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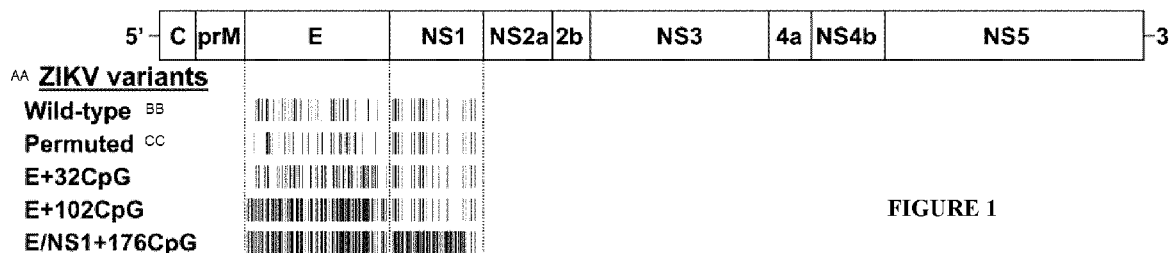


FIGURE 1

(57) Abstract: Provided herein are cytosine-phosphate-guanine (CpG) recoded oncolytic flaviviruses having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type, and methods and uses thereof for the treatment of cancer, such as brain cancer, and particularly glioblastomas such as SOX2-negative glioblastoma. Pharmaceutical compositions and kits are also provided, as well as glioblastoma stem cell-derived tumor models, methods for the production thereof, and methods and uses thereof for identifying anti-cancer agents targeting glioblastoma.

RECODED ONCOLYTIC VIRUSES FOR TREATMENT OF CANCER

FIELD OF INVENTION

The present invention relates generally to oncolytic viruses. More specifically, the present invention relates to CpG recoded oncolytic flaviviruses for treatment of cancer.

BACKGROUND

Cytosine-phosphate-guanine (CpG) dinucleotide frequencies are suppressed in vertebrate genomes and most RNA viruses [1,2]. The rational increase of CpG dinucleotide numbers in viral genomes showed the potential to become a cutting-edge approach for vaccine development and alternative to traditional live attenuated vaccines. The concept is to increase the number of CpG dinucleotides in an RNA viral genome while retaining the amino acid composition of encoded proteins that leads to impaired infection but robust protective host immune responses. Mechanistically, it has been demonstrated that cellular Zinc-finger antiviral protein (ZAP) targets recoded viruses by specifically binding to genomic regions enriched for CpG dinucleotides [3,4]. Subsequently, synergy or complementation of ZAP function by oligoadenylate synthetase 3, RNase L and cytoplasmic protein KHNYN inhibits replication of viruses containing the elevated number of CpG dinucleotides [5,6]. Efficacy of the CpG-recoded influenza virus vaccine has been demonstrated in mice; we also showed full protection evoked by CpG-recoded Zika virus (ZIKV) vaccine candidates in mice challenged with lethal heterologous ZIKV [7].

Zika virus emerged in the Americas in 2015 evoking a great concern around the world with fetal death, microencephaly, severe brain lesions and developmental abnormalities in fetuses and offspring. Paradoxically, neurotropic ZIKV was explored with an idea to apply ZIKV in a surgical area of the brain after glioblastoma removal to suppress the growth of glioblastoma stem cells (GSCs) and tumor. The authors showed oncolytic activity of ZIKV in GSCs *in vitro* and in a glioblastoma mouse model [8,9]. The approach is relevant because patients suffer inevitable

relapses after glioblastoma surgery and accumulating evidence indicates that GSCs play a central role in tumor recurrence [10,11]. Glioblastomas rarely metastasize beyond the brain, and patients usually suffer a recurrence within proximity of the surgical zone [12] that supports the proposed locally-targeted therapeutic approach. Also, glioblastoma therapies with local delivery of viral vectors showed feasibility [13]. Later, attenuated ZIKV vaccine candidate—with 10 nucleotide deletions in 3' UTR—was re-purposed for oncolytic therapy showing efficacy in GSCs *in vitro* and in the mouse model [14]. Moreover, ZIKV oncolytic activity was demonstrated in embryonal central nervous system tumor xenografts and in dogs with large brain tumors [15,16].

Unfortunately, treatment of brain cancer, and particularly glioblastoma, has remained particularly challenging, and effective treatments are still highly desired in the field. Development of virus-based therapies is complex, and identification of viruses specifically targeting cancer cells over host cells has proven difficult. Many virus attenuation approaches have been developed in the field of vaccines; however, it is difficult to predict whether any such attenuation strategies could provide, or could be adapted to provide, viruses with reduced infectivity toward healthy cells and yet still effectively target cancer cells, particularly brain cancer cells such as glioblastomas.

Alternative, additional, and/or improved oncolytic viruses, and methods and uses thereof for treatment of cancer, are desirable.

SUMMARY OF INVENTION

Using cytosine-phosphate-guanine (CpG) recoding techniques, recoded oncolytic flaviviruses have been developed for the treatment of cancer, such as brain cancer, and particularly glioblastomas such as SOX2-negative glioblastoma. Oncolytic flaviviruses having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type are described. Surprisingly, it has been found that the extent, and positioning, of CpG recoding instances within the viral genome can have an impact on viral infectivity toward healthy and cancerous brain cells, and CpG recoded oncolytic flaviviruses having high selectivity and oncolytic activity toward brain/spinal cord cancer cells (particularly,

glioblastomas) have been identified herein. *In ovo* glioblastoma stem cell-derived tumor models, and methods for the production thereof, have also been developed, as well as methods and uses thereof for identifying anti-cancer agents targeting glioblastoma.

In an embodiment, there is provided herein a use of an oncolytic flavivirus for the treatment of cancer, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.

In another embodiment, there is provided herein a use of an oncolytic flavivirus in the manufacture of a medicament for the treatment of cancer, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.

In still another embodiment, there is provided herein a method for treating cancer in a subject in need thereof, said method comprising:

administering an oncolytic flavivirus to the subject, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type;

thereby killing one or more cancer cells in the subject.

In yet another embodiment, there is provide herein an oncolytic flavivirus, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type, for use in the treatment of cancer in a subject in need thereof.

In still another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the CpG recoded oncolytic flavivirus may have reduced infection kinetics toward healthy cells as compared to wild-type virus, and may be oncolytic toward cancer cells.

In yet another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the cancer may be brain or spinal cord cancer.

In another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the cancer may be glioblastoma or any other brain tumor.

In still another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the cancer may be or may comprise SOX2-negative glioblastoma or glioblastoma stem cells (GSCs), TGM2-positive glioblastoma or GSCs, or SOX-2 negative and TGM2-positive glioblastoma or GSCs, or any combinations thereof.

In yet another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the oncolytic flavivirus may be a cytosine-phosphate-guanine (CpG) recoded Zika virus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.

In another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the frequency of CpG dinucleotide instances within the viral genome may be increased by about 30 to about 180 instances as compared to wild-type, such as about 50 to about 110 instances as compared to wild-type; about 80 to about 110 instances as compared to wild-type; about 95-105 instances as compared to wild-type; or about 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, or 105 instances as compared to wild-type.

In yet another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the silent mutations which increase frequency of CpG dinucleotide instances within the viral genome may be primarily, or entirely, localized to the E genomic region of the viral genome.

In another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, any one or more of the NS1 region, the C region, the prM region, the NS2a region, the NS2b region, the NS3 region, the NS4a region, the NS4b region, and/or the NS5 region of the viral genome, or any combinations thereof, may be free, or substantially free, of the silent mutations which increase frequency of CpG dinucleotide

instances within the viral genome.

In still another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the NS1 region of the viral genome may be free, or substantially free, of the silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.

In another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the size of the viral genome of the oncolytic virus may be the same, or substantially the same, as the size of the wild-type viral genome.

In yet another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the oncolytic flavivirus may be a cytosine-phosphate-guanine (CpG) recoded Zika virus of an African or Asian lineage, having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.

In still another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the oncolytic flavivirus may be a cytosine-phosphate-guanine (CpG) recoded Zika virus of contemporary Asian ZIKV H/PF/2013 strain, having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.

In another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the viral genome of the oncolytic virus may be or may comprise a sequence of any one of SEQ ID Nos: 5-7, or a sequence having at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity therewith.

In another embodiment of any of the above use or uses, method or methods, or oncolytic flavivirus or oncolytic flaviviruses, the viral genome of the oncolytic virus may be or may comprise a sequence of SEQ ID NO: 6, or a sequence having at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity therewith.

In another embodiment, there is provided herein an oncolytic flavivirus, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to

wild-type.

In still another embodiment, there is provided herein a pharmaceutical composition comprising any of the oncolytic virus or oncolytic viruses as described herein, and a pharmaceutically acceptable carrier, excipient, buffer, or diluent.

In another embodiment, there is provided herein a glioblastoma stem cell-derived tumor model, said tumor model comprising glioblastoma stem cell(s) implanted *in ovo* on chicken chorioallantoic membrane (CAM).

In yet another embodiment, there is provided herein a method for preparing a glioblastoma stem cell-derived tumor model, said method comprising:

incubating a fertilized egg;

windowing and CAM-dropping the fertilized egg to generate an artificial air sac;

damaging the CAM blood vessel;

placing a retaining member around the damaged blood vessel;

introducing glioblastoma stem cell(s) to a region of the CAM bounded by the retaining member; and

incubating the fertilized egg to allow tumor growth on the CAM.

In still another embodiment, there is provided herein a glioblastoma stem cell-derived tumor model prepared according to any of the method or methods described herein.

In yet another embodiment, there is provided herein a method for identifying anti-cancer agents targeting glioblastoma, said method comprising:

treating any of the glioblastoma stem cell-derived tumor model or models as described herein with a candidate anticancer agent; and

determining whether tumor size and/or proliferation in the glioblastoma stem cell-derived tumor model is reduced by treatment with the candidate anticancer agent;

wherein a reduction in tumor size and/or proliferation in the glioblastoma stem cell-derived tumor model identifies the candidate anticancer agent as an anti-cancer agent targeting glioblastoma.

In another embodiment, there is provided herein a kit comprising any one or more of any of the oncolytic virus or oncolytic viruses as described herein, any of the pharmaceutical composition or pharmaceutical compositions as described herein, any of the glioblastoma stem cell-derived tumor model or models as described herein, instructions for performing any of the method or methods described herein, or any combinations thereof.

BRIEF DESCRIPTION OF DRAWINGS

These and other features will become further understood with regard to the following description and accompanying drawings, wherein:

FIGURE 1 shows the genome of ZIKV and the CpG-recoding strategy used in the Examples. ZIKV genomic regions encoding E and NS1 proteins were recoded to increase the number of CpG dinucleotides. A barcode schematically represents the number of CpG dinucleotides. The actual number of CpG dinucleotides are in Table 1;

FIGURE 2 shows infection kinetics in nonmalignant human brain cells (HMC3 (a) and NPC (b)) and tumor glioblastoma stem cells (528 (e) and 157 (f)) after inoculation at an MOI of 0.01. Cell culture supernatants in 96-well plates were collected and viral titers were measured using the endpoint dilution assay. The dotted line represents the limit of detection (LOD). Cell proliferation assay after inoculation of cells (HMC3 (c) and NPC (d), GSC 528 (g) and GSC 157 (h)) with MOI of 1. Whiskers represent standard error of the mean (SE) from three biologically independent replicates with three technical replicates. “dpi”– days post-inoculation. The asterisk (*) indicates $P < 0.05$ vs. WT (a, b, e, f) and control (c, d, g, h): (c) WT and E+32CpG at 3-7 dpi, Permuted at 5-7 dpi; (e) E/NS1+176CpG at 3 dpi; (f) E+32CpG and E/NS1+176CpG at 4 dpi; (g) WT, Permuted, E+102CpG at 3-7 dpi;

FIGURE 3 shows morphology of GSCs (a: 528; b: 157) *in vitro*; phase-contrast microscopy. (c) Positive TGM2 and negative SOX2 staining in GSC 528. (d) Negative TGM2 and positive SOX2 staining in GSC 157. Morphology of GSC tumors (e 528; f: 157) in *in ovo* cultures by bright-field microscopy. (g) Vascularization of GSC tumor. (h) Intact CAM in a control egg. (i) The volume of tumors formed at sampling (ED 19; Mann-Whitney test). Scale bars are 0.1 (a-d) and 1 mm (e-h). Implantation efficiency of GSC cell cultures (GSC 528: n = 27, GSC 157: n = 26) on chicken embryo CAM (GSC 528: 96%, GSC 157: 88%) and egg viability at ED 19 (GSC 528: 92%, GSC 157: 93%), were comparable in both GSC models;

FIGURE 4 shows H&E staining in GSC 528 (a) and GSC 157 (b) tumors at ED 19. CE: chorionic epithelium, AE: allantoic epithelium, M: intermediate vascularized mesenchyme, BV: blood vessel, and T: tumor. TGM2 (c: GSC 528; d: GSC 157) and SOX2 (e: GSC 528; f: GSC 157) protein expression in tumor cells; TGM2-positive staining is in red (c). Scale bars are 1 (a-b) and 0.1 mm (c-f);

FIGURE 5 shows Zika virus quantification in tumors (a: GSC 528; b: GSC 157). The dotted line (a, b) represents LOD. The volume of tumors inoculated with ZIKV variants (c: GSC 528; d: GSC 157). Relative reduction of tumors (e: GSC 528; f: GSC 157); FC: fold change. *: $P < 0.05$; tumor volumes in ZIKV groups were compared to volumes in the Control group. The dashed line (e, f) represents the base tumor volume in the Control group. Sampling was performed at ED 19;

FIGURE 6 shows immunohistochemistry for ZIKV antigen: (a) Isotype control staining of GSC 528 tumor; (b) Mock-inoculated GSC 157 tumor; (c) GSC 528 inoculated with ZIKV E+102CpG; (d) GSC 157 inoculated with ZIKV E+102CpG (arrows). H&E staining of mock (e: GSC 528; f: GSC 157) and ZIKV-inoculated tumors (g: GSC 528 inoculated with ZIKV E+102CpG; h: GSC 157 inoculated with ZIKV E+102CpG). Scale bars are 0.1 mm;

FIGURE 7 shows infection kinetics in nonmalignant human brain cells (HMC3 (a) and NPC (b)) and tumor glioblastoma stem cells (528 (c) and 157 (d)) after inoculation at an MOI of 0.01. The 96-well plates with cell monolayers were stained with ZIKV-specific Abs and infected cells were counted in the whole well with bright-field microscopy at 200x. Whiskers represent standard error of the mean (SE) from three biologically independent replicates with three technical

replicates. “dpi”– days post-inoculation. The asterisk (*) indicates $P < 0.05$ vs. WT: (a) E+102CpG and E/NS1+176CpG at 4 dpi; and

FIGURE 8 shows genome sequences for each of the ZIKV variants shown in Table 1. The complete genome sequences of Wild-type (WT) (SEQ ID NO: 3), Permuted (SEQ ID NO: 4), E+32CpG (SEQ ID NO: 5), E+102CpG (SEQ ID NO: 6), and E/NS1+176CpG (SEQ ID NO: 7) ZIKV variants are shown. CpG dinucleotides in the regions encoding E and NS1 proteins (highlighted in yellow and green, respectively) are shown in capitals and highlighted in bold.

DETAILED DESCRIPTION

Described herein are cytosine-phosphate-guanine (CpG) recoded oncolytic flaviviruses for the treatment of cancer, such as brain and/or spinal cord cancer. *In ovo* glioblastoma stem cell-derived tumor models, and methods for the production thereof, have also been developed, as well as methods and uses thereof for identifying anti-cancer agents targeting glioblastoma. It will be appreciated that embodiments and examples are provided for illustrative purposes intended for those skilled in the art, and are not meant to be limiting in any way.

Using cytosine-phosphate-guanine (CpG) recoding techniques, recoded oncolytic flaviviruses have been developed for the treatment of cancer, such as brain cancer, and particularly glioblastomas such as SOX2-negative glioblastoma. Oncolytic flaviviruses having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type are described. Surprisingly, it has been found that the extent, and positioning, of CpG recoding instances within the viral genome can have an impact on viral infectivity toward healthy and cancerous brain cells, and CpG recoded oncolytic flaviviruses having high selectivity and oncolytic activity toward brain/spinal cord cancer cells (particularly, glioblastomas) have been identified herein. *In ovo* glioblastoma stem cell-derived tumor models, and methods for the production thereof, have also been developed, as well as methods and uses thereof for identifying anti-cancer agents targeting glioblastoma.

In an embodiment, there is provided herein a use of an oncolytic flavivirus for the treatment of

cancer, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.

In another embodiment, there is provided herein a use of an oncolytic flavivirus in the manufacture of a medicament for the treatment of cancer, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.

In another embodiment, there is provided herein an oncolytic flavivirus, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.

In still another embodiment, there is provided herein a method for treating cancer in a subject in need thereof, said method comprising:

administering an oncolytic flavivirus to the subject, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type;

thereby killing (either directly, or indirectly, or both) one or more cancer cells in the subject.

In yet another embodiment, there is provide herein an oncolytic flavivirus, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type, for use in the treatment of cancer in a subject in need thereof.

As will be understood, flaviviruses may include any suitable flavivirus capable of infecting one or more types of cancer cell, resulting in reduced proliferation and/or increased death of the cancer cell(s), either by lysis, stimulation of host anti-tumor immune responses, or both. The

flavivirus may typically be selected based on the intended application, and more specifically based ability to infect at least some cells of the cancer or tumour to be targeted, and/or characteristics of the subject or patient afflicted with the cancer. Flaviviruses are a genus of viruses in the family *Flaviviridae*, the genus including viruses such as West Nile virus, dengue virus, tick-borne encephalitis virus, yellow fever virus, Japanese encephalitis virus, and Zika virus, for example. As will be understood, there are many different lineages and strains of such viruses. As will be understood, the flavivirus (or other naturally occurring, synthetic, mutated, or genetically modified virus) may be selected based on the intended application, and will typically be selected based on an ability (once recoded) to target/kill cancer cells while having minimal/reduced or no infectivity toward healthy/non-cancerous cells as a result of CpG recoding. Many flaviviruses can be dangerous toward human subjects, and so CpG recoding may be performed and carefully assessed to verify that the chosen CpG recoding of the virus was sufficient to attenuate/minimize infectivity of the virus toward healthy/non-cancerous cells prior to use or administration. The skilled person having regard to the teachings herein will be aware of a variety of suitable assays/tests for evaluating safety and cancer-selectivity of a given recoded virus to determine suitability for a particular scenario or application of interest. In certain embodiments, the flavivirus is preferably a Zika virus, such as a Zika virus of an African or Asian lineage, for example a Zika virus of contemporary Asian ZIKV H/PF/2013 strain or other strain, for example.

In certain embodiments, the oncolytic flavivirus may comprise a cytosine-phosphate-guanine (CpG) recoded flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type counterpart virus. CpG dinucleotide frequencies are suppressed in vertebrate genomes and most wild-type RNA viruses. In CpG recoded oncolytic flavivirus as described herein, the number of CpG dinucleotides occurring in an RNA viral genome may be increased, while retaining or substantially retaining the amino acid composition of encoded proteins (due to codon redundancy). Such CpG recoded flavivirus may be considered as oncolytic flavivirus variants, which may be chosen for selectivity for one or more cancer cells over one or more non-cancerous or healthy cells, so as to suit the intended application. In certain embodiments, the CpG recoded oncolytic flavivirus may have reduced infection kinetics toward one or more healthy or non-cancerous cells as compared to wild-type

virus, and may be oncolytic toward one or more cancer cells. As will be understood, oncolytic activity toward cancer cells may include any one or more of reducing proliferation of one or more cancer cells, death of one or more cancer cells, or both, and may arise from lysis of the cancer cells, stimulation of host anti-tumor immune responses, or both, for example. Typically, oncolytic activity toward cancer cells may include infection of cancer cells by the CpG recoded oncolytic flavivirus, resulting in viral replication, killing the cancer cell and releasing further oncolytic flaviviruses.

In certain embodiments, the oncolytic flavivirus may be a cytosine-phosphate-guanine (CpG) recoded Zika virus of an African or Asian lineage, having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome. By way of example, in certain embodiments the oncolytic flavivirus may be a cytosine-phosphate-guanine (CpG) recoded Zika virus of contemporary Asian ZIKV H/PF/2013 strain, having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.

In certain embodiments, introduced nucleotide mutations of CpG recoding may not substantially alter the translated viral proteins. In certain embodiments, frequencies of UpA dinucleotides in recoded flavivirus variants may be renormalized to the initial level. In certain embodiments, recoded variants may show a modest reduction in codon pair bias scores in particular genomic regions (such as the E and NS1 genomic regions), or may show minimal changes in the complete ORF, for example. In certain embodiments, CpG recoding may be based on *in silico* recoding using previously described algorithms and/or programs (see, for example, [7], [23], which are herein incorporated by reference in their entireties), which may be adapted for the particular virus and or application, for example. In certain embodiments, the size of the viral genome of the oncolytic virus may be the same, or substantially the same, as the size of the wild-type viral genome.

In certain embodiments the frequency of CpG dinucleotide instances within the oncolytic flavivirus viral genome may be increased by about 30 to about 180 instances as compared to wild-type, or any integer value therebetween, or any sub-range spanning between any two of these integer values. By way of example, in certain embodiments, the number of CpG dinucleotide instances within the oncolytic flavivirus viral genome may be increased by about 50

to about 110 instances as compared to wild-type; about 80 to about 110 instances as compared to wild-type; about 95-105 instances as compared to wild-type; or about 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, or 105 instances as compared to wild-type, for example.

In yet another embodiment the silent mutations of the CpG recoded oncolytic flavivirus which increase frequency of CpG dinucleotide instances within the viral genome may be primarily, or entirely, localized to the E genomic region of the viral genome (with reference to, for example, Zika virus or other flavivirus).

In yet another embodiment, any one or more of the NS1 region, the C region, the prM region, the NS2a region, the NS2b region, the NS3 region, the NS4a region, the NS4b region, and/or the NS5 region of the viral genome (with reference to, for example, Zika virus or other flavivirus), or any combinations thereof, may be free, or substantially free, of the silent mutations which increase frequency of CpG dinucleotide instances within the viral genome. By way of example, in certain embodiments, at least the NS1 region of the viral genome may be free, or substantially free, of the silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.

In certain embodiments, the viral genome of the CpG recoded oncolytic flavivirus may be or may comprise a sequence of any one of SEQ ID Nos: 5-7 (see Figure 8), or a sequence having at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity therewith. By way of example, in certain embodiments the viral genome of the CpG recoded oncolytic virus may be or may comprise a sequence of SEQ ID NO: 6, or a sequence having at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity therewith.

As will be understood, oncolytic flaviviruses as described herein may be for administration to a subject or patient in need thereof via any suitable administration route or technique known to the person of skill in the art having regard to the teachings herein, which may be selected based on the cancer to be treated, the tissue or organ affected by the cancer, the location of the cancer, characteristics of the patient or subject, characteristics of the oncolytic flavivirus being used, and/or other treatments or surgical interventions which the subject or patient is or will be

receiving, for example. In certain embodiments, for example where the cancer is a brain cancer, administration may involve administering, contacting, delivering, or applying the oncolytic virus to an affected region of the brain before, during, or after surgical removal of the brain cancer or tumour. In certain embodiments, the oncolytic virus may be administered, contacted, delivered, or applied to an affected region of the body, such as a region of the brain, from which a cancer or tumor has been surgically removed, so as to target residual cancer cells and/or to prevent or reduce recurrence of the cancer. In certain embodiments, the oncolytic flavivirus may be for administration orally, intravenously, subcutaneously, by inhalation, by local injection, by systemic administration, or any combination thereof, depending on the intended application. In certain embodiments, the oncolytic flavivirus may be for administration intracerebrally.

As will be understood, the recoded oncolytic flavivirus may be for use in treating cancer. In certain embodiments, treatment of cancer may comprise reducing proliferation of one or more cancer cells, increasing death of one or more cancer cells, or both, and may arise from lysis of the cancer cells, stimulation of host anti-tumor immune responses, or both, for example.

In certain embodiments, the cancer to be treated may comprise generally any type(s) of cancer(s) or related disease(s) involving abnormal cell growth/proliferation, for which one or more cells of the cancer can be targeted by a recoded oncolytic virus. In certain embodiments, the cancer or tumor to be treated may comprise brain cancer, spinal cord cancer, pancreatic cancer, sarcoma, leukemia, lymphoma, myeloma, or melanoma, or any combinations thereof. In certain embodiments, the cancer to be treated may comprise a cancer or tumor metastasized from any one or more of these cancers. While many of the examples herein are focused mainly toward treatment of brain cancer and glioblastomas with recoded oncolytic flaviviruses, it will be understood that a wide variety of other cancers and other recoded oncolytic viruses (flaviviruses or otherwise) are also contemplated. Results described herein show that viral selectivity for cancer cells over non-cancer cells may be enhanced by CpG recoding, and it is contemplated that such principles may extend to a wide variety of different cancers and different viruses.

In certain embodiments, the cancer may be brain or spinal cord cancer. By way of example, in certain embodiments, the cancer may be or comprise a glioblastoma. In certain embodiments, the cancer may comprise a glioblastoma or other cancer comprising glioblastoma stem cells (GSCs).

In certain embodiments, the cancer may comprise brain glioblastoma stem cells, and may be localized in the brain, or may be metastasized to another part of the body, such as the spinal cord. In certain embodiments, the cancer may be or comprise SOX2-negative glioblastoma or GSCs, TGM2-positive glioblastoma or GSCs, or SOX-2 negative and TGM2-positive glioblastoma or GSCs, or any combinations thereof.

In still another embodiment, there is provided herein a pharmaceutical composition comprising any of the oncolytic virus or oncolytic viruses as described herein, and a pharmaceutically acceptable carrier, excipient, buffer, or diluent. The person of skill in the art having regard to the teachings herein will be aware of a variety of suitable pharmaceutically acceptable carriers, excipients, buffers, and/or diluents, which may be selected to suit the particular formulation and/or intended application, for example.

In yet another embodiment, there is provided herein a glioblastoma stem cell-derived tumor model, said tumor model comprising glioblastoma stem cell(s) implanted *in ovo* on chicken chorioallantoic membrane (CAM).

In still another embodiment, there is provided herein a method for preparing a glioblastoma stem cell-derived tumor model, said method comprising:

- incubating a fertilized egg (such as a chicken egg, a duck egg, or a goose egg, or another suitable egg);
- windowing and CAM-dropping the fertilized egg to generate an artificial air sac;
- damaging the CAM blood vessel;
- placing a retaining member around the damaged blood vessel;
- introducing glioblastoma stem cell(s) to a region of the CAM bounded by the retaining member; and
- incubating the fertilized egg to allow tumor growth on the CAM.

In another embodiment, the fertilized egg may comprise a chicken egg, a goose egg, a duck egg, an ostrich egg, a platypus egg, or another suitable egg.

In still another embodiment, there is provided herein a glioblastoma stem cell-derived tumor model prepared according to any of the method or methods described herein.

In yet another embodiment, there is provided herein a method for identifying anti-cancer agents targeting glioblastoma, said method comprising:

treating any of the glioblastoma stem cell-derived tumor model or models as described herein with a candidate anticancer agent; and

determining whether tumor size and/or proliferation in the glioblastoma stem cell-derived tumor model is reduced by treatment with the candidate anticancer agent;

wherein a reduction in tumor size and/or proliferation in the glioblastoma stem cell-derived tumor model identifies the candidate anticancer agent as an anti-cancer agent targeting glioblastoma.

In another embodiment, there is provided herein a kit comprising any one or more of any of the oncolytic virus or oncolytic viruses as described herein, any of the pharmaceutical composition or pharmaceutical compositions as described herein, any of the glioblastoma stem cell-derived tumor model or models as described herein, instructions and/or reagents and/or tools for performing any of the method or methods described herein, or any combinations thereof.

While the descriptions and examples herein are mainly directed to CpG recoded oncolytic flaviviruses for treatment of brain cancer, it will be understood that it a wide variety of other CpG recoded oncolytic viruses (i.e. not only flaviviruses), as well as a wide variety of other cancers (i.e. not only brain cancers) are also contemplated. Results described herein show that viral selectivity for cancer cells over non-cancer cells may be enhanced by CpG recoding, and it is contemplated that such principles may extend to a wide variety of different viruses and cancers.

One or more illustrative Examples have been described in detail below. It will be understood to

persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention described herein.

EXAMPLE 1 – Zika Virus with Increased CpG Dinucleotide Frequencies Shows Oncolytic Activity in Glioblastoma Stem Cells

Pioneering ZIKV studies and previous findings that ZAP—the host protein targeting CpG-enriched regions in recoded viruses—is underrepresented in some cancer cells [17], led the present inventors to develop and test whether CpG recoding in viral genomes has an oncolytic potential. A main goal of the present studies was to investigate whether CpG-recoding in a viral genome may provide an oncolytic candidate with reduced infection kinetics in healthy brain cells but with high virulence in GSCs. As a model, we used ZIKV in these studies: first, we tested the oncolytic activity of CpG-recoded ZIKV variants in two human primary GSCs *in vitro*; second, we established an *in ovo* glioblastoma model and tested how CpG-recoded ZIKV variants affect growth of GSC-derived tumors.

Indeed, this example sought to investigate whether cytosine phosphate-guanine (CpG) recoding in a viral genome may provide oncolytic candidates with reduced infection kinetics in nonmalignant brain cells, but with high virulence in glioblastoma stem cells (GSCs). As a model, we used well-characterized CpG-recoded Zika virus vaccine candidates that previously showed genetic stability and safety in animal models. *In vitro*, one of the CpG-recoded Zika virus variants had reduced infection kinetics in nonmalignant brain cells but high infectivity and oncolytic activity in GSCs as represented by reduced cell proliferation. The recoded virus also efficiently replicated in GSC-derived tumors *in ovo* with a significant reduction of tumor growth. We also showed that some GSCs may be resistant to Zika virus oncolytic activity, highlighting the desirability of personalized oncolytic therapy and/or strategies to overcome resistance in GSCs. We demonstrated CpG recoding approaches for oncolytic virus development, and results contribute to a better understanding of host–tumor–CpG-recoded virus interactions.

Materials and Methods

Cell cultures

C6/36 cells (ATCC #CRL-1660) were maintained in Minimum Essential Medium (MEM; Sigma #M4655) supplemented with 10% fetal bovine serum (FBS; Sigma #12103c) and 1x P/S (Penicillin-Streptomycin; Gibco #15140-122). VERO E6 cells (ATCC #CRL-1586) were maintained in DMEM supplemented with 3% FBS, 1x P/S, and 2.67 mM Sodium Bicarbonate (Gibco #25080-094). The human microglial HMC3 cells (ATCC #CRL-3304) were maintained in MEM supplemented with 10% FBS, and 1x P/S. The human NPCs were differentiated from human induced pluripotent stem cells that were reprogrammed from fibroblasts obtained from a healthy individual [18,19] (the University of Saskatchewan's Biomedical Research Ethics Board Number: 17-181); NPCs were cultured in medium consisting of 50% Dulbecco's modified Eagle's medium/F12 (DMEM/F12; HyClone #SH3002301) and 50% Neurobasal medium (Gibco #21103-049) containing 1x B27-RA, 1x N-2 (Gibco #17502-048), 1x P/S, 20 ng/ml basic Fibroblast Growth Factor (bFGF, PeproTech #100-18B), 2 μ M SB431542 (Stemgent #04-0010-10), 10 ng/ml Leukemia Inhibitory Factor (PeproTech #300-05), 3 μ M CHIR99021 (StemCell Technologies #72052), and 10 μ M Y-27632 (Tocris Bioscience #12-541) [20]. For NPCs, plates were precoated with growth factor-reduced Matrigel (BD Biosciences #354230). Previously well-characterized GSC 157 and 528 (obtained from patients with high-grade gliomas and characterized as a proneural and mixed subtype, respectively) [21,22] were cultured in DMEM/F12 with 1x B27-RA (Fisher #12-587-010), 1x P/S, 3 IU/ml Sodium Heparin (Fisher #H19), 20 ng/ml bFGF, and 20 ng/ml Epidermal Growth Factor (StemCell Technologies #78006). Low passages (<10) of NPC and GSC cells were used in the study. Cells were cultured at +37°C (C6/36 cells were cultured at +28°C) in a 5% CO₂ humidified incubator. For a detachment of C6/36 cells, we used cell scrappers (Fisher #08-100-242), HMC3 and VERO—Trypsin-EDTA (Gibco #25200-072), NPCs—TrypLE (Gibco #LS12604021), GSCs—Accumax (StemCell Technologies #07921).

Design and Recovery of CpG-recoded ZIKV Variants

In silico recoding and recovery of CpG-modified ZIKV variants were previously described [7]. The MUTATE SEQUENCES program in SSE 1.3 software package [23] was used to modify the sequence of the contemporary Asian ZIKV H/PF/2013 strain [GenBank: KJ776791.2] [24] and to generate variants with increased CpG numbers in regions encoding envelope (E) and

nonstructural 1 (NS1) proteins (Figure 1). The actual number of CpG dinucleotides are in Table 1. Introduced nucleotide mutations did not alter the translated viral proteins. We also renormalized frequencies of UpA dinucleotides in recoded ZIKV variants to the initial level. Recoded variants showed a modest reduction in codon pair bias scores in the E and NS1 genomic regions or minimal changes in the complete ORF [7].

Table 1: CpG dinucleotide composition in ZIKV variants

Genomic region	ZIKV variant	Number of CpGs
E	Wild-type	35
	Permuted	35
	E-32CpG	67
	E+102CpG	137
	E/NS1+176CpG	137
NS1	Wild-type	25
	Permuted	25
	E-32CpG	25
	E+102CpG	25
	E/NS1+176CpG	99
ORF ¹	Wild-type	316
	Permuted	316
	E-32CpG	348
	E+102CpG	418
	E/NS1+176CpG	492

¹ ORF: open reading frame

Genome sequences for each of the ZIKV variants shown in Table 1 are provided in Figure 8. As will be understood by the skilled person having regard to the teachings herein, these sequences in Figure 8 are depicted with “t” nucleotides, however these “t”s are intended to represent “u” nucleotides in the genome, given that Zika is an RNA virus. In this Figure, the complete genome sequences of Wild-type (WT), Permuted, E+32CpG, E+102CpG, and E/NS1+176CpG ZIKV variants are shown. CpG dinucleotides in the regions encoding E and NS1 proteins (highlighted in yellow and green, respectively) are shown in capitals and highlighted in bold.

To ensure sequence disruption did not damage or destroy the unknown replication element(s), we designed a permuted control (Figure 1); the sequence region was permuted using the CDLR method in the SSE software package [25-27].

To recover ZIKV variants we used infectious subgenomic amplicons (ISA) [24,28,29] as previously described [7]. Recoded fragments were *de novo* synthesized (GenScript), amplified with high fidelity PCR (Invitrogen Platinum PCR SuperMix, High Fidelity #12532016) and transfected into C6/36 *Aedes albopictus* mosquito cells at +37 °C for 12 h, and incubated for 7 days at +28 °C [28]. Media from virus-negative C6/36 cells was used as a control for transfection. After passaging twice in C6/36 cells, cell culture media containing ZIKV was centrifuged (12000 g, 20 min, +4 °C), and frozen (-80 °C). Viral titers were quantified in triplicates in VERO cells with the endpoint dilution assay described below.

All recovered ZIKV variants showed stability of *de novo* introduced CpG dinucleotides after ten passages in VERO cells and infection in neonatal mice [7]. All virus stocks and cell cultures were free of mycoplasma contaminations as confirmed by PCR Detection Kit (Sigma #MP0035).

Replication Phenotypes of CpG-recoded ZIKV Variants In Vitro

We evaluated the viral replication kinetics in cell cultures of human origin (HMC3, NPCs, GSC 157, and GSC 528) as previously described [7]. Cells in suspension were inoculated at MOI of 0.01 in 100 µl of appropriate cell culture medium. Eppendorf tubes with inoculated cells were incubated at +37°C for 1 h and shaken gently every 10 min. Afterward, cells were washed three times with serum-free media and seeded in 96-well plates. Wells were first coated with growth factor-reduced Matrigel and prefilled with 150 µl of cell culture medium. Then 50 µl of cell suspension was added on top to get a resulting concentration of 4×10^4 (HMC3) or 10^5 (NPC, GSC 157, and GSC 528) cells per well. Plates corresponding to different experimental time points were infected at the same initial time. Mock-infected cells were included as controls in each plate.

Infected plates were incubated (5% CO₂, +37°C) until the sampling time point. Then supernatants were collected, clarified (2000g, 5 min) and frozen (-80°C) until subsequent infectious virus quantification with the endpoint dilution assay described below [7,30-34]. Cell culture media were serially diluted fivefold in four replicates starting from 1:5, and 50 µl of each

dilution was added to confluent VERO cells cultured in 96-well plates. Dilutions were made in complete cell culture media. After 2 h, 150 µl of fresh media was added to each well. The cells were incubated for 7 days. After washing and drying, the plates were kept at -20°C at least for 2 h or until use. Cell fixation and staining with virus-specific 4G2 Abs were done, as previously described [7,30-34]. Fifty percent tissue culture infective dose (TCID₅₀) endpoint titers were calculated by the Spearman-Kärber formula and expressed in a decimal logarithm. Media from mock-inoculated cells were used as negative controls.

After the supernatant collection, the plate with infected cells was dried and frozen (-20°C). Plates were stained with anti-pan flavivirus E protein monoclonal 4G2 antibodies (Abs; ATCC #HB-112), and infected cells were counted in the well with bright-field microscopy at 200x magnification and expressed per cm² [7,30]. Cell culture supernatants and fixed plates were collected at 0-5 days post-inoculation, with three technical replicates and three biological replicates per time point for each ZIKV variant.

Cell Proliferation Assay

Cells in suspension were inoculated at MOI of 1 in 100 µl of appropriate cell culture medium. Eppendorfs with inoculated cells were incubated at +37°C for 1 h and shaken gently every 10 min. Afterward, cells were washed with media and seeded in 96-well plates. Wells were first prefilled with 50 µl of cell culture medium and 50 µl of cell suspension was added on top to get a resulting concentration of 5x10³ cells per well. Plates corresponding to different experimental time points were infected at the same initial time. Mock-infected cells were included as controls in each plate.

On days 0, 1, 3, 5 and 7, cell proliferation was analyzed with the CellTiter-Glo Luminescent Assay (Promega #G7571) according to the manufacturer's instructions. For analysis, 96-well black plates (PerkinElmer #6005660) and CellTiter-Glo reagent were equilibrated to room temperature (+22°C, 30 min). Then CellTiter-Glo reagent was added to each well, and plates were placed on an orbital shaker (+22°C, 12 min). Luminescence was quantified on a Promega GloMax Explorer microplate reader. All data were normalized to day 0 and expressed as relative cell proliferation.

Chicken Chorioallantoic Membrane (CAM) Assay for GSCs

Chicken CAM assays are commonly used in cancer research [35,36]. Here, to develop an *in ovo* model for glioblastoma, we implanted GSCs on chicken CAM.

Experiments were performed following the Canadian Council on Animal Care guidelines for humane animal use and were approved by the University of Saskatchewan's Animal Research Ethics Board (#004CatA2017). Fertilized eggs of Lohmann Selected Leghorn layers (LSL-Lite) were placed in an incubator (GQF #1502) and maintained at $37.8 \pm 0.1^\circ\text{C}$ and $50 \pm 2\%$ of relative humidity with turning every 2 h. The day on which eggs were placed in the incubator was considered as day 0 of embryonic development (ED).

On ED 6, eggs were candled and infertile eggs were excluded. Viable eggs (94.1%) were turned into a horizontal position and the upper surface was marked with a pencil. The automatic rotation was turned off starting from this day. On ED 7, windowing and CAM dropping were done as previously described with a rotary tool and generating an artificial air sac [37-40]. The created window was closed with a semipermeable adhesive film (3M Tegaderm Roll #16004). Eggs were placed back to an incubator, and relative humidity was increased to $54 \pm 2\%$.

On ED 10, we verified egg viability and implanted GSC as previously described [41,42]. First, the adhesive film was removed with sterile scissors. Then CAM blood vessel was damaged by squeezing with forceps until mild bleeding was visually observed and sterile Teflon O-ring (the O-Ring Store #AS568-010 TEF010) was placed with sterile forceps on CAM with the ruptured vessel in the middle [43]. One million live cells were resuspended in 50 μl of fresh ice-cold cell culture medium containing 25% of Matrigel HC (Corning #354262). Then ice-cold cell suspension was slowly placed inside the O-ring (25 $\mu\text{l}/\text{min}$) to allow gel solidifying and preventing leakage of cell suspension outside the ring.

The window was closed again with the adhesive film, and eggs were placed to the egg incubator (37°C) with windows facing to the top.

On ED 19, viable embryos were placed at $+4^\circ\text{C}$ for 2h. Macro photos of opened eggs with tumors were taken with a stereomicroscope (Leica #M80 with #MC170 HD digital camera) and tumor volumes were calculated according to Hagedorn *et al.* [44]:

$$V = \frac{4}{3}\pi \left(\frac{1}{2} \sqrt{(d_1 d_2)} \right)^3,$$

where d1 and d2 are diameters of the tumor measured with ImageJ 1.51r.

Tumors were separated from surrounding tissues with sterile forceps and scissors and placed in 10% paraformaldehyde for hematoxylin and eosin (H&E) staining, embedded in NEG-50 medium (ThermoFisher #6502) and snap-frozen (-80°C) for immunohistochemistry, or preserved in PCR-grade Eppendorfs (-80°C) for ZIKV quantification.

Oncolytic Phenotypes of CpG-recoded ZIKV Variants in the Glioblastoma CAM Model

We evaluated ZIKV loads and the tumor growth reduction in CAM assay. For inoculation, cells were resuspended in 200 µl of appropriate cell culture media as described above; media contained ZIKV variants at MOI of 0.25. Eppendorf tubes with cells were incubated at +37°C for 1 h and shaken gently every 10 min. Afterward, cells were washed three times with media and seeded on CAM (10⁶ cells/egg) at ED 10 as described above. Eggs were placed in incubators until the sampling time point at ED 19.

At sampling, the size of tumors was measured as described above. To assess size reduction in ZIKV-infected tumors, the average size of the tumors in the mock-infected group was divided by the average size in ZIKV-infected tumors.

RNA Extraction and Reverse Transcriptase Quantitative Polymerase Chain Reaction Assay (RT-qPCR)

Single-use scalpel blade and sterile forceps were used to separate 9-54 mg of tumor tissue. Tissue samples were weighed on analytical balances, and after adding 0.6 ml of lysis buffer were homogenized using RNase-free stainless steel beads and TissueLyser II (QIAGEN) operating for 5 min at 25 Hz. Then RNA extraction was continued with the PureLink RNA Mini Kit (Invitrogen #12183025) according to the manufacturer's instructions.

ZIKV specific SYBR Green-based one-step RT-qPCR was used for ZIKV RNA quantification in tumors [45]. PCR reactions were conducted on the StepOne Plus platform (Life Technologies) and analyzed using StepOne 2.3 software. The reaction mixture (20 µl) consisted of 10 µl 2x SensiFAST SYBR Hi-ROX One-Step Mix (Bioline #BIO-73005), 0.4 µl RiboSafe RNase

Inhibitor, 0.2 µl reverse transcriptase, 0.8 µl (400 nM) of each primer (ZIKV-F10287: 5'-AGGATCATAGGTGATGAAGAAAAGT-3' (SEQ ID NO: 1); ZIKV-R10402: 5'-CCTGACAACACTAAGATTGGTGC-3' (SEQ ID NO: 2)), 3.8 µl nuclease-free water and 4 µl RNA template. A reverse transcription step of 10 min at 45°C and an enzyme activation step of 2 min at 95°C were followed by 40 amplification cycles (5 s at 95°C and 34 s at 60°C). RNA (10238-10444 = 207 nt amplicon) from a stock of the ZIKV PRVABC59 strain [GenBank: KU501215.1] was used to generate a standard curve and quantify viral RNA loads. The standard curve had a wide dynamic range (10^2 - 10^9 copies/reaction) with a high linear correlation ($R^2 = 0.9997$) between the cycle threshold (Cq) value and template concentration. The slope of the standard curve (-3.4351) corresponded to the 95.5% reaction efficiency level. PCR values were corrected for tissue weights and upon logarithmical transformation expressed as ZIKV RNA genome copies per gram. In all PCR tests, we used VERO cell culture media containing ZIKV as a positive PCR control. As a negative control, we used samples from mock-inoculated cells. Strict precautions were taken to prevent PCR contamination. Aerosol-resistant filter pipette tips and disposable gloves were used. Kit reagent controls were included in every RNA isolation and PCR run.

Histopathology and Immunohistochemistry (IHC)

Tumors collected from chicken eggs were fixed in formalin for subsequent H&E staining.

For immunohistochemistry, staining was performed as previously described [7,30,46] with some modifications. Briefly, slide chambers were covered with Matrigel and seeded with cells. Slide chambers after reaching cell confluence or 10 µm cryosections of tumors were fixed in 10% buffered formalin at +4°C for 15 minutes. After treatment with 1% H₂O₂ and 1% Triton X-100 (20 min, RT), chambers and tissue sections were incubated with primary monoclonal Abs (mouse anti-ZIKV: ATCC #HB-112, 1/20; mouse anti-TGM2: ThermoFisher #MA5-12739, 1/50; rat anti-SOX2: eBioscience #14-9811-80, 1/50) for 1h at +37°C. Afterward, chambers and cryosections were incubated with a horseradish peroxidase-conjugated reagents (ZIKV and TGM2: anti-mouse Envision HRP labelled polymer, Agilent #K4001; SOX2: rabbit anti-rat, Abcam #ab6734, 1/200) following Lab Vision Ready-To-Use AEC Substrate System (Abcam #ab64252) according to the manufacturer's instructions. Subsequently, tissues were

counterstained with hematoxylin. Examination and imaging were performed under a microscope (Leica #DM2000 LED with #MC170 HD digital camera).

Statistical Analysis

Zika virus infectious titers in cell culture supernatants from NPC, HMC3, and GSCs and cell proliferation data were assessed with non-parametric analysis of variance after aligned rank transformation [47]. Results of statistical analyses are provided in text (main effects), figures or figure legends (interaction effects in a two-way model).

Zika virus loads and tumor growth *in ovo* were compared using the Kruskal-Wallis H test and Dunn's multiplicity-adjusted post-test.

Results were considered significantly different when $P < 0.05$.

Results

CpG-recoded ZIKV Variants Show Reduced Infection Kinetics in Nonmalignant Human Brain Cells and Distinct Oncolytic Activity in Different Glioblastoma Stem Cells In Vitro

We compared infection kinetics caused by WT and CpG-recoded ZIKV variants in HMC3 and NPCs representing human nonmalignant brain cells, and in GSC 528 and GSC 157 representing human glioblastoma stem cells (Figure 2) [21,22].

Wild-type, Permuted, and E+32CpG variant—the variant with the lowest CpG content among all recoded variants—showed similarly high infectious viral loads ($P = 0.87-0.99$) and kinetics in the HMC3 cell line (Figure 2a). In contrast, other CpG-recoded variants with the higher CpG content—ZIKV E+102CpG ($P = 0.059$) and ZIKV E/NS1+176CpG ($P = 0.001$; only $0.7 \log_{10}$ above the detection limit)—showed reduced infectious titers (Figure 2a). All ZIKV variants, except ZIKV E/NS1+176CpG ($P = 0.018$), replicated more slowly in NPCs producing low infectious titers ($P = 0.96-0.99$) (Figure 2b). The ZIKV NS1/E+176CpG variant—one with the highest CpG content among all recoded viruses—did not show infectious titers in NPCs (Figure 2b). Quantification of virus-positive cells was in accordance with the endpoint dilution assay (Figure 7, particularly Figure 7a,b).

Results of the proliferation assay in nonmalignant brain cells were in a high agreement with infection kinetics: HMC3 cells infected with both ZIKV E+102CpG and ZIKV E/NS1+176CpG

showed high proliferation—close to the mock-infected control ($P = 0.29-0.46$; Figure 2c). In contrast, HMC3 cells infected with WT, Permuted, and ZIKV E+32CpG did not show proliferation ($P < 0.001$). Infection with any ZIKV variant did not affect the proliferation of NPCs ($P > 0.99$; Figure 2d).

Zika virus variants showed distinct infection phenotypes in different GSCs. In GSC 528, only the E/NS1+176CpG variant—the variant with the highest CpG content—showed a considerable reduction in infectious titers ($P \leq 0.002$; Figure 2e) and in the number of ZIKV-infected cells (Figure 7b). All other variants, including ZIKV E+102CpG—the variant with the second-largest CpG content, showed similar infection kinetics with high infectious titers ($P = 0.15-0.44$). In GSC 157, however, infection with all ZIKV variants resulted in infectious titers close or below the detection limit (Figure 2f).

In agreement with infection phenotypes, all ZIKV variants (except ZIKV NS1/E+176CpG) considerably reduced proliferation of GSC 528 ($P \leq 0.005$; Figure 2g). More resistant to infection GSC 157 did not show changes in proliferation kinetics ($P \geq 0.19$; Figure 2h) under the conditions tested.

In summary, while increasing the ZIKV genomic CpG content reduced infection kinetics in nonmalignant brain cells (Figures 2a,b), the recoded ZIKV E+102CpG variant in particular showed oncolytic activity in glioblastoma stem cells as represented by high viral loads and reduced GSC proliferation. The *in vitro* oncolytic activity, however, was induced only in GSC 528 (Figure 2e).

Implantation of Human GSCs on CAM Results in Tumor Growth

To further assess whether CpG-recoded ZIKV variants show oncolytic activity in GSC-derived tumors, we developed a CAM model.

GSC 528 and GSC 157 had a different growth pattern and cell marker expression. *In vitro*, GSC 528 formed loose spheres (Figure 3a), while GSC 157 formed compact spheres that stayed integrated after gentle pipetting (Figure 3b). We stained both cell types with SOX2 and TGM2 markers; these markers have been previously used to characterize GSCs 528 and 157 [22]. GSC 528 were positive for TGM2 (Figure 3c), but negative for SOX2. While SOX2 expression was previously described in GSC 528, the loss of this marker during passaging was also reported that

highlights a mixed composition of these cells [48,49]. In accordance with the previous report [22], GSC 157 showed no TGM2 and strong SOX2 expression (Figure 3d).

In agreement with different *in vitro* growth patterns and distinct cell phenotypes, GSC 528 and GSC 157 showed different tumor formation phenotypes *in ovo*. Both cell types formed compact round-shaped solid tumors (Figures 3e,f) with the vascular network (Figures 3g); however, GSC 528 tumors were on average 6.2 times larger than GSC 157 tumors (Figures 3i). Histologically, GSC-derived tumors were vascularized (Figures 4a,b). GSC 528 tumors were encapsulated in the CAM mesenchyme, while GSC 157 had multiple nuclear-free zones with an unstructured background stained with eosin (Figures 4a,b). In accordance with *in vitro* staining, IHC in GSC 528 tumors showed expression of TGM2 (Figure 4c), while GSC 157 tumors lost the SOX2 marker (Figures 4d,f).

In summary, we demonstrated GSC growth and tumor formation in CAM. In accordance with distinct *in vitro* growth and protein marker expression, GSC 528 and GSC 157 showed different patterns of tumor growth and formation *in ovo*.

CpG-recoded ZIKV Variants Show Distinct Oncolytic Activity in Different Glioblastoma Stem Cells In Ovo

After the *in ovo* model was established, we compared infection kinetics and oncolytic activity of ZIKV variants in GSC-derived tumors. As in the previous mouse study [14], we used *in ovo* transplantation of GSCs pre-treated with ZIKV; this approach partially reproduces the prevention of glioblastoma recurrence in the clinical setting, where tumor is surgically removed and then chemotherapy and radiation are applied to eliminate residual malignant cells [50]. Because *in vitro* infection phenotypes caused by Permuted and ZIKV E+32CpG variants did not differ from the WT variant, we only focused on WT, ZIKV E+102CpG, and E/NS1+176CpG variants.

Interestingly, both CpG-recoded variants showed higher, on 0.8-1.3 log₁₀, viral loads than WT variant in GSC 528 and 157 tumor tissues (Figure 5a,b).

The GSC 528 tumor size was considerably reduced—16 and 13 times—in ZIKV-WT ($P < 0.0001$) and ZIKV E+102CpG groups ($P < 0.0001$), respectively (Figures 5c,e). In the ZIKV E/NS1+176 CpG group, the tumor size reduction was lower—3.4 times—but still significant ($P = 0.03$; Figures 5c,e). Despite high viral titers in GSC 157 tumors (Figure 5b), the tumor size

reduction was only 1.8-2.3 times in all groups ($P = 0.22-0.50$; Figures 5d,f). Zika virus antigens, phenotypical alterations, and size reduction in tumors were also revealed by IHC and H&E staining (Figure 6).

To summarize, all ZIKV variants replicated in GSC-derived tumors showing high viral titers. All ZIKV variants significantly reduced the growth of GSC 528-derived tumors; in contrast, GSC 157-derived tumors were relatively more resistant to oncolytic activity.

Discussion

A main goal of these studies was to probe CpG recoding in a viral genome for the development of oncolytic candidates. We studied whether ZIKV variants with the increased CpG content have reduced infection kinetics in nonmalignant brain cells while retaining virulence in GSCs. *In vitro*, ZIKV E+102CpG and E/NS1+176CpG variants with the increased CpG content showed reduced infection kinetics in nonmalignant microglia cells; the proliferation activity of nonmalignant cells was also mostly not affected. In contrast, similar to wild-type virus, the recoded ZIKV E+102CpG—the variant with the second-largest CpG content—showed oncolytic activity in GSCs 528 as represented by high viral loads and reduced cell proliferation. Next, we established, to our knowledge the first, CAM model for GSC-derived tumors, and demonstrated high oncolytic activity of the ZIKV E+102CpG variant. In accordance to *in vitro* results, *in ovo*, oncolytic activity also depended on the viral CpG content: while GSC 528-derived tumors infected with ZIKV E/NS1+176CpG showed only moderate volume reduction (3.4 times), the ZIKV E+102CpG variant showed oncolytic activity with high tumor volume reduction comparable to WT ZIKV (13-16 times; Figure 5e). This dissonance of different CpG-recoded variants demonstrates that oncolytic activity of a virus may be tuned by adjusting the number of *de novo* introduced CpG dinucleotides within a viral genome.

Oncolytic activity of WT and recoded ZIKV variants varied in GSCs derived from different patients and with different cell phenotype. While ZIKV variants showed oncolytic activity in GSC 528 *in vitro* and *in ovo*, GSC 157 were relatively more resistant to oncolytic activity. A recent study showed that ZIKV preferentially infects and kills GSCs in a SOX2-dependent manner [51]; however, our data suggest that SOX2-negative GSCs (like GSC 528) can be susceptible to ZIKV oncolytic activity and *in vitro* SOX2-positive GSCs (like GSC 157) can be

relatively more resistant. The heterogeneity in susceptibility and resistance of GSCs from different patients to chemotherapy is well-known [21]. The resistance of some GSCs to ZIKV oncolytic activity is an important finding, emphasizing the desirability of personalized oncolytic therapy and/or strategies to overcome resistance mechanisms in GSCs.

An important consideration for therapy is the safety of oncolytic viruses for patients and public health. Recoded oncolytic candidates with hundreds extra CpG dinucleotides most probably will show rare or no reversion to virulence; in support, the stability of *de novo* introduced CpGs in the ZIKV genome during *in vitro* and *in vivo* infection has been demonstrated in our recent study [7]. Moreover, in contrast to the WT virus, the present most promising ZIKV E+102CpG oncolytic candidate previously showed excellent safety pattern in mouse pregnancy model [7]. Another potential perspective for the safe CpG recoding oncolytic approach is in combination with other established oncolytic strategies; for example, CpG recoding may serve as an additional safety level. A small number of nucleotide mutations or deletions may determine attenuation in efficient oncolytic viruses—e.g., in a modified ZIKV with only 10 nucleotide deletions in 3' UTR [52]. This reliance on a small number of critical mutations/deletions in oncolytic viruses might lead to reversion to virulence during highly efficient replication in GSCs that are more conducive to infection than nonmalignant host cells. One more example—Seneca Valley virus—is a promising oncolytic candidate that does not cause infection in humans but poses a significant threat to livestock [53]. A rational strategy for CpG recoding may reduce the potential for zoonotic spillover of oncolytic viruses and outbreaks in livestock.

Towards efficacy, novel therapeutic approaches against glioblastoma are formulated to modulate the immune response towards the tumor and surrounding microenvironment [54]. In DNA molecules, CpG dinucleotides can directly activate B cells, natural killer cells, dendritic cells, monocytes, and macrophages through TLR9 stimulation [55]; introduced CpG dinucleotides in synthetic RNA molecules also may activate cellular immune responses, however, the mechanisms of activation remain unclear [56]. Without wishing to be bound by theory, it is contemplated that the increased CpG content in recoded RNA viruses may, perhaps, lead to local brain immune activation which may augment oncolytic efficacy against glioblastoma.

In this study, we established an *in vitro* and *in ovo* experimental toolbox with ZIKV-sensitive (GSC 528) and ZIKV-resistant (GSC 157) cells. This experimental system may be used to

identify GSC factors that determine resistance level to ZIKV oncolytic activity, for example.

Collectively, these results support the CpG recoding approach for oncolytic therapy. These findings contribute to a better understanding of interactions between CpG-recoded viruses, tumor, tumor environment, and host responses. These results support the CpG-recoding technology developed herein as being adaptable, and may allow for oncolytic viruses with desirable therapeutic activity.

One or more illustrative embodiments have been described by way of example. It will be understood to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as defined in the claims.

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All references cited herein and elsewhere in the specification are herein incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. Use of an oncolytic flavivirus for the treatment of cancer, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.
2. Use of an oncolytic flavivirus in the manufacture of a medicament for the treatment of cancer, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.
3. A method for treating cancer in a subject in need thereof, said method comprising:

administering an oncolytic flavivirus to the subject, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type;

thereby killing one or more cancer cells in the subject.
4. An oncolytic flavivirus, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type, for use in the treatment of cancer in a subject in need thereof.
5. The use of claim 1 or 2, the method of claim 3, or the oncolytic flavivirus of claim 4, wherein the CpG recoded oncolytic flavivirus has reduced infection kinetics toward healthy cells as compared to wild-type virus, and is oncolytic toward cancer cells.
6. The use, method, or oncolytic flavivirus of any one of claims 1-5, wherein the cancer is brain cancer or spinal cord cancer.
7. The use, method, or oncolytic flavivirus of any one of claims 1-6, wherein the cancer is glioblastoma or other brain tumor.

8. The use, method, or oncolytic flavivirus of claim 6 or 7, wherein the cancer is or comprises SOX2-negative glioblastoma or glioblastoma stem cells (GSCs), TGM2-positive glioblastoma or GSCs, or SOX-2 negative and TGM2-positive glioblastoma or GSCs, or any combinations thereof.
9. The use, method, or oncolytic flavivirus of any one of claims 1-8, wherein the oncolytic flavivirus is a cytosine-phosphate-guanine (CpG) recoded Zika virus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.
10. The use, method, or oncolytic virus of any one of claims 1-9, wherein the frequency of CpG dinucleotide instances within the viral genome is increased by about 30 to about 180 instances as compared to wild-type, such as about 50 to about 110 instances as compared to wild-type; about 80 to about 110 instances as compared to wild-type; about 95-105 instances as compared to wild-type; or about 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, or 105 instances as compared to wild-type.
11. The use, method, or oncolytic virus of any one of claims 1-10, wherein the silent mutations which increase frequency of CpG dinucleotide instances within the viral genome are primarily, or entirely, localized to the E genomic region of the viral genome.
12. The use, method, or oncolytic virus of any one of claims 1-11, wherein any one or more of the NS1 region, the C region, the prM region, the NS2a region, the NS2b region, the NS3 region, the NS4a region, the NS4b region, and/or the NS5 region of the viral genome, or any combinations thereof, are free, or substantially free, of the silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.
13. The use, method, or oncolytic virus of any one of claims 1-12, wherein the NS1 region of the viral genome is free, or substantially free, of the silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.
14. The use, method, or oncolytic virus of any one of claims 1-13, wherein the size of the viral genome of the oncolytic virus is the same, or substantially the same, as the size of the wild-type viral genome.

15. The use, method, or oncolytic virus of any one of claims 1-14, wherein the oncolytic flavivirus is a cytosine-phosphate-guanine (CpG) recoded Zika virus of an African or Asian lineage, having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.
16. The use, method, or oncolytic virus of any one of claims 1-15, wherein the oncolytic flavivirus is a cytosine-phosphate-guanine (CpG) recoded Zika virus of contemporary Asian ZIKV H/PF/2013 strain, having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome.
17. The use, method, or oncolytic virus of any one of claims 1-16, wherein the viral genome of the oncolytic virus is or comprises a sequence of any one of SEQ ID Nos: 5-7, or a sequence having at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity therewith.
18. The use, method, or oncolytic virus of any one of claims 1-17, wherein the viral genome of the oncolytic virus is or comprises a sequence of SEQ ID NO: 6, or a sequence having at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity therewith.
19. An oncolytic flavivirus, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type.
20. A pharmaceutical composition comprising an oncolytic virus as defined in any one of claims 1-19, and a pharmaceutically acceptable carrier, excipient, buffer, or diluent.
21. A glioblastoma stem cell-derived tumor model, said tumor model comprising glioblastoma stem cell(s) implanted *in ovo* on chicken chorioallantoic membrane (CAM).
22. A method for preparing a glioblastoma stem cell-derived tumor model, said method comprising:

incubating a fertilized egg;

- windowing and CAM-dropping the fertilized egg to generate an artificial air sac;
- damaging the CAM blood vessel;
- placing a retaining member around the damaged blood vessel;
- introducing glioblastoma stem cell(s) to a region of the CAM bounded by the retaining member; and
- incubating the fertilized egg to allow tumor growth on the CAM.
23. A glioblastoma stem cell-derived tumor model prepared according to the method of claim 22.
24. A method for identifying anti-cancer agents targeting glioblastoma, said method comprising:
- treating a glioblastoma stem cell-derived tumor model according to claim 21 or 23 with a candidate anticancer agent; and
- determining whether tumor size and/or proliferation in the glioblastoma stem cell-derived tumor model is reduced by treatment with the candidate anticancer agent;
- wherein a reduction in tumor size and/or proliferation in the glioblastoma stem cell-derived tumor model identifies the candidate anticancer agent as an anti-cancer agent targeting glioblastoma.
25. A kit comprising any one or more of an oncolytic virus as defined in any one of claims 1-19, a pharmaceutical composition according to claim 20, a glioblastoma stem cell-derived tumor model according to claim 21 or 23, instructions for performing a method according to any one of claims 3, 22, or 24, or any combinations thereof.

FIGURE 1

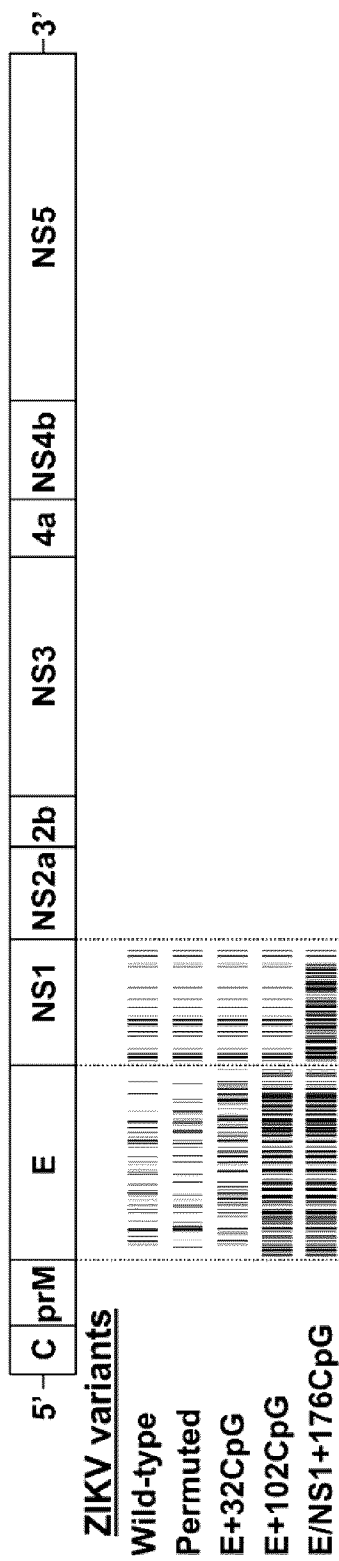


FIGURE 2

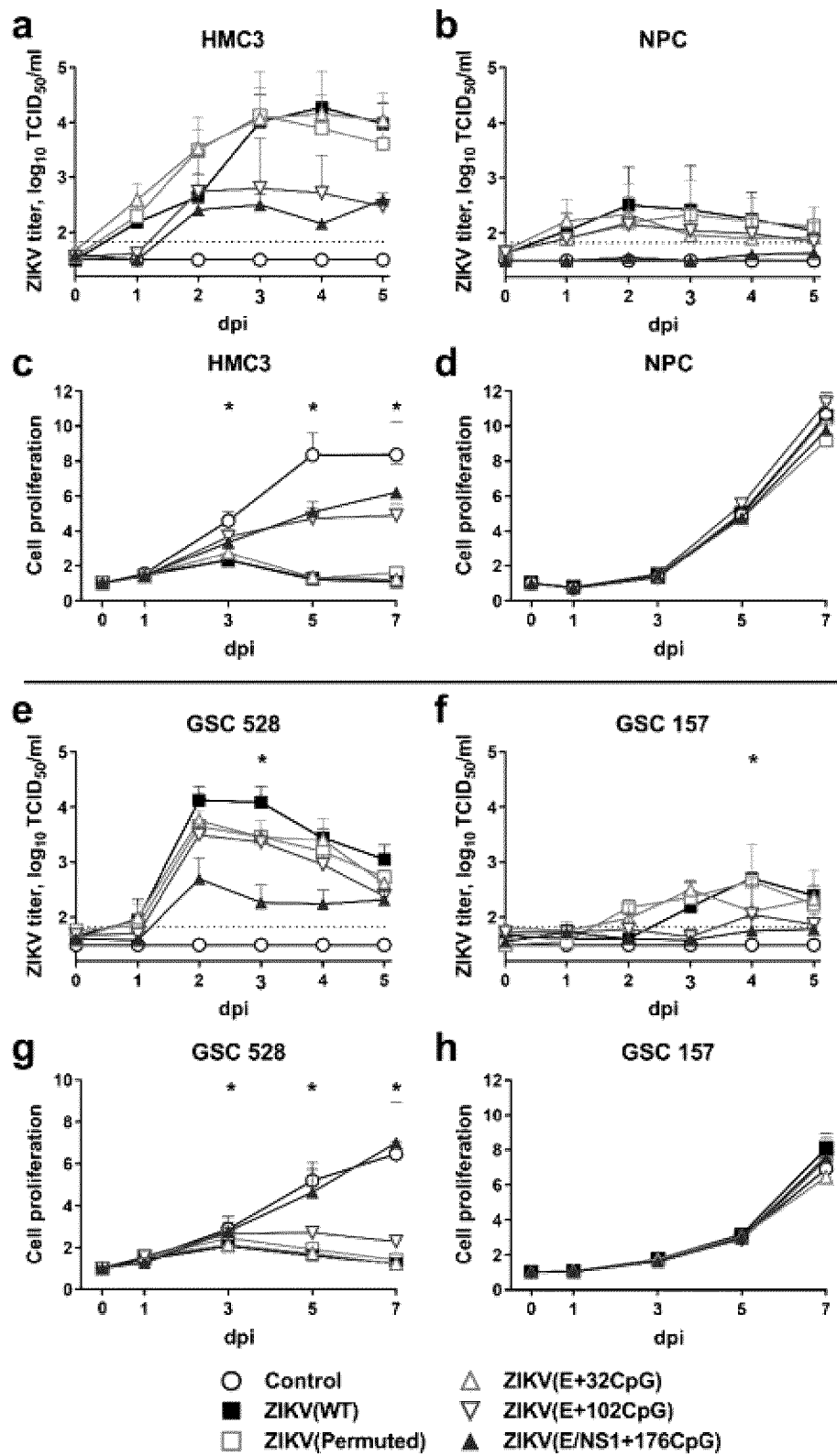


FIGURE 3

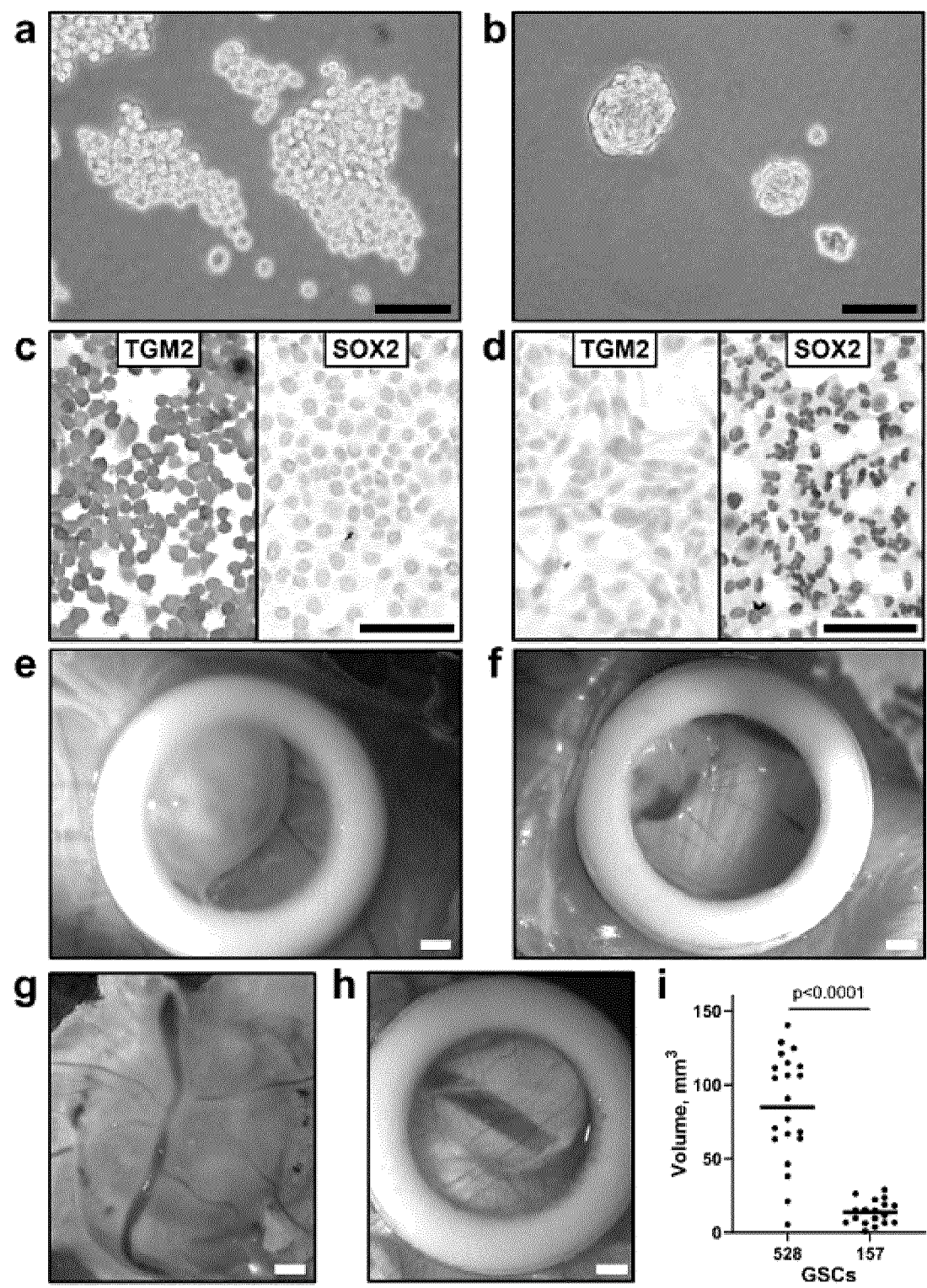


FIGURE 4

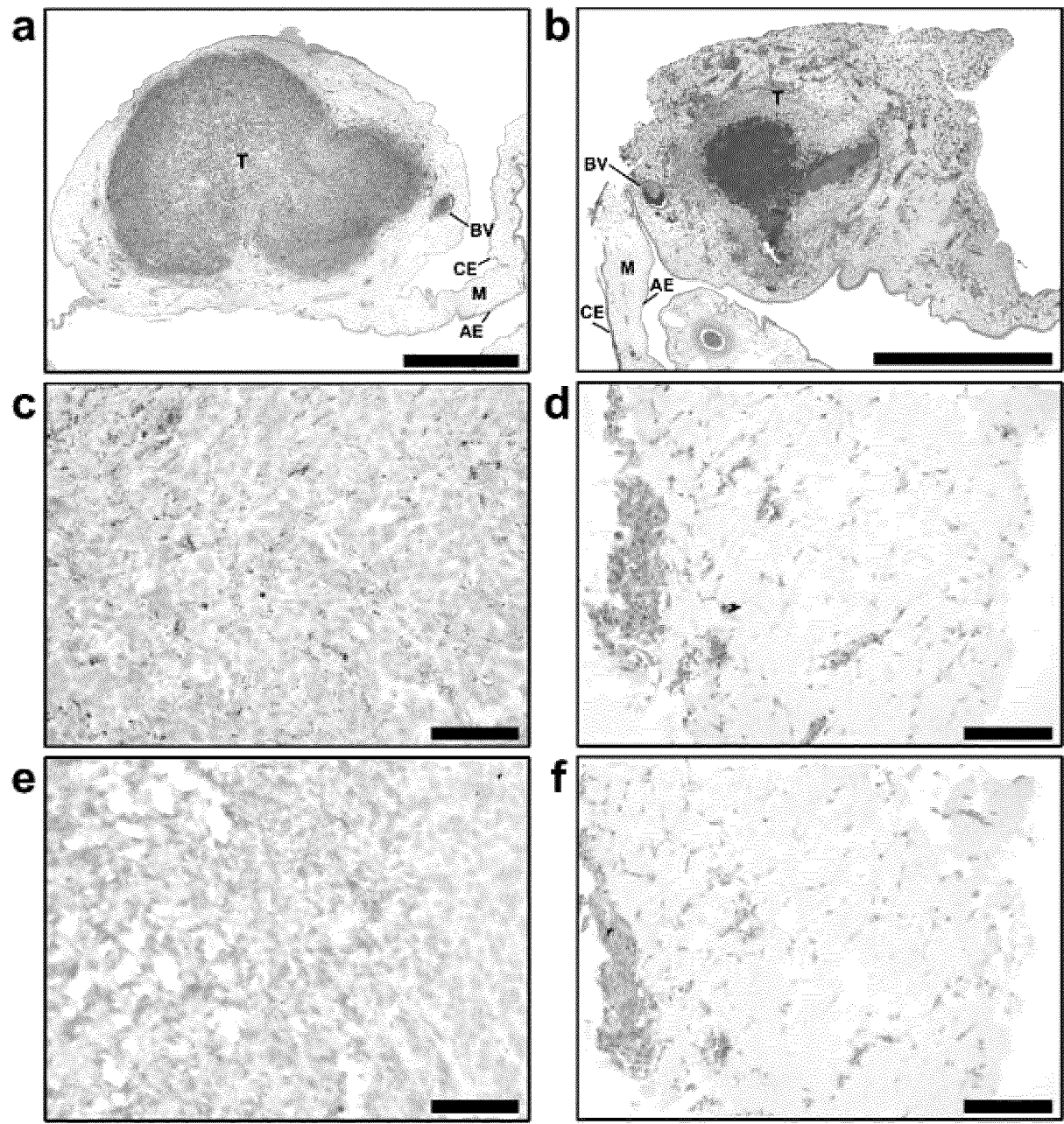


FIGURE 5

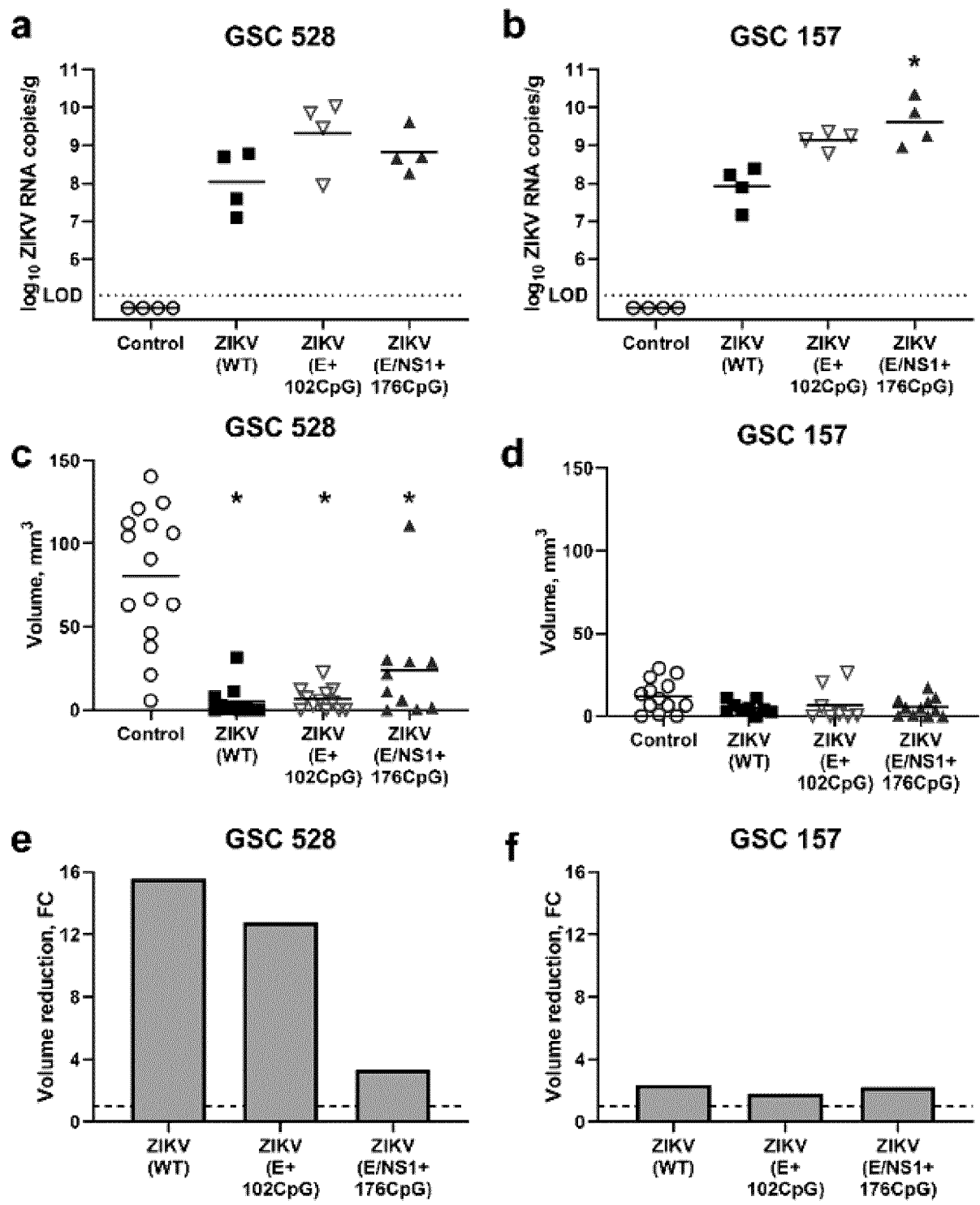


FIGURE 6

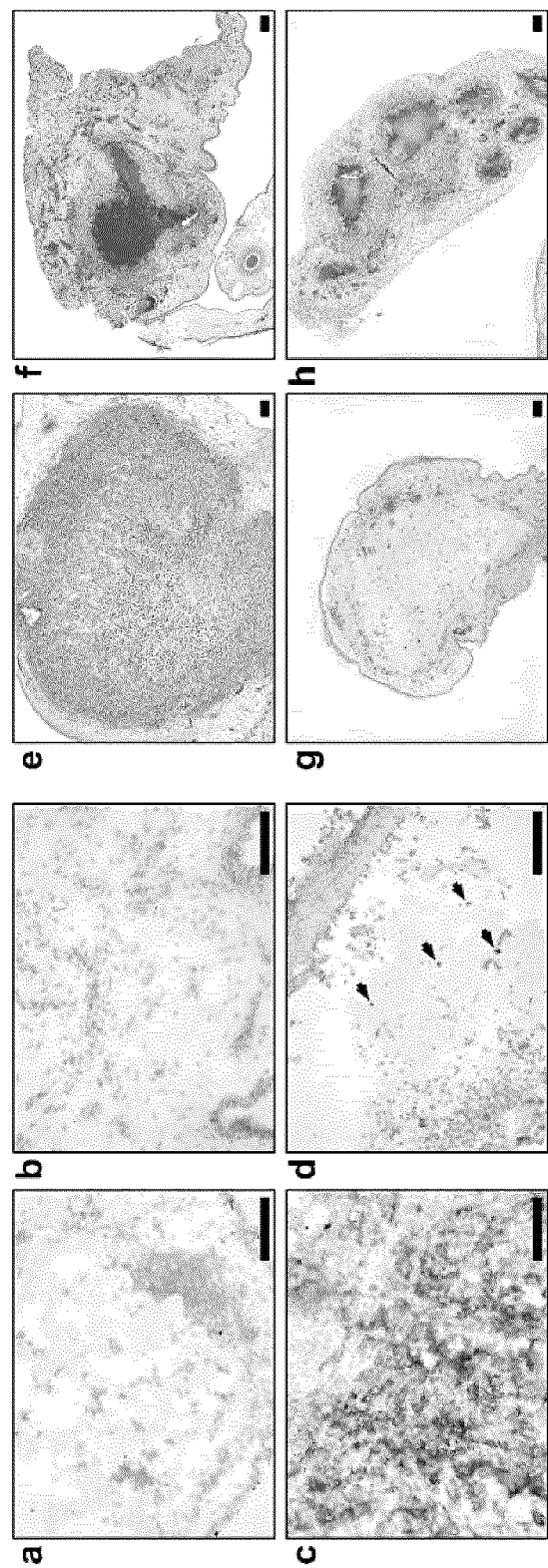


FIGURE 7

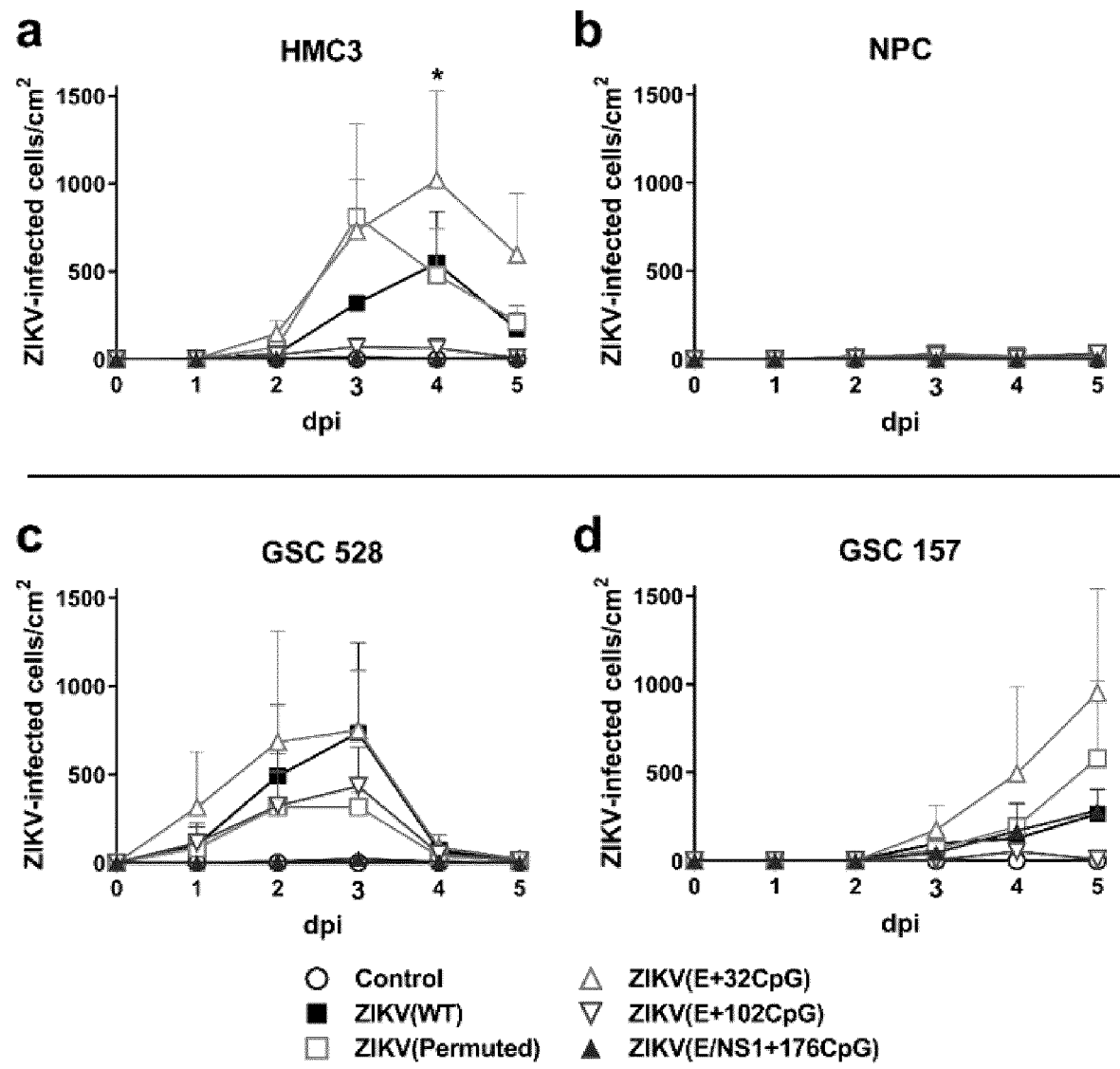


FIGURE 8

Genome Sequence of Wild-Type ZIKV Variant (SEQ ID NO: 3):

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E

NS1

FIGURE 8 CON'T

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FIGURE 8 CON'T

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Genome Sequence of Permuted ZIKV Variant (SEQ ID NO: 4):**>Permuted**

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FIGURE 8 CON'T

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NS1

FIGURE 8 CON'T

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FIGURE 8 CON'T

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Genome Sequence of E+32CpG ZIKV Variant (SEQ ID NO: 5):

>E+32CpG

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E

FIGURE 8 CON'T

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NS1

FIGURE 8 CON'T

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FIGURE 8 CON'T

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Genome Sequence of E+102CpG ZIKV Variant (SEQ ID NO: 6):**>E+102CpG**

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E

NS1

FIGURE 8 CON'T

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FIGURE 8 CON'T

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FIGURE 8 CON'T

Genome Sequence of E/NS1+176CpG ZIKV Variant (SEQ ID NO: 7):

>E/NS1+176CpG

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 tcacac**CGCG**ct**CGcCG**g**CGCG**ctagaagctgagatgga**CGgCGCG**aaggga**CG**actgt**CGT**ctgggtcatttgaaat
 gt**CG**actgaaaatggacaaactt**CG**attaaaagggtgtatcatatt**CGT**tgtgca**CGgCG**gtttcactttcacaaag
 atcc**CG**gcagagacacttca**CG**gcac**CGT**gac**CGT****CG**agggtccaatatg**CG**ggcac**CGaCG**ac**CGT**gtaaagttcc
CGCGcagatggc**CGT**ggacatgcaaa**CG**ctga**CG**ccagt**CG**gtagggttgatca**CGgCG**aat**CG**gtgatca**CG**gaaa
 gca**CG**gaaaact**CG**aagatgatgctagaacttgatc**CG**ccatt**CG**gagactcttatattgtatttg**CG**tagg**CG**ag
 aaaaaataa**CG**catcattggcat**CGA**ag**CG**ggagca**CG**attggaaaagcatttgaag**CG**actgtt**CG**agg**CG**caaa
 a**CG**aatggcagtttttgg**CG**acac**CGCG**gtgggactt**CG**gattcagt**CGgCG**gagcactgaatt**CGT**gggaaaaggaa
 ttcatacaaat**CGgCG**ag**CGT**tcaaatcattgttggagggaatgt**CGT**gggtttcacaaattctcataggaa**CG**
 ttgctgatgtggttgggtctgaacacaaagaa**CG**gatcaattt**CG**ctcatgtgtttag**CGT**ttgg**CG**gtgtgtgat
 attttgtcaactg**CG**gtttctgctgatgtgggatgtt**CG**gtagattttt**CG**aaaaaagaaa**CGCG**atg**CG**gtac**CG**
 gagtgtttgtttacaa**CGaCGT****CGA**ag**CGT**gg**CGCG**at**CG**ttacaaatatcatcc**CG**att**CGcCGCGT****CG**attggc**CG**
Gcagc**CGT**gaagcaagcatgggaaga**CG**gcac**CG**gatttcatc**CGT**tt**CGCG**aatggaaaacataatgtgg**CG**
 atc**CGT****CGA**aggagaactaaatg**CG**atcctggaagaaaa**CG**gggttcaactgac**CGT**gggtgg**CG**gat**CG**gtgaaaa
 atc**CG**atgtgg**CGCG**gtc**CG**cag**CG**attgcc**CGT**ttcc**CGT**aaatgaactt**CG**catggatggaaaagcttgggggaaa
 t**CG**tattt**CGTgCGCGCG**caagacaaacaacagttt**CGT**ttgga**CG**gagata**CG**ctgaaagaatgoc**CG**ctgaa
 acat**CGTgCGT**ggaatagttttctgggtggaagatca**CG**gttt**CGgCG**tatttcata**CG**ag**CGT**gtggctgaaagtt**CG**
GCGaagattatt**CGT**ttggaatgtgatc**CG**gcagttattggaac**CGCG**gtgaaaggaaaagaagc**CGT**tcacagtgat
 ctaggatactggat**CG**aaag**CG**aaaaaaa**CG**aca**CGT**gg**CG**gtgaaa**CGCGCG**catctgat**CG**agatgaaaa**CGTg**
CGaatggc**CG**aat**CG**caca**CGT**gtggac**CGaCG**aat**CGA**agagag**CG**atctgatcatto**CG**aaatcatttagc**CG**
 gtc**CG**ctcagtcataata**CGCGCG**aaggatat**CG**aactcaaatgaaaggac**CGT**ggcacag**CGA**agaactagaa
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 aaccactgcaag**CG**gaaggggtgat**CG**aggaatgggtgctgcagggagtgcacaatgccccactgt**CGT**tc**CG**ggcta
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 gcagatcaactgatcacatggatcacttctcccttggagtgttgtgattctgctcatggtgcaggaagggctgaa
 gaagagaatgaccacaaagatcatcataagcacatcgatggcagtgctggtagctatgatcctgggaggattttcaa
 tgagtgcactggctaagccttgcaattttgatgggtgccaccttcgoggaaatgaacactggaggagatgtagctcat

E

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FIGURE 8 CON'T

gatggttggtggagcggggatacctgcagccctatggaaaggtcattgatcttggatgtggcagagggggctggagt
 tactacgcccaccatccgcaaagttcaagaagtgaaggatacacaaaaggaggccctgggtcatgaagaacccat
 gttggtgcaaagctatgggtggaacatagtcctgtcttaagagtgggggtggacgtctttcatatggcggtgagccgt
 gtgacacgttgctgtgtgacataggtgagtcacatctagtcctgaagtgaagaagcacggacgtcagagtcctc
 tccatggtgggggattggcttgaaaaagaccaggagccttttgtataaaagtgtgtgcccatacaccagcactat
 gatggaaacccctggagcgaactgcagcgtaggtatgggggaggactggtcagagtgccactctcccgaactctacac
 atgagatgtactgggtctctggagcgaagcaacaccataaaaagtgtgtccaccacgagccagctcctcttgggg
 cgcatggacgggcccaggaggccagtgaatatgaggaggatgtgaatctcggtcttggcagcggggtgtggttaag
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 tctttgacgagaaccacccatataggacatgggcttaccatggaagctatgaggccccacacaagggtcagcgtcc
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 aaagaagagttcatcaacaaggttcgtagcaatgcagcattaggggcaatatttgaagaggaaaaagagtggaaagac
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 caaagtggtaaaaggtccttagaccagctgaaaaaggaagacagttatggacattatttcgagacaagaccaaaggg
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 gaggaagttctagagatgcaagacttgtggctgctgaggaggtcagagaaagtgaaccaactggttgagagcaacgg
 atgggataggctcaaacgaatggcagtcagtgagatgattgctgtgtgaagccaattgatgataggtttgcacatg
 ccctcaggttcttgaatgatatgggaaaagtttaggaaggacacacaagagtggaaacccctcaactggatgggacaac
 tgggaagaagttccgttttgcctcccaccacttcaacaagctccatctcaaggacgggaggtccattgtggttccctg
 ccgccaccaagatgaactgattggccgggcccgcgtctctccaggggcccggatggagcatccgggagactgcttgcc
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 cgaaatggacagacattccctatttgggaaaaaggaagacttgtggtgtggatctctcatagggcacagaccgcgc
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 ctacctatccaccaagttcgctacttgggtgaagaaggtctacacctggagtgtgtgaagcaaccaatcttagtgt
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 agcctatagtcaggccgagaacgccatggcacggaagaagccatgctgcctgtgagccccctcagaggacactgagtc
 aaaaaaccccacgcgcttggaggcgcaggatgggaaaaagaaggtggcgaccttccccaccccttcaatctggggcctg
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 attgacgctgggaaagaccagagactccatgagtttccaccacgctggccggccaggcacagatcgccgaatagcggc
 ggccggtgtggggaatccatgggtct

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2021/050693

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **C12N 7/01** (2006.01), **A61K 35/768** (2015.01), **A61P 35/00** (2006.01), **C12N 15/40** (2006.01), **C12N 5/09** (2010.01), **C12Q 1/02** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Keywords used across the whole IPC

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Canadian Patent Database, Genome Quest, STN

Keywords: Flavivirus, Zika, CpG, codon optimization, glioblastoma, SEQ ID NOs: 5-7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2019/126690 A1 (COLEMAN, J. R. et al.) 27 June 2019 (27-06-2019)	1-16, 19, 20, 25 17, 18
Y	WO 2019/0389912 A1 (HOURIOUX, C. et al.) 26 December 2019 (26-12-2019)	17, 18
X	BALČIŪNIENĖ, N. et al., " <i>Histology of human glioblastoma transplanted on chicken chorioallantoic membrane</i> ". Medicina, 2009, Vol. 45(2), pp. 123-131, ISSN Electronic ISSN 1648-9144 (abstract) [online] [retrieved on 15 July 2021 (15-07-2021)]. Retrieved from the Internet: < https://medicina.lsmuni.lt/med/0902/0902-05e.htm >	21-24

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* "A" "D" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance document cited by the applicant in the international application earlier application or patent but published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search
16 July 2021 (16-07-2021)

Date of mailing of the international search report
05 August 2021 (05-08-2021)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
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50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 819-953-2476

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2021/050693**Box No. 1** **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2021/050693**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2021/050693

The claims are directed to a plurality of inventive concepts as follows:

Group A ++- Claims 1-20, 25 are directed to an oncolytic flavivirus wherein, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type, as well as the use of said oncolytic flavivirus in the treatment of a cancer. It should be noted that Group A is directed to multiple groups, wherein each Group is defined by one specific oncolytic flavivirus (e.g. the flavivirus designated as GSC 157, or GSC 528 each define one group); and

Group B - Claims 21-24 are directed to a glioblastoma stem cell-derived tumor model, said tumor model comprising glioblastoma stem cell(s) implanted in ovo on chicken chorioallantoic membrane (CAM).

The claims must be limited to one inventive concept as set out in PCT Rule 13.

Since oncolytic flavivirus wherein, said oncolytic flavivirus being a cytosine-phosphate-guanine (CpG) recoded oncolytic flavivirus having silent mutations which increase frequency of CpG dinucleotide instances within the viral genome as compared to wild-type, as well as the use of said oncolytic flavivirus in the treatment of a cancer are known in the art (see D1), further, since a glioblastoma stem cell-derived tumor model, said tumor model comprising glioblastoma stem cell(s) implanted in ovo on chicken chorioallantoic membrane are known in the art (see D3) there is no common inventive concept between claims of Groups A ++ (as noted above, Group A is directed to multiple groups, wherein each Group is defined by one specific oncolytic flavivirus (e.g. the flavivirus designated as GSC 157, or GSC 528 each define one group) and Group B.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2021/050693

Patent Document Cited in Search Report	Publication Date	Patent Family Members	Publication Date
WO 2019/126690 A1	27-06-2019	AU2018392799 A1 CN111511397 A IL275550 A KR10-2020-0103020 A BR112020012241 A2 EP3727442 A1 JP2021508696 A IN202027030412 A	27 June 2019 (27-06-2019) 07 August 2020 (07-08-2020) 31 August 2020 (31-08-2020) 20 October 2020 (20-10-2020) 24 November 2020 (24-11-2020) 28 October 2020 (28-10-2020) 11 March 2021 (11-03-2021) 02 October 2020 (02-10-2020)
WO 2019/0389912 A1	26-12-2019	FR3052779 B1 WO2017/216505 A1 EP3472182 A1 CN109715649 A US20190389912 A1 AR108844 A1	22 June 2017 (22-06-2017) 21 December 2018 (21-12-2018) 24 April 2019 (24-04-2019) 03 May 2019 (03-05-2019) 26 December 2019 (26-12-2019) 03 October 2018 (03-10-2018)