when cre recombinase is transformed, the antibiotic marker is excised, leaving a negligible amount of foreign DNA remaining in the BAC. After PCR verification and extraction of the rescue BAC via maxipreparation, the ORFX rescue/ORFY mutant VZV BAC DNA is chemically transfected into human cells. The virus is then grown and analyzed for comparison to WT VZV$_{LUC}$ (Figure 3B).

Generation of VZV deletion mutant using *galK* (galactokinase)-based selection system

Although only a small amount of foreign DNA remains on the rescue virus created by the antibiotic selection system, a vaccine produced by this method would not be an acceptable candidate. Therefore, an alternate method for generating VZV mutation clones and the corresponding rescue viruses is the *galK*-based mutagenesis selection/counter selection system. The *galK*-based mutagenesis also relies on the homologous recombination system mentioned above, but this system possesses an advantage over the antibiotic selection system in that no foreign DNA will remain in the rescue virus. The *E. coli* SW102 strain is used because the *galK* gene of the galactose operon in these bacteria is defective. As a result, the strain cannot survive in a medium where galactose is the only source of carbon. This system supplies the *galK* gene *in trans* (within the BAC) so that the bacteria will only grow when a homologous recombination event replaces ORFX with *galK* [24,44,45]. The procedure to generate a deletion mutant via this selection process is nearly identical to the antibiotic-based system from Generation of VZV Deletion Mutants using Drug-based Selection System, differing only in the *E. coli* strain and growth medium used, and consequently, the selection and verification process as well (Figure 4A).

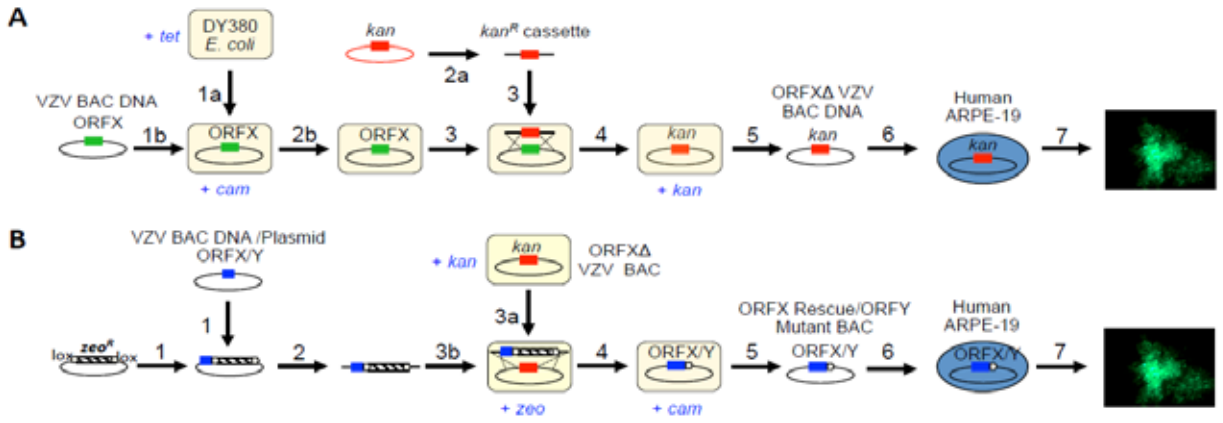

Figure 3: Generating recombined VZV ORF deletion mutant and rescue/mutant generation viruses by drug-based mutagenesis system. A. Generation of ORF deletion mutant virus:

DY380 are made to be electrocompetent (E.C.) (1a) for VZV BAC DNA transformation (1b). Next, a kanamycin resistant gene is PCR amplified by primers containing 40-bp sequences homologous to the flanking regions of the gene or region of interest (ORFX) in VZV to create the kanamycin resistance cassette (2a). In order to generate a deletion mutant clone (ORFXΔ VZV), DY380 *E. coli* carrying WT VZV BAC undergo E.C. cell preparation with recombinase system activated (2b). The kanamycin resistance cassette is then transformed into the E.C. DY380 harboring WT VZV BAC (3). Upon homologous recombination, ORFX will be replaced by the kanamycin resistant gene, generating the VZV ORFXΔ mutant BAC clone (4). The deletion clone is then verified by PCR and sensitivity to antibiotics, and extracted via BAC Maxipreparation (5).The mutant BAC is then chemically transfected into human ARPE-19 cells (6) and grown for mutant analysis (7). B. Generation of ORF rescue/mutant VZV virus. A plasmid containing a zeocin resistant gene flanked by loxP sites is used for cloning of either ORFX (for rescue) or ORFY (for introduction of new gene or mutated ORFX) (1). The ORFX/Y flanked by the zeocin resistant gene is PCR amplified (2), conferring 40-bp homology to the regions flanking the kanamycin resistance gene in the VZV ORFXΔ mutant BAC. DY380 harboring VZV ORFXΔ mutant BAC are made electrocompetent and its recombination system activated (3a) for transformation of rescue cassette and cre recombinase (3b). Upon homologous recombination (4), a VZV ORFX/Y rescue BAC is generated. Furthermore, when cre recombinase is transformed, the zeocin marker is excised, leaving little foreign DNA remaining in the BAC. After PCR verification and extraction of the rescue BAC via Maxipreparation (5), the ORFX Rescue/ORFY Mutant BAC DNA is chemically transfected into human ARPE-19 cells (6). The virus that results is then grown and analyzed for comparison to WT VZV (7).

*Images adapted from Dulal et al. (2012).

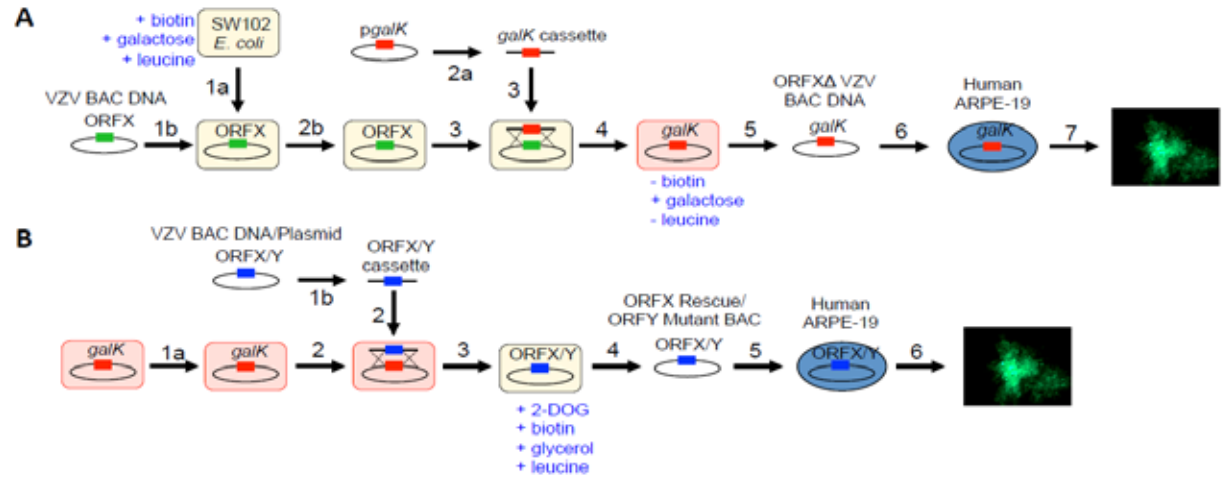

Figure 4: VZV mutational analysis and rescue/mutant generation by *galK*-based mutagenesis system. A. Generation of ORF deletion mutant virus: SW102 *E. coli* are made to be electrocompetent (E.C.) (1a) for VZV BAC DNA transformation (1b). Next, a *galK* gene is PCR amplified by primers containing 40-bp sequences homologous to the flanking regions of the gene or region of interest (ORFX) in VZV to create the *galK* cassette (2a). In order to generate a deletion mutant clone (ORFXΔ), SW102 *E. coli* carrying WT VZV BAC undergo E.C. cell preparation with recombinase system activated (2b). The *galK* cassette is then electroporated into the E.C. SW102

harboring WT VZV BAC (3). Upon homologous recombination, ORFX will be replaced by the *galK* gene, generating the VZV ORFXΔ mutant BAC clone (4). The deletion clone is then verified by PCR and sensitivity to antibiotics, and extracted via BAC Maxipreparation (5). The mutant BAC is then chemically transfected into human ARPE-19 cells (6) and grown for mutant analysis (7). B. Generation of ORF rescue/mutant VZV virus: SW102 *E. coli* harboring VZV_{LUC} ORFXΔ mutant BAC is made electrocompetent and recombination system activated (1a). ORFX (for rescue generation) or ORFY (any other gene, or mutated ORFX) is PCR amplified with primers conferring homology to flanking regions in ORFXΔ mutant BAC (1b), so that upon transformation (2) and homologous recombination (3), the ORFX/Y cassette will replace the *galK* gene in the ORFXΔ mutant BAC. After PCR verification, growth property confirmation, and extraction of the rescue/mutant BAC via Maxi preparation (4), the ORFX Rescue/ORFY Mutant BAC is chemically transfected into human ARPE-19 cells (5). The virus that results is then grown and analyzed for comparison to WT VZV (6).

*Images adapted from Dulal et al. (2012).

Generation of VZV rescue/mutant clones in *galK*-based counter selection system

The method of producing a rescue/mutant clone in the *galk*-based system is procedurally the same as with the antibiotic selection system (Generation of VZV Rescue/Mutant Clones in a Drug-based Selection System) with the small difference being the *E. coli* strain used and therefore growth medium used, and the lack of an antibiotic selection marker inserted along with the WT or mutated ORF. When the *galK* is removed from the BAC and replaced with either the WT ORF (for rescue analysis) or a mutated form of the viral gene (for further mutational analysis) by homologous recombination, any remaining clones containing *galK* are counter selected for by growing the bacteria on a medium that is toxic to bacteria containing a functional *galK*. This method of counter selection has a key advantage over the antibiotic selection rescue system, where a second antibiotic marker (zeocin in the previous case) is used to confirm the rescued or mutated ORF replacing the initial antibiotic marker. Although this second antibiotic marker can be excised due to the presence of loxP sites, a small amount of foreign DNA still remains, whereas the *galK* counter selection system leaves no foreign DNA present in the BAC (Figure 4B).

Xenotransplantation of Human Tissue

Many obstacles exist toward gaining knowledge concerning VZV pathogenesis and virulence within living hosts because VZV cannot replicate in any animal with the exception of humans [46]. To counter this issue, a small animal model using human tissue xenografts has been developed to provide a suitable *in vivo* model for studying VZV [46]. This model uses human tissue such as skin, thymus/liver and dorsal root ganglion (DRG). The tissue xenografts are implanted under the kidney capsule of severely combined immunodeficient (SCID) mice. After the tissue becomes vascularized and viable, the xenograft can be inoculated with different strains of VZV_{LUC} and analyzed via the IVIS system for *in vivo* studies [47-49]. These SCID mice xenotransplanted with human fetal tissue are known as humanized SCID mice, or SCID-hu mice.

With the use of photon detecting devices, viral infection can be observed and quantified within living animals. Usually, small animals are preferred; the more tissue between the site of bioluminescence and the site of detection, the more photons are scattered causing the signal to be lost. Dark fur or heavily pigmented skin can also present issues because it may block the optical signal. Thus, SCID mice are the ideal candidates for animal model testing with BLI due to their small size and light pigmentation. More importantly, their lack of an immune system eliminates the possibility of immune-based xenograft rejection.

The SCID-hu mouse model and IVIS enable researchers to monitor the continuous *in vivo* spread of viral infection over a sustained period of time. This is much more practical than the previous practice that required sacrificing multiple animals for each virus studied at each time point in order to analyze infected tissues and perform plaque assays [50-52]. The use of SCID-hu mice has shed light on VZV infection and pathogenesis *in vivo*, using human thymus xenografts to study immunity and pathogeneses, skin xenografts to study skin tropism, and human DRG xenografts to study neurotropism.

Profiling the VZV Genome

Functional analysis of each ORF can be performed by mutational analysis of deletion mutants. PCR methods are employed to replace each ORF with a selectable marker via homologous recombination in bacteria and the resulting BAC clone can be transfected into human cells for virus propagation and mutational analysis. Despite having the smallest genome of the eight herpesviruses, not even half of VZV's ORFs have been characterized previously [10]. Creating deletion mutants using the aforementioned methods has allowed for a functional study of VZV's 70 unique ORFs in MeWo cells and skin organ culture (SOC) [53]. Understanding the roles and essentiality of each ORF is the first step to identify VZV tissue tropic factors, which will contribute to the development of a skin- and neuro-attenuated vaccine.

The function of each ORF is determined by transfecting ARPE-19 cells with mutant DNA and observing viral replication and plaque growth. Through the systematic deletion of each ORF and the transfection of mutants into ARPE-19 cells, the essentiality of each ORF can be determined. If the ORF is nonessential, a viral plaque will be observable within 3-5 days post-infection. A viral plaque that is much smaller than that of a WT infection suggests that the respective ORF strongly influences growth. If no plaque formation is observed, the ORF is likely to be essential for viral replication. A color-coded map of the VZV genome is presented in Figure 5 to represent the results of the global scale mutational analysis of the individual ORF essentiality for viral replication [53].

Tissue Tropic Factors of VZV and the Search for a Neuroattenuated Vaccine

A previous global scale mutational analysis indicated that of VZV's 70 unique ORFs, 44 are essential and 26 are nonessential in human melanoma (MeWo) cells and skin organ culture (SOC). Most essential ORFs encode proteins imperative to the viral life cycle [53]. Of the 26 nonessential ORFs, eight ORF deletion mutants demonstrated severe growth defects in MeWo cells, indicating that their functions are also important for viral replication. The remaining 18 nonessential ORFs show no defects for viral replication in MeWo cells. All dispensable ORF deletion mutants were also tested in human fetal SOCs for their essentiality for viral infection in the skin [54]. Among these 18 dispensable VZV ORFs, four ORFs were found to be essential for VZV replication in skin: ORF 7, 10, 14, and 47. Three of these ORFs (10, 14, and 47) had already been known to impact viral replication in skin tissue [53,55-57]. Recent studies have implicated the role of ORF11 as essential in VZV infection in skin xenografts. ORF11 is a tegument layer protein of unknown function [58] that forms a complex with ORF9, and without this protein-protein interaction, ORF11's intracellular distribution in the *trans*-golgi network is affected, leading to attenuation in skin infection *in vivo*. The authors posit that this is due to the necessity of ORF11 to be included in the virion tegument, and without a proper ORF9 interaction, ORF11 is not included in the tegument and results in failure to infect skin xenografts *in vivo* [58]. Interestingly, the ORF11 deletion mutant was found to be dispensable for infection of MeWo cells *in vitro* [59], and was previously found to be dispensable in skin organ culture *ex vivo* [53], indicating an altered VZV infection and adaptation in cultured cells/tissue that is not seen *in vivo* (Figure 6).

A previously undiscovered fourth skin tropic factor, ORF7, was a novel tissue tropic gene discovery in the global scale mutational analysis [53]. Although the current VZV vaccine is attenuated in skin, the fact that it remains neurovirulent results in a specific focus on developing a neuroattenuated vaccine. In the search for a VZV neurotropic factor that could be removed or altered to form the foundation of a viable neuroattenuated vaccine, the dispensable VZV ORFs were screened, as tissue tropic factors are often dispensable since they are not required

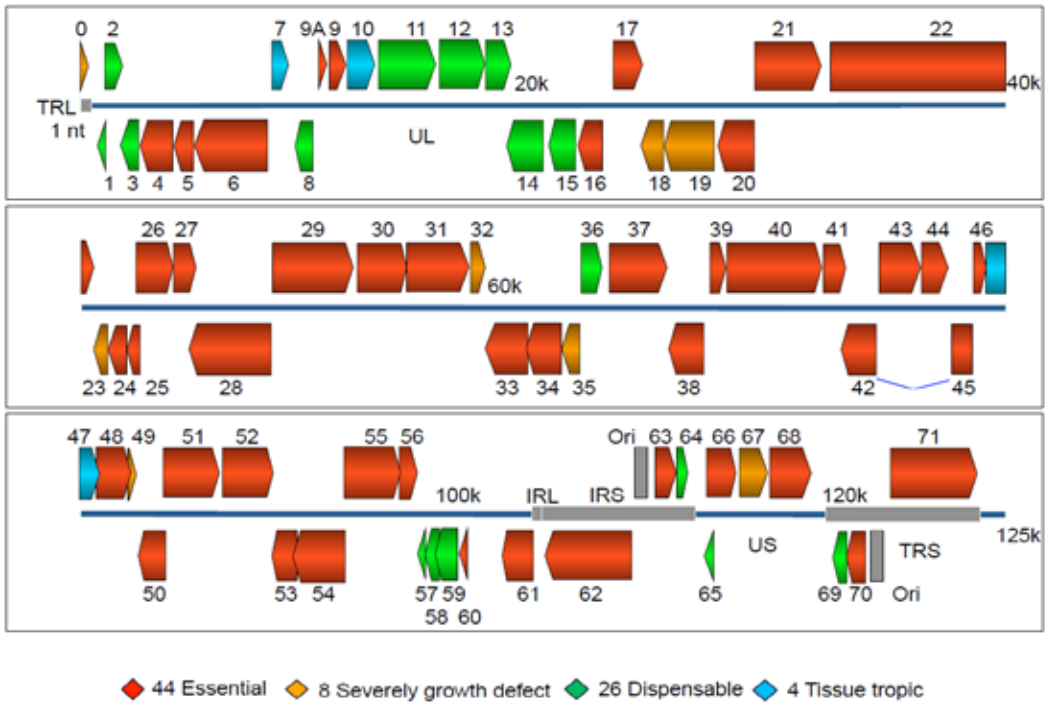

Figure 5: Map of VZV open reading frames organized by essentiality and tissue tropism.

Genomic organization and ORFs arrangement are based on the viral sequence of the VZV pOka strain (parental strain of WT VZV). Each VZV ORF is color-coded according to the growth properties of its corresponding virus gene-deletion mutant in cultured MeWo cells and human fetal skin organ cultures. The blue lines for ORF42 represent a splicing junction. For all growth curves, wild-type infections served as positive controls and mock infections served as negative controls.

*Image adapted from Selariu et al. (2010).

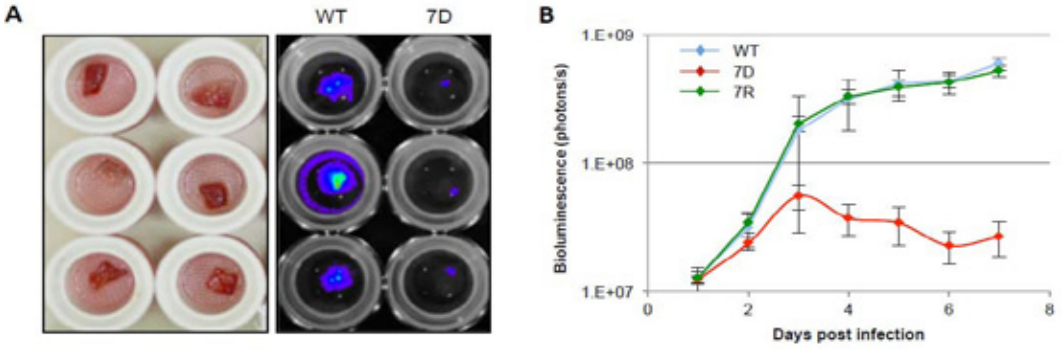

Figure 6: VZV ORF7 deletion virus in skin organ culture. **A.** Left panel is an image of *ex vivo* skin organ culture (SOC) maintained in Netwell inserts. Right panel is an image from the IVIS assay of the same SOC tissue infected with parental and ORF7 deletion (ORF7D) VZV_{LUC} viruses. **B.** *Ex vivo* growth curve analysis of ORF7D VZV_{LUC} in human fetal skin organ cultures (SOC). Skin tissues were inoculated with 5×10^3 PFU of either WT VZV_{LUC}, ORF7D VZV_{LUC}, or ORF7 rescue (ORF7R) VZV_{LUC} viruses, in parallel. VZV replication was monitored daily by IVIS for one week as bioluminescence emitting from each skin culture was measured. Each line represents an average of the data from 3 different skin tissue samples, all infected with the same virus.

for replication but do result in attenuation in specific tissues. Therefore, the 18 dispensable genes discovered in the VZV mutational library study were screened in neural models for possible identification of a neurotropic factor. The 18 ORF deletion BAC DNA clones were individually transfected into a differentiated human neuroblastoma line, SH-SY5Y. ORF7D VZV was shown to be the only dispensable mutant virus unable to form plaques in differentiated SH-SY5Y cells [60,61]. To further affirm the neurotropic properties of ORF7, differentiated SH-SY5Y and human embryonic stem cells (hESC)-derived neurons were infected with WT and 7D cell-free viral particles. No evidence of plaque formation at any point post infection in the SH-SY5Y and hESC *in vitro* studies for ORF7D VZV were noted [58].

Previous investigations of *in vivo* VZV neurotropism relied on tissues acquired from deceased individuals [5,46,62]. Because tissues from postmortem individuals who died from primary varicella or herpes zoster are extremely rare, information regarding VZV pathogenesis in the sensory nerve ganglia is limited. *In vitro* model systems, on the other hand, do not survive long term and can undergo physiological changes due to culture conditions. To counter these problems, a SCID-hu DRG xenograft model was developed to provide an opportunity to

explore VZV infection in human neurons [46,47,51,62]. Examination of VZV replication in human DRG xenografts reveals cell-cell fusion with polykaryon formation, a hallmark of VZV infection. The neurons and their surrounding satellite cells form a neuron-satellite cell complex [62]. In addition, one site of VZV latency is established in the DRG cells. Clusters of neurons and satellite cells remain despite demonstrating no CPE. Though infectious VZV can no longer be recovered from the xenografts, the VZV genome persists. These latent genomes can potentially replicate and cause disease upon reactivation [46,49,62,63].

A SCID-hu DRG model was used to demonstrate the neurotropic properties of ORF7. To create the SCID-hu DRG model, the DRG tissue was harvested from fetal spinal tissue at 18-24 gestational weeks. The tissue is then implanted under the kidney capsule of a SCID mouse. Visualization at 12 weeks post transplantation reveals a graft attached to the mouse kidney (Figure 7A). BLI is used to monitor and quantify WT VZV and ORF7D VZV growth *in vivo* within the SCID-hu DRG xenografts (Figure 7B). A growth kinetic analysis collected for the ORF7 mutant in the DRG xenograft further confirms the *in vitro* model where ORF7 fails to replicate and/or spread in neural cells (Figure 7C). Lastly, an ORF7 rescue virus (ORF7R VZV) was used for the growth analysis to validate the sole responsibility of the neuroattenuation to ORF7 (Figure 7C).

Conclusion and Implications

Reactivation of VZV in an immunocompromised host later in life can cause herpes zoster, characterized by belt-like rashes and, often, post-herpetic neuralgia. The current v-Oka vaccine is effective in preventing chickenpox, but leaves patients susceptible to shingles, as the live attenuated virus retains its neurovirulence and establishes latency in the host [13,64,65]. To this end, it is imperative that a neuroattenuated vaccine be developed to eliminate the establishment of VZV latency.

The use of the VZV BAC has greatly improved the efficiency and accuracy of creating recombination viruses necessary for the study of VZV pathogenesis. With the added luciferase marker, VZVLUC can be visualized both *in vitro* and *in vivo* using SCID-hu mice for further analysis.

A global profile was created on the essentiality of each ORF for VZV replication *in vitro* in MeWo cells and in SOC. ORF7 was found to be a novel skin-tropic factor. Its deletion virus was later screened in neurons and discovered to also be a novel neurotropic factor and potential vaccine candidate to eradicate VZV altogether. ORF7 encodes a 29-kDa tegument protein, but its function remains unknown. Further functional studies and homolog analyses are required to elucidate the exact mechanism behind ORF7 as both a skin- and neuro-tropic factor. Furthermore, although ORF11 was later found to be a skintropic viral gene [58], the screening of the ORF11 deletion mutant did not alter VZV growth in differentiated SH-SY5Y and since it does not result in neuroattenuation, its prospects as a standalone vaccine candidate are in doubt [61].

In addition to VZV ORF deletion mutant studies, there have been numerous molecular advances made in the field of VZV pathogenesis that will help elucidate the underpinnings of VZV infection, latency, and reactivation [66,67]. Current research is also focusing on the use of SVV as a model for VZV pathogenesis since VZV and SVV are very closely related alphaherpesviruses [68,69]. Alternatively, a novel VZV vaccine could also be utilized as a vaccine vector for other human viruses such as Human Immunodeficiency Virus (HIV). Previous studies in primates using cell-associated VZV as an adjuvant for Simian Immunodeficiency Virus (SIV) vaccination, a primate homolog to HIV, has actually been shown to exacerbate disease upon challenge when compared to unimmunized animals due to up regulation of

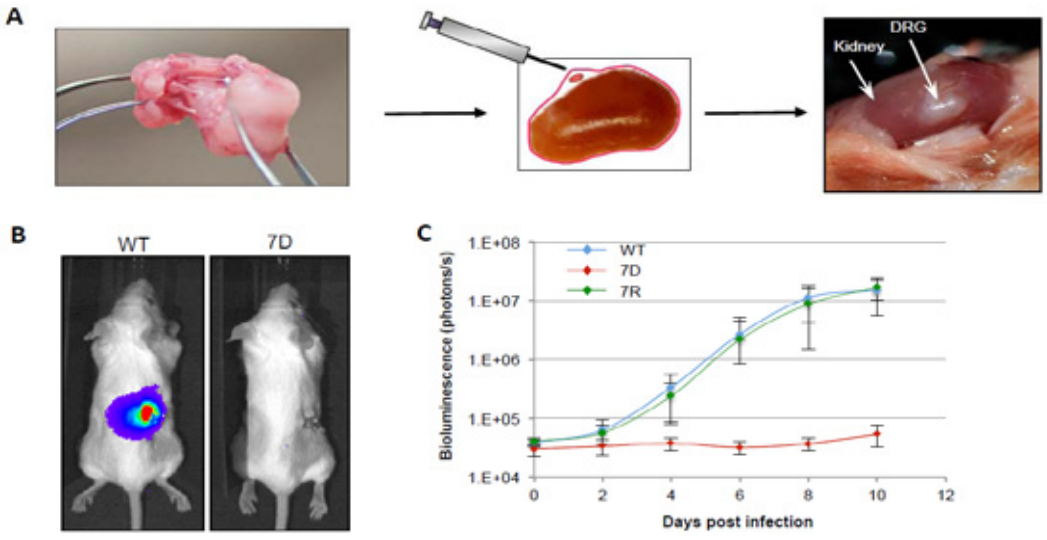

Figure 7: VZV ORF7 deletion virus in SCID-hu dorsal root ganglion (DRG) Model.

A. Schematic of DRG tissue xenotransplantation. Fetal DRG harvested from spinal cord (left panel) is implanted under the kidney capsule of a SCID mouse (middle panel). After vascularization, the implant is exposed and injected with virus for *in vivo* growth analysis. **B.** Image of SCID-hu mouse used for *in vivo* growth analysis depicting lack of ORF7D VZV_{LUC} growth in a DRG xenotransplant. Image adapted from Zhang et al. (2007). **C.** *In vivo* (SCID-hu mice with DRG xenotransplants) growth curve analysis of ORF7D VZV_{LUC} in parallel with WT VZV_{LUC} and ORF7 Rescue (7R) VZV_{LUC}. The DRG was inoculated with 5×10^3 PFU WT VZV_{LUC}, ORF7D VZV_{LUC} and ORF7R VZV_{LUC}, as indicated. VZV replication was monitored daily by IVIS for one week as bioluminescence emitting from each DRG was measured. Each line represents an average of the data from 3 different DRG samples, all infected with the same virus.

SIV-specific CD4+ T cells and co-receptors for SIV. Current research is attempting to optimize the VZV, or other herpesvirus, adjuvant composition by determining whether cell-free VZV can impart similar or improved immunogenicity and infection without the negative effects of increasing the targets and reservoirs for SIV [70].

Ultimately, the goal of this review is to highlight the advancements made in the field of VZV pathogenesis that will one day produce a novel vaccine that will not only function to prevent the childhood chickenpox disease, but more importantly, completely prevent the establishment of VZV latency and therefore eliminate the risk of reactivation and the debilitating herpes zoster manifestation in the vaccinated population.

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