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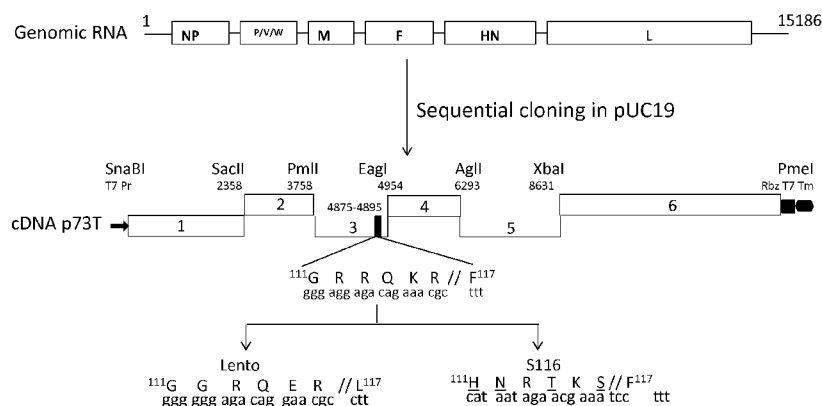
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(54) Title: COMPOSITIONS FEATURING AN ATTENUATED NEWCASTLE DISEASE VIRUS AND METHODS OF USE FOR TREATING NEOPLASIA

Figure 1. Construction of NDV 73T antigenomic cDNA



(57) Abstract: The present invention provides methods for inducing regression of tumors in human subjects, the methods utilize a modified mesogenic strain of Newcastle disease virus (NDV) with modified F protein cleavage site, which is non-pathogenic to poultry (lentogenic), but exhibits oncolytic properties. The disclosed methods provide safe, effective and reliable means to induce regression of a tumor in an individual in need thereof. These methods overcome the drawbacks of using pathogenic strains of viruses for human therapy.



COMPOSITIONS FEATURING AN ATTENUATED NEWCASTLE DISEASE VIRUS AND METHODS OF USE FOR TREATING NEOPLASIA

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims benefit of U.S. Provisional Application Serial No. 61/873,039, filed on September 3, 2013, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

10 Newcastle disease virus (NDV) is an avian virus causing a contagious bird disease affecting many domestic and wild avian species. Exposure of humans to infected birds (*e.g.*, in poultry processing plants) can cause mild conjunctivitis and influenza-like symptoms, but NDV otherwise poses no hazard to human health and most people are seronegative for NDV. Based on viral pathogenicity in chickens, NDV pathogenicity is classified as high (velogenic), medium (mesogenic) or low (lentogenic) as determined by the intracerebral pathogenicity index (ICPI). Due to agricultural concerns, the mesogenic and velogenic NDV having chicken virulence (ICPI>0.7) have been classified by the USDA as “select agents” since 2008. The Select Agents and Toxin List includes biological agents having the potential to pose a severe threat to human and animal health, to plant health, or to animal and plant products

20 Naturally occurring forms of NDV have been used in clinical studies as an immunotherapeutic and virotherapeutic biologic. NDV shows promise as an anticancer agent because of the virus’ ability to selectively kill human tumor cells with limited toxicity to normal cells. However, due to the reclassification of NDV as a select agent, the development of NDV as an anti-cancer agent has failed to progress. Other oncolytic viruses have shown considerable promise in clinical trials. To facilitate the development of NDV as a cancer therapy, new forms of the virus are required. Ideally, such new forms would retain their ability to target tumor cells, but would no longer cause disease in birds.

SUMMARY OF THE INVENTION

30 As described below, the present invention features compositions and methods for the treatment of neoplasia.

In one aspect the invention generally provides an attenuated Newcastle disease virus (NDV) having an F protein cleavage site of NDV LaSota strain or glycoprotein B (gB) of cytomegalovirus (CMV) (S116). In one embodiment of the invention the modified F protein cleavage sequence (FPCS) has one of the following sequence modifications S116: ¹¹¹H-N-R-T-K-S/F¹¹⁷; S116K: ¹¹¹H-N-K-T-K-S/F¹¹⁷; S116M: ¹¹¹H-N-R-M-K-S/F¹¹⁷; S116KM: ¹¹¹H-N-K-M-K-S/F-I¹¹⁸; or R116: ¹¹¹H-N-R-T-K-R/F-I¹¹⁸. In another embodiment the attenuated virus strain is a modified 73T strain. In yet another embodiment the attenuated NDV virus is r73T-R116 virus. In further embodiments the virus has an increased HN-L intergenic region. In yet other embodiments the HN-L intergenic region is a non-coding sequence between at least about 50-300 amino nucleotides in length. In further embodiments the non-coding sequence is derived from a paramyxoviruses type -1 (APMV-1), a respiratory syncytial virus (RSV) or a random sequence. In yet another embodiment the HN and L intergenic non-coding sequence is 60, 102, 144, 198, or 318 nt in length. In additional embodiments the virus has one or more heterologous polynucleotide sequences inserted at the P-M junction and/or the HN-L junction. In further embodiments the virus has two or more heterologous polynucleotide sequences, wherein at least one heterologous polynucleotide sequence is inserted at the P-M junction and at least one is inserted at the HN-L junction. In other embodiments the heterologous polynucleotide sequence is a transgene encoding a polypeptide that enhances the oncolytic properties of the virus. In yet another embodiment the transgene encodes a cytokine, cell surface ligand, and/or chemokine. In other embodiments the cytokine is selected from the group consisting of GM-CSF, IL-2, IL-21, IL-15, IL-12, and IL-12p70. In particular embodiments the cytokine is human GM-CSF. In other embodiments the heterologous polynucleotide sequence is a transgene encoding a detectable moiety. In certain embodiments the expression level of the detectable moiety correlates with virus replication. In yet another embodiment the F and HN genes of NDV are replaced by corresponding extracellular domains of canine Parainfluenza virus 5 (PIV 5) or pigeon paramyxovirus type 1 (PPMV-1). In another particular embodiment the virus is 73T-R116i-hGM-CSF. In other embodiments the attenuated virus has a Mean death time in eggs (MDT) of greater than 90hr or about 90-156 hours. In another embodiment the attenuated virus has an intracerebral pathogenicity index between about 0-0.7. In additional embodiments the attenuated virus has an intracerebral pathogenicity index of about 0. In yet another embodiment the attenuated virus has less than about 15% cytotoxicity in HT1080 cells. In further embodiments the attenuated virus selectively kills tumor cells with killing

efficiency at least 10 or 15%. In another embodiment the tumor cell killing efficiency in between about 75%-100%.

Another aspect of the invention generally features a method of selectively killing tumor cells, involving contacting a tumor cell with the attenuated Newcastle disease virus described herein. In another embodiment of the invention the tumor cell is a cell of a cancer of bladder, ovarian, brain, pancreas, prostate, sarcoma, lung, breast, cervical, liver, head and neck, gastric, kidney, melanoma, lymphoma, leukemia, thyroid, colon, and melanoma cancer cells. In yet another embodiment the method involves administering to the subject an effective amount of an attenuated Newcastle disease virus described herein. In yet another embodiment the attenuated Newcastle disease virus is delivered systemically, intraperitoneally, or intratumorally. In further embodiments virus is administered at a dose of about 10^7 pfu to about 10^9 pfu. In additional embodiments the virus is administered intravenously at a dose of about 10^9 pfu to about 10^{11} pfu. In further embodiments the subject has a cancer selected from the group consisting of bladder, ovarian, brain, pancreas, prostate, sarcoma, lung, breast, cervical, liver, head and neck, gastric, kidney, melanoma, lymphoma, leukemia, thyroid, colon, and melanoma cancer.

In yet another aspect the invention generally features a method of treating a neoplasia in a subject that has developed an anti-NDV immune response, the method involving administering to the subject an effective amount of an attenuated chimeric Newcastle disease virus described herein, wherein the virus is a chimeric virus comprising a F and/or HN gene of a canine Parainfluenza virus 5 (PIV 5) or Pigeon paramyxovirus type 1 (PPMV-1), wherein the chimeric Newcastle disease virus is antigenically distinct from NDV. In an embodiment of the invention the method increases the level of oncolytic viruses present in the subject relative to the level of oncolytic viruses present in a control subject that has developed an anti-NDV immune response, but that is not receiving a chimeric Newcastle disease virus.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, Dictionary of Microbiology and Molecular Biology

(2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

5 By “attenuated Newcastle disease virus” is meant a Newcastle disease virus that selectively kills tumor cells but that does not pose a threat to poultry. In one embodiment, an attenuated Newcastle disease virus has an ICPI less than about 0.4 or 0.7. In other embodiments, attenuated Newcastle disease virus has an ICPI of between about 0 and 0.1.

10 By “heterologous polynucleotide sequence” is meant a recombinant polynucleotide that is not present in the wild-type condition.

By “detectable label” is meant a composition that when linked to a molecule of interest renders the latter detectable, via spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, electron-dense reagents, enzymes (for example, as commonly used in an ELISA), biotin, digoxigenin, or haptens.

By “agent” is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

20 By “alteration” or “change” is meant an increase or decrease. An alteration may be by as little as 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, or by 40%, 50%, 60%, or even by as much as 70%, 75%, 80%, 90%, or 100%.

As used herein, the term “antibody” refers to a polypeptide or group of polypeptides that are comprised of at least one binding domain that is formed from the folding of polypeptide chains having three-dimensional binding spaces with internal surface shapes and charge distributions complementary to the features of an antigenic determinant of an antigen. An antibody typically has a tetrameric form, comprising two identical pairs of polypeptide chains, each pair having one “light” and one “heavy” chain. The variable regions, or variable chain polypeptides, of each light/heavy chain pair form an antibody binding site.

30 The term “mAb” refers to monoclonal antibody. Antibodies of the invention comprise without limitation whole native antibodies, bispecific antibodies; chimeric antibodies; Fab, Fab', single chain V region fragments (scFv), fusion polypeptides, and unconventional antibodies.

By "biologic sample" is meant a sample obtained from a subject including a sample of biological tissue or fluid origin, obtained or collected *in vivo* or *in situ*. In particular embodiments, a biological sample includes any cell, tissue, fluid, or other material derived from an organism.

5 By "capture reagent" is meant a reagent that specifically binds a nucleic acid molecule or polypeptide to select or isolate the nucleic acid molecule or polypeptide.

By "clinical aggressiveness" is meant the severity of the neoplasia. Aggressive neoplasia are more likely to metastasize than less aggressive neoplasia. While conservative methods of treatment are appropriate for less aggressive neoplasia, more aggressive neoplasia
10 require more aggressive therapeutic regimens.

As used herein, the terms "determining", "assessing", "assaying", "measuring" and "detecting" refer to both quantitative and qualitative determinations, and as such, the term "determining" is used interchangeably herein with "assaying," "measuring," and the like. Where a quantitative determination is intended, the phrase "determining an amount" of an
15 analyte and the like is used. Where a qualitative and/or quantitative determination is intended, the phrase "determining a level" of an analyte or "detecting" an analyte is used.

The term "subject" or "patient" refers to an animal which is the object of treatment, observation, or experiment. By way of example only, a subject includes, but is not limited to, a mammal, including, but not limited to, a human or a non-human mammal, such as a non-
20 human primate, murine, bovine, equine, canine, ovine, or feline.

The term "reduce" or "increase" is meant to alter negatively or positively, respectively. An alteration may be by 5%, 10%, 25%, 30%, 50%, 75%, or even by 100%.

By "reference" is meant a standard of comparison.

By "periodic" is meant at regular intervals. Periodic patient monitoring includes, for
25 example, a schedule of tests that are administered daily, bi-weekly, bi-monthly, monthly, bi-annually, or annually.

By "severity of neoplasia" is meant the degree of pathology. The severity of a neoplasia increases, for example, as the stage or grade of the neoplasia increases.

Nucleic acid molecules useful in the methods of the invention include any nucleic
30 acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a

double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (*e.g.*, a gene described herein), or portions thereof, under various conditions of stringency. (See, *e.g.*, Wahl, G. M. and S. L. Berger (1987) *Methods Enzymol.* 152:399; Kimmel, A. R. (1987) *Methods Enzymol.* 152:507).

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, *e.g.*, formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, *e.g.*, sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 µg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C. in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 µg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42° C in 15

mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS.

Additional variations on these conditions will be readily apparent to those skilled in the art.

Hybridization techniques are well known to those skilled in the art and are described, for

5 example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel *et al.* (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

10 By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%, more preferably 80% or 85%, and more preferably 90%, 95%, 96%, 97%, 98%, or even 99%
15 or more identical at the amino acid level or nucleic acid to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches
20 identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a
25 BLAST program may be used, with a probability score between e^{-3} and e^{-100} indicating a closely related sequence.

As used herein, "substantially pure" means that a species of interest is the predominant species present (*i.e.*, on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a
30 composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 percent of all macromolecular species present in the composition, more preferably more than about 85%, 90%, 95%, and 99%. Most preferably,

the species of interest is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

As used herein, the terms "treat," "treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated. Thus, a successful treatment may prolong the survival of a patient or alleviate an undesirable symptom.

As used herein, the terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like refer to reducing the probability of developing a disorder or condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

A dose refers to a single administration of a therapeutic composition. Dosage refers to the amount of a therapeutically active molecule in a dose. A treatment regimen refers to the dosage, schedule, and mode of administration of one or more doses. A cycle refers to a repeatable unit of one or more doses within a treatment regimen. In some treatment regimens dosages are uniform for each dose. In other treatment regimens, the dosages may not be uniform. For example, one or more loading doses may be used to raise the concentration of a therapeutic molecule to a desired level in a patient. Loading doses may be followed by one or more maintenance doses, generally comprising lower dosages (for example one half or less of a loading dose) which are sufficient to maintain a desired concentration of a therapeutic molecule in a patient. One or more tapering doses may be used to gradually reduce the concentration of a therapeutic molecule in a patient.

By "specifically binds" is meant a compound (*e.g.*, antibody) that recognizes and binds a molecule (*e.g.*, polypeptide), but which does not substantially recognize and bind other molecules.

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of

numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Any compounds, compositions, or methods provided herein can be combined with
 5 one or more of any of the other compositions and methods provided herein.

As used herein, the singular forms “a”, “an”, and “the” include plural forms unless the context clearly dictates otherwise. Thus, for example, reference to “a biomarker” includes reference to more than one biomarker.

Unless specifically stated or obvious from context, as used herein, the term “or” is
 10 understood to be inclusive.

The term “including” is used herein to mean, and is used interchangeably with, the phrase “including but not limited to.”

As used herein, the terms “comprises,” “comprising,” “containing,” “having” and the like can have the meaning ascribed to them in U.S. Patent law and can mean “includes,”
 15 “including,” and the like; “consisting essentially of” or “consists essentially” likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

20 SEQUENCES

An exemplary nucleotide sequence of full-length NDV virus 73T is:

tacgtataatacgcactcactatagggacaaacagagaatccgtaggttacgataaaaggcgaaggagca
 attgaagtggacgggtagaaggtgtgaatctcgagtgcgagcccgaagcacaaactcgagaaagccttc
 25 tgccaacatgtcttcgtatttgacgagtacgaacagctcctcgcgttcagactcgcccaatggagct
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 30 acaataggagtggagtgtctgaagagagacacagagattcgcgatgatagcagggtctctccctcgggc
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 ctggagaggatcctctctatccaggcccaagtatgggtcacagtagcaaaagccatgactgcgtatgaga
 ctgcagatgagtcagaaacaagacgaatcaataagtatatgcagcaaggcagggtccaaaagaaatacat
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 5 gatggggagaccaattcctggatctgatgagagcggtagcaaatagcatgagggaagcgccaaactctg
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 aagtctgacagcggatactatgctgacctcttctgcatattggacttatgaccacgtagatagggaagg
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 10 ccaggtgtttagaagaactggctagattgaggtccctgattcaaccaacaatttcggaagattgagaa
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 cgtcaggtacattgtcaggagatttctcgcattcaacctgcaggagcttaagacattctaactcggcct
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 45 gtctgactcctgccaatttagggggacttagtaaccttcaatactcaaggctctacactagaaatatcg
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 15 gggaagcacctgttgcgggtcttctgagctacttgggtggcaccggagctaaggacagtgcactcaaa
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gctgctgccaccgttgagcaataactagcataacccttggggcctctaaacgggtcttgaggggtttt
tgctgaaaggagtcgtggagacgtgtttaaac

10

An exemplary nucleotide sequence of wt F protein is, wherein the underlined sequence denotes the nucleotide sequence of the F protein cleavage site:

atgggccccagaccttctaccaagaaccagcacctatgatgctgactgtccgggtcgcgctggtactgagttgcatctgtccggcaa
15 actccattgatggcaggcctcttgcggctgcaggaattgtgtaacaggagacaaagcagtcacatatacctcatcccagacagg
atcaatcatagttaagctcctccaaacctgcccaggataaggagggcatgtcgaaagcccccttggatgcatacaacaggacattg
accactttgctaccccccttggtgactctatccgtaggatacaagagctctgtaactacatctggagggaggagacagaaacgctttat
aggcgccattattggcggttggtccttggagttgcaactgctgcacaaataacagcgccgcagctctgatacaagccaaacaaat
gctgccaacatcctccgacttaagagagcattgccgcaaccaatgaggcgtgcatgaggtcactgacggattatcgcaactagca
20 gtggcagttgggaagatgcagcagtttgcattgaccaatttaataaaacaactcaggaattaggctgcatcagaattgcacagcaagt
ggcgtagagctcaacctgtatctaaccgaattgactacagtattcgaccacaaatcactcacctgccttaacaagctgactattcag
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tatgctgccacacttggaaacctatccgtaagcacaaccaggggatttgctcggcactgtcccaaaagtggtagacaggtcg
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gctcagtcacgctaactgcaagatgacaacatgtagatgtgtaaacccccgggtatcatatcgcaaaactatggggaagccgtgtct
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ctcaatacaagattctcaagtaataataacaggcaactctgatatctcaactgagcttgggaatgtcaacaactcgatcagtaatgcttga
30 ataagttagaggaaagcaacagcaactagacaaagtcaatgtcaactgaccagcacatctgctctattacatatcgtttgactat
catatctctgttttggatacttagcctggttctagcatgctacctaagtacaagcaaaaggcgcaacaaagacctattatgcttgg
gaataataccctagatcagatgagagccactacaaaaatgtga

35 An exemplary amino acid sequence of wild type F protein wherein the underlined sequence denotes the amino acid sequence of the F protein cleavage site:

mgprpstknppmmmltvrvalvlsicpansidgrplaaagivvtgdkavniytssqtsiivkllpnlpkdkeacakapldaynr
tltlltplgdsirriqesvttsggrrqkrfigaiiggvalgvataaqitaaaaliqakqnaanilrlkesiaatneavhevtgdlsq lavav
gkmqqfvndqfnkttelgciriaqqvgvelnlylteltvfgpqitspalnklitqalynlaggnmdhlltklgvgnnqlssliggli
tgnpilydsqtllgiqvtlpsvgnlnnmratylettsvstrgfasalvpkvvtqvgsvieeldtsycietldlyctrivtfpmspgiy
40 sclsgntsacmysktegaltpymtikgsvia nckmttcrvnp pgiisqnygeavslidkqscnvlsl dgitlrsgefdatyqkni
siqdsqviitgnldistelgnvnnsisalnkleesnskldkvnvklstsalityivltiislvgilslvlacylmykqkaqqktilwlg
nntldqmrattkm

An exemplary nucleotide sequence of mouse GM-CSF is:

45 Atgtggctgcagaacctgctgttctgggcacgtggtgtacagcctgagcggccctaccagatcccccatcaccgtgaccagacct
ggaaacatgtggaagccatcaaagaggccctgaatctgctggacgacatgccctgaccctgaacgaagaggtggaagtgtgtcc

aacgagttcagcttcaagaaactgacctgctgcagacccggctgaagatctttgagcagggcctgagaggcaacttcaccaagctg
aaggcgctctgaacatgaccgccagctactaccagacactgccccccaccccgagacagattgcgagacacaagtgaccac
ctacgccgacttcacgacagcctgaaaaccttctgaccgacatcccttcgagtgcagaacccggccagaagtga

5 An exemplary amino acid sequence of mouse GM-CSF is:

mwlqnlflglivvyslaptspitvtrpwkhveaikealnllddmpvtlneevvvsnefsfkkltcvqtrlkifeqglrgnftklkg
alnmtasyyyqtycpptpetdctqvttyadfidslkftldipfeckkpggk

An exemplary nucleotide sequence of human GM-CSF is:

10 Atgtggtgcagagcctgctgctgctgggcacagtggcctgtagcatctctgccctgccagaagccctagccctagcacacagccc
tgggagcatgtgaacgccatccaggaagccagacggctgctgaacctgagcagagacacagccgagatgaacgagacagtg
gaagtgatctccgagatgttcgatctgcaagagcctacctgctgcagacccggctggaactgtacaagcagggcctgagaggcag
cctgaccaagctgaagggacccctgaccatgatggccagccactacaagcagcactgccccccacaccgagacaagctgtgcc
accagatcatcaccttcgagagcttcaaagagaacctgaaggacttctgctgtagtcccttcgactgctgggagcccgtgcagg
15 aatga

An exemplary amino acid sequence of human GM-CSF is:

mwlqsllllgtvacsisaparspspstqpwehvnaiqearrllnlsrdaaemnetvevisemfdlqptclqrlelykqglrgsltkl
kgpltmashykqhcptpescatqiitfesfkenlkdfllvipfdcwepvqe

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the construction of NDV 73T antigenomic cDNA strain 73T. NDV sequences in GenBank were aligned to obtain consensus sequences to design DNA oligonucleotides for RT-PCR of the viral RNA. Six subgenomic cDNA fragments generated
25 by high-fidelity RT-PCR were assembled in the pUC19 vector. The full length cDNA of NDV 73T was designated as p73T. The nucleotide and deduced amino acid sequence of the F protein cleavage site (FPCS) in the 73T were modified as that of the NDV LaSota strain (lentogenic, lento) and gB of cytomegalovirus (CMV) (S116). The double slash indicates the site of cleavage of the F protein. In addition, the 73T strain cDNA plasmid (p73T) contains a
30 27 nucleotide (nt) T7 RNA polymerase promoter at 5' end and a 189 nt containing HDV antigenome ribozyme sequence and a T7 RNA polymerase transcription-termination signal at the 3' end. To generate non-virulent NDV, the sequence encoding the protease cleavage site of the fusion protein was modified by site-directed mutagenesis as those of the non-virulent NDV LaSota strain (lentogenic, lento) or glycoprotein B (gB) of cytomegalovirus (S116).

35 Figures 2A and 2B depict insertion of transgene cassette(s) into the NDV 73T genome. Figure 2A shows the insertion of a transgene at the P-M junction. An AfeI restriction site was introduced at nt 3148 in the subclone plasmid containing SacII-PmlI

fragment. cDNAs encoding codon-optimized human or mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin 2 (IL-2). The inserted gene cassette contains the gene end (GE; 5'-TTAAGAAAAAA -3'), intergenic nucleotide (T), the gene start sequence (GS; 5'-ACGGGTAGA -3') and open reading frame (ORF) of the transgene.

5 In addition, ten nucleotides (5'-cgccgccacc-3') were inserted upstream of the initiation site to introduce a Kozak sequence. The SacII-PmlI fragment from the resulting plasmid was shuffled into plasmid r73T and named as p73T-P1. Additionally, Figure 2A shows the insertion of a transgene at the HN-L junction between the HN ORF and the gene end signal (GE) sequence of HN, an AfeI restriction site was introduced at nt 8231 in the plasmid
10 containing the AgeI-XbaI fragment. The gene cassette was generated by PCR using a pair of phosphate sense and antisense primers (Table 3) and inserted into AfeI site. The AgeI-XbaI fragment from the resulting plasmid was shuffled into plasmid p73T, yielding p73T-HN1. The full length (FL) 73T cDNA containing the transgene at P-M or HN-L junction was designated as p73T-P1 or p73T-HN1, respectively. Figure 2B shows the insertion of two
15 transcriptional cassettes to the P-M junction. An AfeI site was introduced at the end of the ORF of GM-CSF (nt 3619). The IL-2 ORF was amplified using a pair of phosphate sense and antisense primers containing the GE and GS sequences and inserted at the AfeI site. The SacII-PmlI fragment from the resulting plasmid including GM-CSF and IL-2 transcriptional cassettes was swapped back into plasmid r73T, yielding p73T-P2.

20 Figures 3A-3C show the recovery of infectious recombinant NDV strain 73T (r73T) with modified FPCS and depict F protein cleavage and fusion activity *in vitro*. Figure 3A shows how NDV 73T NP, P, L and antigenic cDNA (p73T-lento or p73T-S116) were cloned under the control of the T7 RNA polymerase promoter and terminator. The four plasmids were co-transfected into an RNA polymerase expressing cell line. The recovered viruses
25 were designated as r73T-lento or r73T-S116. The r73T-lento and r73T-S116 were passaged in Vero cells with media with and without trypsin supplement. The growth of r73T-lento is trypsin dependent whereas r73T-S116 can grow in medium without trypsin supplement. To assess the genetic stability of FPCS in r73T-S116 and transgene, r73T-S116 with and without hGM-CSF at P-M junction were further passaged for 10 passages in Vero and human
30 fibrosarcoma HT1080 cells at MOI 0.01 in media without supplement of trypsin. The mutations (R113K and/or Q114M) in the FPCS appeared at passage 7 and the S116R mutation was detected at passages 9. At passage 10, the F, HN and transgene were sequenced and no additional mutations were found. Figures 3B and 3C show the effect of F protein

cleavage site (FPCS) mutation on cell fusion and F protein cleavage in vitro. For construction of plasmid co-expressing two transgenes, GFP and NDV F or HN genes, the protein open reading frames of NDV F or HN gene were amplified by PCR and cloned into plasmid pViro2-neo-MCS (Invitrogen) under the control of the cytomegalovirus (CMV) promoter. 293T cells were seeded at 5×10^5 cell/well on 6 well plate for transfection the next day. Figure 3B depicts cells transfected with 2 μ g of NDV F plasmid DNA for one day and harvested in protein lysis buffer for Western blot analysis using anti-NDV F specific polyclonal antiserum. The NDV F protein with lentogenic cleavage site and S116 were not cleaved, only F0 was detected. The F proteins of R116 and S116-KM were partially cleaved as indicated by the appearance of the F1 protein band. Figure 3C shows the cells that were cotransfected with different F plasmid with wt HN plasmid and that were examined for fusion formation by florescent microscope. The wt F protein was most efficient in fusion formation.

Figure 4 is a table summarizing characteristics of r73T-lento and r73T-S116 derivatives. ^a All viruses contain hGM-CSF at the P-M junction. ^b The amino acids in the FPCS that are different from FPCS-S116 are underlined. ^c Plaque formation in Vero cells without trypsin in the overlay after 36 hrs incubation and visualized under x10 magnification. ^d Mean death time in eggs (MDT). ^e Pathogenicity of NDV in 1-day-old pathogen-free chicks by the intracerebral pathogenicity index (ICPI). The ICPI assay was performed at National Veterinary Service Laboratories (NVSL) (Ames, Iowa). ^f Cytotoxicity effect of the viruses on human fibrosarcoma HT1080 cells after infection at multiplicities of infection (MOI) of 0.01 at 72 hours postinfection. The relative percentage of surviving cells is determined by comparing each sample with untreated cells that were considered 100% viable. Data presented in the table is relative percentage of dead cells. ^g Virus grown in Vero cells after infection with MOI of 0.01 and cultured in OPTI-MEM without trypsin supplement for 3-5 days at 37 °C. ^h Virus grown in chicken embryonated eggs. 10-11-day old embryonated eggs were infected with 1,000 pfu of r73T. The amniotic fluid was harvested after incubation at 37 °C for 72 hrs. The infectious virus titer was determined in Vero cells by plaque assay.

Figures 5A and 5B depict strategies to attenuate r73T-R116 virus virulence in chicken. Figure 5A depicts insertion of transgenes at the P-M junction (1) and HN-L junction (2), and extension of the HN-L intergenic region by insertion of non-coding sequence (3). Insertion of the transgene cassette at P-M junction is the same as that shown in Figure 2A. The 2nd transgene cassette contains the L gene start sequence (GS; 5'-ACGGGTAGA-3'), open reading frame (ORF) of the transgene, sequences from 3'

untranslated region of the L gene (in italics) and the L gene end sequence (GE; 5'-TTAAGAAAAA-3'). The non-coding sequences used for extending the HN-L junction were taken from paramyxoviruses type -1 (APMV-1), respiratory syncytial virus (RSV) or random sequence which does not have sequence identity or homology with known sequences. The insertion sequence can be in the range of 60-318 nt. Insertion of a 2nd transgene at HN-L allows the virus to express two transgenes (*e.g.*, hGM-CSF and GFP). Figure 5B depicts sequences that were inserted at the HN-L junction.

Figure 6A is a table summarizing characteristics of r73T-R116 derivatives. ^a All viruses contain hGM-CSF at the P-M junction. ^b Sequences inserted at the HN-L junction as shown in Figure 5B. ^c Plaque formation in Vero cells without trypsin in the overlay after 36 hrs incubation and visualized under x10 magnification. ^d Mean death time in eggs (MDT). ^e Pathogenicity of NDV in 1-day-old pathogen-free chicks by the intracerebral pathogenicity index (ICPI). The ICPI assay was performed at National Veterinary Service Laboratories (NVSL) (Ames, Iowa). ^f Cytotoxicity effect of the viruses on human fibrosarcoma HT1080 cells after infection at multiplicities of infection (MOI) of 0.01 at 72 hours postinfection. The relative percentage of surviving cells is determined by comparing each sample with untreated cells that were considered 100% viable. Data presented in the table is relative percentage of dead cells. ^g Virus grown in Vero cells after infection with MOI of 0.01 and cultured in OPTI-MEM without trypsin supplement for 3-5 days at 37 °C. ^h Virus grown in chicken embryonated eggs. 10-11-day old embryonated eggs were infected with 1000 pfu of r73T. The amniotic fluid was harvested after incubation at 37 °C for 72 hrs. The infectious titer was determined in Vero cells by plaque assay.

Figures 6B-6E are graphs showing the growth kinetics of recombinant NDV in DF-1 and Vero cells. DF-1 and Vero cells in six-well plates were infected with each indicated virus at a multiplicity of infection (m.o.i.) of 5.0 (single cycle, Figures 6B and 6C) or 0.001 (multi cycle, Figures 6C and 6D). The infected cell culture supernatants were collected at 10 hour intervals until 50 hours post infection, and virus titers were determined by plaque assay.

Figures 6F and 6G show viral RNA and protein synthesis in DF-1 cells. DF-1 cells were infected with each virus as indicated at m.o.i of 5.0, incubated for 20 h, total intracellular RNAs were extracted for Northern blot analysis (Figure 6F) and a second set of infected cells were examined for protein synthesis by Western blotting (Figure 6G). The RNAs were separated by electrophoresis in a formaldehyde agarose gel, transferred onto nitrocellulose membranes, and hybridized with biotin labeled RNA probes specific to the HN, NP, P, and L genes. A positive

RNA probe in the L gene was used to detect viral genomic RNA. The total proteins were separated on SDS-PAGE and blotted with anti-NP, F, HN and L serum. The total proteins loaded on the gel were detected by an actin-specific antibody. Recombinant NDV R116i with 198 nt insertion between the HN and L intergenic sequence had an overall reduced RNA and protein synthesis in the DF-1 cells.

Figures 6H and 6I depict viral RNA and protein synthesis in Vero cells. Figure 6H shows Northern blot analysis of RNA synthesis. Vero cells were infected with the viruses at m.o.i of 5.0, incubated for 20 h, total intracellular RNAs were extracted. The RNAs were separated by electrophoresis in a formaldehyde agarose gel, transferred onto nitrocellulose membranes, and hybridized with biotin labeled RNA probes specific to the NP and L genes or a positive sense L gene RNA to detect genomic RNA. Figure 6I depicts Western blot analysis of viral protein synthesis in infected Vero cells. The total proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-NP, F, HN and L serum. The total proteins loaded on the gel were detected by an actin-specific antibody. The R116i viruses with 198nt insertion had reduced genomic RNA, but greatly increased NP mRNA compared to S116 and wt NDV. The L mRNA level was too low to tell the difference. The proteins upstream of the L gene were upregulated but the L protein level was reduced in Vero cells.

Figures 6J and 6K show comparison of protein synthesis in infected DF-1 and Vero cells at low multiplicity of infection by Western blot analysis. DF-1 and Vero cells were infected with each virus as indicated at m.o.i of 0.001, incubated for 72 h and harvested in protein lysis buffer. The total proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-NP, F, HN and L serum. The proteins loaded on the gel were detected by an antibody against actin. The level of L protein was reduced in both cell lines, however, the proteins upstream of the L gene was down regulated in DF-1 cells but upregulated in Vero cells.

Figures 6L shows viral protein synthesis in infected human cells compared to DF-1 cells. Human HT1080, Hela cells and DF-1 cells were infected with each virus as indicated at m.o.i of 5.0, incubated for 20 h and harvested in protein lysis buffer. The proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane and blotted with anti-HN and anti-L serum. The HN protein expression of R116i with 198nt insertion between the HN and L junction is increased in HT1080 and Hela cells, but decreased in DF-1 cells. The L protein was decreased in all three cell lines infected with R116i-198 or 198RSV.

Figure 7 are graphs depicting the growth kinetics of r73T viruses in embryonated chicken eggs. To determine growth conditions for the r73T viruses, growth kinetic studies were performed in eggs. Chicken embryonated eggs were infected with 100, 1000, 10,000 or 100,000

PFU/egg of indicated viruses and incubated for 2, 3 or 4 days (73T wt, top left; R116, top right; R116i-318, middle left; R116i-198-RSV, middle right; R116i-198-random, bottom left; S116-KM, bottom right). The allantoic fluid was harvested and viral titers were determined by FFA. Inoculation of 100 FFU/egg had low titer on day 1, but reach peak titer on day 2.

- 5 Overall, most viruses reached peak titers of ~ 8 logs/mL on Day 2 regardless of the inoculate dose used. R116i-198 has the lowest titer, low inoculum dose generated the highest yield on day 2, indicating that there might be accumulation of defective particles. R116i-318-APMV-insertion also had a lower peak titer of ~7 logs. R116i-198-RSV reached peak titer of 7.5 logs. S116 rederived by reverse genetics had no reduction in virus production in eggs.
- 10 Therefore, among the R116 derivatives, the virus with RSV-198nt insertion was a top oncolytic virus candidate in terms of growth properties in eggs.

Figure 8 are graphs depicting the growth kinetics of r73T viruses in Vero cells. The viruses were also evaluated in sero-free Vero cell clone 51D11, a proprietary cell line generated by MedImmune. All the viruses replicated similarly well under both moi

15 conditions (0.001, top; 0.0001, bottom). The modification of 73T FPCS and intergenic insertion in the HN-L junction of R116 did not affect virus growth in Vero cells.

Figures 9A-9D show that r73T viruses selectively replicate in tumor cells and have cytotoxicity for tumor cells, compared to non-neoplastic cells. Data were obtained after infection with r73T derivatives at different dose ranging from 1 to 100,000 PFU for 72 hour.

- 20 Figure 9A is a graph showing the evaluation of rT3T and its derivatives for cell killing in human fibrosarcoma HT1080 relative to untreated control cells. In HT1080 cancer cells, the virus with lentogenic FPCS had the least killing, S116 at the FPCS was intermediate, and viruses with R116 at FPCS had cell killing as efficient as r73T wt virus. Figure 9B is a graph showing the evaluation of rT3T and its derivatives for cell killing in normal human skin
- 25 fibroblast CCD1122Sk cells relative to untreated control cells. In normal CCD1122SK cells, all viruses did not kill the cells as efficiently as the cancer cells, with reduction of killing efficiency of ~100-fold. Regardless of the FPCS sequences, all the viruses had similar killing in normal cells, likely due to a single cycle of replication (no virus spread). Figure 9C is a table showing the cell killing efficiency in cancer and normal cells, expressed as 50%
- 30 effective concentration (EC_{50}) interpolated from the dose response curve using Prism 6.0. The R116 derivative had a similar EC_{50} value as r73T wt with an EC_{50} of 10 PFU, indicating that the modification of FPCS did not affect virus replication in cancer cells and cell killing efficiency. Figure 9D is a graph showing replication of the viruses in the HT1080 and

CCD1122SK cells at MOI 0.01 at day 3 post infection. All viruses preferentially replicated in cancer cells than in normal cells, with a difference of about 1.5-2.0 logs.

Figures 10A and 10B are graphs showing r73T derivatives are effective in tumor regression upon local and systemic administration. To evaluate oncolytic activity *in vivo*, an HT1080 xenograft model was established by injecting HT1080 cells at a concentration of 5×10^6 cells/0.1ml subcutaneously into Balb/C athymic nude mice at age of 5-6 week old.

Figure 10A is a graph showing the effect of R116i-318-hGM-CSF administered intratumorally (it) or intravenously (iv). The data show that R116i-318-hGM-CSF derivatives had anti-tumor activity *in vivo* when delivered either systemically or

intratumorally to immunodeficient mice carrying human tumor xenografts. Tumor growth rate was compared between the treatment and the control groups. The tumor regressions induced by the two routes of administration were both significantly different from the control group. Mice were randomized into groups (n=10) as indicated when tumor volume reached approximately 65 mm^3 . Mice received a single dose of either PBS or 2×10^7 Pfu of r73T-

hGM-CSF-R116i-198 administered intratumorally (IT) or 1×10^8 PFU administered intravenously (IV) via tail vein injection. * $P < 0.05$, un-paired student T test. Figure 10B is a graph comparing the oncolytic activities of r73T derivatives in the HT1080 xenografts by IV injection of 1×10^8 PFU. Two doses of r73T derivatives were capable of inducing significant tumor regression with varying degrees of effectiveness. r73T-lento was the least effective in tumor regression whereas r73T wt was the most effective in tumor regression. r73T-lento had a similar effect as the S116 virus, although S116 virus has a 10-fold lower EC_{50} in the *in vitro* cell killing (see Figure 9A). r73T-R116i-318 was as potent as 73T wt in inhibition of tumor growth until 9 days post the 2nd dose (day 19 post tumor implantation), the tumor grew back in R116i treated mice but not in the 73T wt treated group. Mice were randomized into groups

(n=7) when tumor volume reached approximately 180 mm^3 . Mice received either PBS or 1×10^8 PFU of r73T-hGM-CSF-lento (lento) or r73T-hGM-CSF-S116K113M114 (S116 KM) or r73T-hGM-CSF-R116i-318 nt APMV-N (R116i) or r73T-hGM-CSF (r73T wt) administered by IV. Tumor size was measured every 3-4 days. * $P < 0.05$, un-paired student T test. The data show that r73T derivatives had anti-tumor activity *in vivo* when delivered either

systemically or intratumorally to immunodeficient mice carrying human tumor xenografts. The efficient cleavage of the F protein is important for virus replication *in vitro* and *in vivo*. The viruses with the R116 at the FPCS were more potent in cell killing *in vitro* and *in vivo*.

Figures 11A-G depict the tissue biodistribution of r73T derivatives following intravenous delivery and the effect of mouse versus human GM-CSF on tumor growth inhibition. To determine if the oncolytic NDV virus selectively replicates in tumor tissues and viral clearance, virus distribution in different organs was determined. Athymic nude mice bearing subcutaneous HT1080 tumors with size of $\sim 250 \text{ mm}^3$ were treated with R116i (r73T-hGM-CSF-R116i-318 nt APMV-N) at a dose of 1×10^8 PFU intravenously and sacrificed on day 1, 4 or 8 (n=3 per time point). Serum, lungs, spleen, ovaries and tumor were collected and the presence of virus was quantified. Viral replication in tumor and organs were assessed at days 1, 4 and 8 post infection. Figure 11A is a graph depicting quantification of virus in tissues by plaque assay in Vero cells. Virus in organs was only detected on day 1 (virus was not detected in ovary at all time points, data not shown) and virus load in tumor tissues was ~ 100 -fold higher than lungs and spleens. The presence of virus in tumor persisted for at least 8 days, indicating that the virus selectively replicated in tumor tissues.

Figure 11B is a graph depicting quantification of GM-CSF expressed by virus in tissues by ELISA assay of hGM-CSF transgene expression. Consistent with the viral replication data obtained by plaque assay, the level of hGM-CSF was the highest and lasted more than 8 days in the tumor tissue. These data demonstrated that the NDV virus effectively replicated in tumor tissue and that the transgene was effectively delivered to local tumor tissue.

Figure 11C depicts graphs showing the effect of mGM-CSF expression on tumor growth inhibition in HT1080 xenograft mouse tumor model. Athymic nude mice at 5-6 weeks old in groups of seven were implanted subcutaneously (s.c.) with 5×10^6 HT1080 cells in the right flank. When tumors reached a volume of 110 mm^3 (day 6), the tumor were injected with a single dose of 1×10^8 pfu rNDV, R116i-198RSV with mGM-CSF or hGM-CSF. The tumor size was measured every 3-4 days. R116i-198RSV with mGM-CSF was less potent in tumor growth inhibition than hGM-CSF transgene. However, no difference in tumor growth inhibition was observed for S116 with either hGM-CSF or mGM-CSF.

Figure 11 D depicts graphs showing the effect of mGM-CSF on virus clearance from tumors in HT1080 xenograft mouse tumor model. Athymic nude mice in groups of three were implanted subcutaneously (s.c.) with 5×10^6 HT1080 cells into the right flank. When tumors reached a volume of 180 mm^3 (day 10), the mice were intravenously treated with one dose of 1×10^8 pfu of R116i-198RSV or S116KM. The tumors were collected on day 4 or 7 and viral titers in the tumor tissue were determined by plaque assay. Both R116i-198RSV

and S116KM with either mGM-CSF or hGM-CSF had comparable titer on day 4. On day 7, R116i-198RSV with mGM-CSF was greatly reduced compared to R116i-198RSV with hGM-CSF. In comparison, the titers of S116 mGM-CSF and hGM-CSF were comparable.

Figure 11E depicts a graph showing the effect of R116i-198RSV or S116KM infection on immune cell infiltration into tumors in HT1080 xenograft mouse tumor model. Athymic nude mice in groups of three were implanted subcutaneously (s.c.) with 5×10^6 HT1080 cells into the right flank. When tumors reached a volume of 180 mm^3 (day 10), the mice were intravenously treated with one dose of 1×10^8 pfu of virus as indicated. The tumors were collected on day 4 and the tissues were processed for neutrophil, NK cells and macrophage staining by FACS analysis. R: R116i-198-RSV, S: S116-KM. R116i-198RSV with mGM-CSF had more immune cell infiltration.

Figure 11F depicts a table showing that cytokines and chemokines were up-regulated in HT1080 xenograft mouse tumor model. Athymic nude mice in groups of three were implanted subcutaneously (s.c.) with 5×10^6 HT1080 cells into the right flank. When the tumors reached a volume of 180 mm^3 (day 10), the mice were intravenously treated with one dose of 1×10^8 pfu of R116i-198RSV or S116KM with hGM-CSF or mGM-CSF. The tumors were collected on day 4 and the tissues were processed for levels of cytokines and chemokines by Luminex analysis. Virus infection induced cytokines and chemokines production in the local tumor tissues, their levels varied based on virus backbone and human or mouse GM-CSF.

Figure 11G depicts a graph showing that R116i-198RSV-hGM-CSF and R116i-318APMV-hGM-CSF were comparable in tumor growth inhibition in HT1080 xenograft mouse tumor model. Athymic nude mice in groups of seven were implanted subcutaneously (s.c.) with 5×10^6 HT1080 cells into the right flank. When tumors reached a volume of approximately 110 mm^3 (day 6), a single dose of virus at 1×10^8 pfu was injected intratumorally. The tumor size was measured every 3-4 days and graphed. The insertion length of 198 and 318nt did not affect R116i oncolytic activity.

Figures 12A and Figure 12B depict construction of antigenome cDNA of 73T containing chimeric F and/or HN genes and their characterization and Figure 12C compares function of RNA polymerase complex activity. Viral surface glycoproteins are important antigens for immunogenicity and virulence in chickens. F and/or HN genes of NDV were replaced by the corresponding extracellular (ecto) domains of other paramyxoviruses which are not virulent in chickens individually or in combination. Parainfluenza virus 5 (PIV 5) is a

canine paramyxovirus and does not cause diseases in human, and pigeon paramyxovirus type 1 (PPMV-1) has been shown to be nonvirulent in chickens. Figure 12A shows that F and/or HN glycoprotein ectodomains of the full-length antigenomic NDV 73T cDNAs were replaced with those of PPMV-1 and/or PIV5. NDV, PIV5, and PPMV-1 sequences are indicated with colored boxes as blue, purple or green, respectively. The amino acid lengths of individual proteins or protein domains are indicated. Figure 12B is a table showing characterization of 73T derivatives containing chimeric F and/or HN genes. Plaque formation in Vero, relative HT1080 cell killing and MDT were performed as described previously. Chimeric viruses, except for PVI-5 F-HN, were recovered and grew in the cells in the absence of exogenous trypsin. All three viruses formed good sized plaques and showed efficient cell to cell spreading. PPMV-1 F-HN or F chimeric viruses had MDT value of 79 and 84 hr, respectively, indicating potential virulence in chickens. Both PPMV-1 chimeric virus killed HT1080 cells efficiently at levels of 71 and 61%. PIV5-F chimera did not grow in eggs and was not virulent in chickens, but killed HT1080 cells at 47%. Serum cross reactivity between the NDV and PPMV-1 or PIV5 chimeras was examined by neutralization assay using the serum collected from mice that were IV administered with 2 doses of 1×10^8 PFU of r73T-R116i. r73T virus can be neutralized by the NDV-infected serum (titer 960) whereas the PPMV-1 and PIV5 chimera were not neutralized (titer < 4), confirming no cross reactivity between NDV and PPMV-1 or PIV5. The chimeric viruses with distinct antigenicity could be to boost oncolytic viruses in patients for whom anti-NDV immune responses have developed during prior NDV treatment.

Figure 12C shows the comparison of NDV RNA polymerase activity with other paramyxoviruses by mini-genome assay. The T7 expressing cells were transfected with the three plasmids expressing the NP, P, L proteins of NDV and the plasmid encoding the NDV anti-minigenomic cDNA encoding the GFP gene using Lipofectamine 2000, or the plasmids encoding N, P, L genes of measles virus (MV) or respiratory syncytial virus (RSV) and respective RSV or MV GFP anti-minigenome cDNA plasmid. Two or three days post transfection, minigenome replication as indicated by the expression of GFP proteins was examined under fluorescent microscope. The NDV had the strongest polymerase activity compared to measles virus and RSV.

Figures 13 A and B show cancer cell line sensitivity to rNDV variants. Cancer cell lines from various tissues of origin were infected with NDV S116 or NDV R116i at an MOI of 0.1 and cell viability was assessed 72hrs post infection using Cell Titre Glo. Sensitivity to

NDV is defined as greater than 30% cell killing at 72hrs post infection. Figure 13A depicts a graph of percentage of NDV sensitive human cancer cell lines across a minimum of 16 broad indications. Numbers of cell lines within each group are indicated numerically and in the table. Figure 13B depicts a graph of sensitivity of 22 cancer cell lines to NDV variants. % cell kill is determined as a percentage of untreated control cells.

Figure 14 are graphs showing permissivity of cancer cell lines to recNDV^{GM-CSF}. Cell killing of R116i-GM-CSF in representative tumor cell lines are presented in Figure 14. Maximum % kill was determined 3 days following infection of tumor cell lines relative to non-infected control at MOI of 0.1. Although certain cell lines were derived from the same tumor types, such as bladder, gastric, prostate, melanoma, breast, ovarian, pancreatic and lung cancers, they showed different sensitivity to the virus killing. The numbers of cell lines from each tumor indication that can be killed by R116i-GM-CSF at rate of >50% are summarized at Table 1.

Figures 15A-F are graphs showing that NDV derivatives inhibit tumor growth in different mouse cancer models. Figures 15A and 15B are graphs showing recNDV^{GM-CSF} strains inhibit tumor growth in syngeneic mouse melanoma model (B16F10 AP3). 73T-R116i-hGM-CSF (Figure 15A left) and R116i-mGM-CSF (Figure 15A right) were evaluated for their oncolytic effect in the B16 melanoma model in a pilot study. As virus tolerability in the B16 mouse was being evaluated, the group size was small (n=3). Each virus was dosed intravenously (i.v) at 2×10^7 pfu twice on days 11 and 14; intraperitoneally (i.p) at 2×10^7 pfu twice on days 11 and 14; or intratumorally (i.t) once at 1.1×10^7 pfu on day 11. The groups treated with R116-hGM-SCF or mGM-SCF by the three routes of administration had slower rate of tumor growth compared to the untreated group. Tumor inhibition rates were statistically significant from the control group.

Figure 15B shows an efficacy study in B16F10 syngeneic murine melanoma model with intratumoural administration of 1×10^8 pfu of S116 NDV variants. Tumour growth inhibition is shown on the left panel and individual animal measurements are shown on the middle panel and a survival plot is shown on the right.

Figure 15C shows that NDV has potent anti-tumour activity in immune-competent mouse CT26 colorectal tumour model. In particular, Figure 15C provides an efficacy study in CT26 syngeneic murine colorectal model with intratumoural administration of 1×10^8 pfu of R116 NDV variant encoding human GM-CSF. Tumour growth inhibition is shown on the right panel and IHC analysis of the remaining tumour is shown on the right hand side with

H&E and NDV staining. Necrotic areas, evidence of multinucleated syncytia and viable areas of tumours are indicated.

Figures 15D-F depict tumor growth inhibition in ovarian xenograft model. Athymic nude mice at were implanted subcutaneously (s.c.) with Ovar4 cells in the right flank. When tumors reached a volume of 100 mm³ mice were randomized into treatment groups. Established tumors were injected with once weekly with of 2.5x 10⁷ pfu rNDV, R116i-318 with mGM-CSF or hGM-CSF. Tumour growth curves (Figure 15D) and histological analyses of tumors (Figure 15E and Figure 15F) are shown.

Figure 16 is a table summarizing characteristics of NDV constructs. Plaque formation in Vero cells without trypsin in the overlay after 36 hrs incubation and visualized under x10 magnification. Cytotoxicity effect of the viruses on human fibrosarcoma HT1080 cells after infection at multiplicities of infection (MOI) of 0.01 at 72 hours postinfection. The relative percentage of surviving cells is determined by comparing each sample with untreated cells that were considered 100% viable. Data presented in the table are relative percentage of dead cells. Pathogenicity of NDV in 1-day-old pathogen-free chicks by the intracerebral pathogenicity index (ICPI). The ICPI assay was performed at National Veterinary Service Laboratories (NVSL) (Ames, Iowa).

Figure 17 is a graph showing that NDV produced from eggs and human cell lines exhibited different sensitivity to complement mediated inactivation. Human serum with confirmed complement (C') activity (Sigma, St. Louis, MO) was serially diluted with PBS and incubated with 100 pfu of NDV for 1 hour at 37°C prior to infecting Vero cells for plaque assay. Following incubation at 37°C for 6 days, the plaques were visualized by crystal violet staining and scored. The virus grown in embryonated chicken eggs was mostly inactivated by serum diluted at 1:10 to 1:40. The 293-grown virus was more resistant to C' than egg-grown virus, approximately 40% infectivity was retained at serum concentration of 1:40. However, the HeLa-grown virus was most resistant to C' mediated viral inactivation. Approximately 90% live virus was infectious at serum concentration of 1:40.

Figures 18A and 18B are Western blots showing comparisons of RCA protein levels in 293 and HeLa cells and in viruses produced from these two cell lines. For Figure 18A equal amount of 293 and HeLa S3 cells were loaded onto the SDS-PAGE for Western blot. HeLa cells contained higher levels of hCD46, hCD55 and hCD59 proteins than 293 cells. Figure 18B shows a Western blot that was performed to examine protein amounts in virus from infected 293 or HeLa cells. Uninfected cells (mock) were served as controls. The three

CD molecules were detected in viruses from the infected Hela cells at levels higher than those from 293 cells.

Figure 19 shows evaluation of membrane bound C' regulators (RCA) proteins in C' mediated viral inactivation. The cDNA encoding hCD55, hCD59 and hCD46 was synthesized by Origene (Rockville, MD) or Genscript (Piscataway, NJ). Each gene cassette was inserted into the P-N intergenic region of NDV antigenomic cDNA and recombinant viruses were generated by reverse genetics. The recombinant viruses were amplified in eggs and purified by 15-60% sucrose gradient and viral band was pelleted by ultracentrifugation. Expression of each RCA protein by recombinant NDV was confirmed by Western blotting.

Figure 20 is a graph showing that CD55 is a major RCA protein for preventing C' inactivation of NDV. NDV with hCD46, hCD55 or hCD59 were amplified in eggs, purified by sucrose gradient, incubated with human plasma diluted from 1:10 to 1:40 for 1 hour and viral infectivity was examined by plaque assay in Vero cells. NDV with hCD55 produced in eggs had similar resistance to C' compared to NDV produced in Hela cells, approximately 65% viable viruses at plasma concentration of 1:20 and ~80% viable virus at plasma concentration of 1:40. hCD46 appeared to slightly or marginally improve viral resistance to C' mediated inactivation, approximately 20% more viable virus when incubating with 1:40 diluted human plasma compared to NDV control. No difference was detected at lower plasma dilution.

DETAILED DESCRIPTION OF THE INVENTION

The invention features compositions comprising an attenuated Newcastle disease virus and methods of using that virus for the treatment of neoplasia.

The invention is based, at least in part, on the discovery of an oncolytic NDV with reduced chicken virulence. As reported in more detail below, the NDV 73T strain was derived from NDV MK-107, which is a commercial poultry vaccine (mesogenic) first marketed in 1948. The NDV MK-107 strain was maintained through 73 passages in Ehrlich ascites tumor cells (Cassel *et al.*, Cancer. 1965 Jul;18:863-8). NDV MK-107 was used in a series of Ph I and Ph II clinical studies in the 1970's. NDV MK-107 was also used in the 1980's as an immunotherapeutic to treat late stage melanoma patients (Cassel *et al.*, Cancer. 1983 1;52:856-860; Murray *et al.*, Cancer. 1977. 40:680-686).

In order to generate an oncolytic NDV with reduced chicken virulence, the recombinant NDV 73T strain includes certain genetic modifications. In particular, the F protein cleavage sequence was altered and the length of the HN-L intergenic sequence was increased. Advantageously, the modified virus can be used to express a transgene(s) of interest. In one embodiment, the NDV 73T strain includes a transgene encoding a polypeptide that enhances the oncolytic properties of recombinant NDV. In another embodiment, the NDV 73T strain includes a transgene encoding a biomarker that provides a read-out useful to monitor virus replication. If desired, NDV 73T strain can be modified to incorporate additional genetic information that disrupts the normal transcriptional polarity of the standard genome and is expected to further reduce viral virulence in chickens. Accordingly, the invention provides a recombinant Newcastle Disease Virus (NDV) generated using reverse genetics to reduce its pathogenesis in chickens while maintaining its selective cancer cell killing ability, and methods of producing such a virus. The invention also provides for the construction and use of NDV as a viral vector to deliver and express heterologous gene products for enhanced cancer treatment. The transgenes encoding exemplary therapeutic agents that can be delivered by NDV are described herein below. In working examples described herein below, novel NDV viral constructs expressing granulocyte macrophage-colony stimulating factor (GM-CSF) selectively killed cancer cells, but did not kill normal cells. This selective cancer cell killing effect was observed in a number of cancer cell lines, as well as *in vivo* when tested in the xerograft HT1080 tumor model. The efficacy and selectivity of the recombinant attenuated Newcastle Disease Virus (NDV) was also demonstrated in a melanoma model where tumor regression was observed. In sum, the invention provides for the insertion of specific transgene(s) into a recombinant attenuated NDV vector and the efficient expression of the encoded protein in a tumor environment.

Newcastle Disease Virus

The Newcastle disease virus (NDV) is an enveloped virus containing a linear, single-strand, nonsegmented, negative sense RNA genome. The negative-sense, single-stranded genome of NDV encodes a RNA-directed RNA polymerase, a fusion (F) protein, a hemagglutinin-neuraminidase (HN) protein, a matrix protein, a phosphoprotein and a nucleoprotein. The genomic RNA contains genes in the following order: 3'-NP-P-M-F-HN-L.

The organization of the NDV RNA genome is described in greater detail herein below. The genomic RNA also contains a leader sequence at the 3' end.

The structural elements of the virion include the virus envelope which is a lipid bilayer derived from the cell plasma membrane. The glycoprotein, hemagglutinin-neuraminidase (HN), protrudes from the envelope allowing the virus to contain both hemagglutinin and neuraminidase activities. The fusion glycoprotein (F), which is an integral membrane protein, is first produced as an inactive precursor, then cleaved post-translationally to produce two disulfide linked polypeptides. The active F protein is involved in penetration of NDV into host cells by facilitating fusion of the viral envelope with the host cell plasma membrane. The matrix protein (M), is involved with viral assembly, and interacts with both the viral membrane as well as the nucleocapsid proteins.

The main protein subunit of the nucleocapsid is the nucleocapsid protein (NP) which confers helical symmetry on the capsid. In association with the nucleocapsid are the P and L proteins. The phosphoprotein (P), which is subject to phosphorylation, is thought to play a regulatory role in transcription, and may also be involved in methylation, phosphorylation and polyadenylation. The L gene, which encodes an RNA-dependent RNA polymerase, is required for viral RNA synthesis together with the P protein. The L protein, which takes up nearly half of the coding capacity of the viral genome, is the largest of the viral proteins, and plays an important role in both transcription and replication.

The replication of all negative-strand RNA viruses, including NDV, is complicated by the absence of cellular machinery required to replicate RNA. Additionally, the negative-strand genome can not be translated directly into protein, but must first be transcribed into a positive-strand (mRNA) copy. Therefore, upon entry into a host cell, the genomic RNA alone cannot synthesize the required RNA-dependent RNA polymerase. The L, P and NP proteins must enter the cell along with the genome upon infection.

Without being bound to theory, it is hypothesized that most or all of the viral proteins that transcribe NDV mRNA also carry out replication. The mechanism that regulates the alternative uses (*i.e.*, transcription or replication) of the same complement of proteins has not been clearly identified. Directly following penetration of the virus, transcription is initiated by the L protein using the negative-sense RNA in the nucleocapsid as a template. Viral RNA synthesis is regulated such that it produces monocistronic mRNAs during transcription. Following transcription, virus genome replication is the second event that occurs upon infection of a cell by negative-strand RNA viruses. As with other negative-strand RNA

viruses, viral genome replication of Newcastle disease virus (NDV) is mediated by virus-specified proteins. The first products of replicative RNA synthesis are complementary copies (*i.e.*, plus-polarity) of NDV genome RNA (cRNA). These plus-stranded copies (anti-genomes) differ from the plus-strand mRNA transcripts in the structure of their termini.

5 Unlike mRNA transcripts, the anti-genomic cRNAs are not capped and methylated at the 5' termini, and are not truncated and polyadenylated at the 3' termini. The cRNAs are coterminal with their negative strand templates and contain all the genetic information in each genomic RNA segment in the complementary form. The cRNAs serve as templates for the synthesis of NDV negative-strand viral genomes (vRNAs).

10 Both the NDV negative strand genomes (vRNAs) and antigenomes (cRNAs) are encapsidated by nucleocapsid proteins; the only unencapsidated RNA species are virus mRNAs. For NDV, the cytoplasm is the site of virus RNA replication, just as it is the site for transcription. Assembly of the viral components likely takes place at the host cell plasma membrane. Mature virus is then released from the cell by budding.

15

Oncolytic Viruses

Viruses are known to exert oncolytic effects on tumor cells and the use of oncolytic viruses as therapeutic agents has been reported. Some effort has been done to use non-human viruses exhibiting medium to high pathogenicity for their natural hosts in the treatment of cancer patients. The present invention discloses methods for inducing regression of tumors in human subjects, the methods utilize a modified mesogenic strain of Newcastle disease virus (NDV) with modified F protein cleavage site, which is non-pathogenic to poultry (lentogenic), but exhibits oncolytic properties. The disclosed methods provide safe, effective and reliable means to induce regression of a tumor in an individual in need thereof. These methods overcome the drawbacks of using pathogenic strains of viruses for human therapy.

25

Accordingly in one aspect, the present invention provides a method for inducing regression of a tumor in a subject, the method comprises the step of administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a lentogenic oncolytic strain of NDV. According to one embodiment, the lentogenic oncolytic strain of NDV is NDV r73T-R116.

30

Oncolytic viruses are capable of exerting a cytotoxic or killing effect *in vitro* and *in vivo* to tumor cells with little or no effect on normal cells. The term "oncolytic activity" refers to the cytotoxic or killing activity of a virus that targets tumor cells. Without wishing to be

bound to any mechanism of action, the oncolytic activity exerted by a lentogenic strain of NDV (*e.g.*, r73T-R116), is probably primarily due to cell apoptosis and to a lesser extent to plasma membrane lysis, the latter is accompanied by release of viable progeny into the cell's milieu that subsequently infect adjacent cells. Without wishing to be bound to a particular theory, it is believed that NDV has direct cytolytic activity on the cancer cells. It is also believed that NDV is capable of specifically differentiating cancer cells from normal, healthy cells. Results have indicated that several oncogenes (H-ras, N-ras, and N-myc) which are known to confer malignant behavior to cancer cells, enhance the susceptibility of cells to killing by NDV. See, Lorence, R. M., Reichard, K. W., Cascino, C. J. *et al.* (1992) Proc. Am. Assoc. Cancer Res., 33, 398; Reichard, K. W., Lorence, R. M., Cascino, C. J., *et al.* (1992) Surg. Forum, 43, 603-606. In addition, it has been observed that treatment of cells with retinoic acid (vitamin A) also enhances lysis of cancer cells by NDV. Reichard, K. W., Lorence, R. M., Katubig, B. B., *et al.* (1993) J. Pediatr. Surg., 28, 1221.

The cytotoxic effects under *in vitro* or *in vivo* conditions can be detected by various means as known in the art, for example, by inhibiting cell proliferation, by detecting tumor size using gadolinium enhanced MRI scanning, by radiolabeling of a tumor, and the like.

For clinical studies, it is desirable to obtain a clonal virus so as to ensure virus homogeneity. Clonal virus can be produced according to any method available to the skilled artisan. For example, clonal virus can be produced by limiting dilution or by plaque purification.

NDV Culture

The virus employed in the invention may be prepared by a variety of methods. For example, NDV may be prepared in 8 to 10 day old fertilized chicken eggs (obtained from SPAFAS, Inc., Roanoke, Ill.). Methods of isolating the virus are known in the art and are described, for example, by Weiss, S. R. & Bratt, M. A. (1974) J. Virol, 13, 1220-1230. This method is further described in Example #1 below. Using this isolation method, NDV may be obtained which is about 90-95% pure.

Alternatively, the virus may be prepared in an *in vitro* cell culture. Preferably, the cell culture comprises mammalian cells, and more preferably, cells can be used for virus manufacture such as Vero cells. The viruses will be purified by chromatograph or other appropriate methods. The cells may be anchorage-dependent or anchorage-independent.

Cell culture techniques that may be employed in the virus preparation are known in

the art and may include use of stationary culture flasks with large surface areas or roller-type flasks. Preferably, the type of culture system selected can support relatively large numbers of cells. To produce large quantity of viruses, a bioreactor process will be deployed whereas the cells are grown in microcarrier beads for virus infection and production.

5 Cell culture mediums that can be employed in the virus production are known to those skilled in the art. The medium typically includes a nutrient source, antibiotic(s) and albumin or a serum source that contains growth factor(s). It is within the skill in the art to select particular mediums and medium constituents suitable for the cells employed in the culture. In certain embodiments trypsin is included in the growth media. In other embodiments,
10 trypsin is not included.

Culture conditions typically include incubation at a desired temperature (such as 37°C), as well as selected concentrations of oxygen and carbon dioxide. The particular culture conditions selected can be determined in accordance with the cells employed in the culture, and determination of such conditions is within the skill in the art.

15 The cells are placed in the culture vessel and allowed to incubate and grow under the selected culture conditions. Preferably, anchorage-dependent cells are allowed to grow to confluence or peak growth. The time required for growth will vary depending upon the size of the initial cell inoculum added to the culture vessel and doubling time of the cell line being employed. Preferably, about 3×10^3 to about 3×10^5 cells are plated per cm^2 and grown for one
20 to five days. For virus inoculation of the cell culture, the medium is removed from the cells (for adherent cells, by aspiration of the culture medium; for cells grown in suspension, by centrifugation of the cell suspension and aspiration of the cell supernatant) and the virus (after reconstitution) is added to the cells in a minimal volume of medium or saline solution (such as Hank's Balanced Salt Solution, Gibco) to prevent dehydration. Preferably, this
25 volume ranges from about 10 to about 2500 microliter per cm^2 culture vessel surface area or 10^5 cells. The preferred dilution of virus inoculum ranges from about 0.001 to about 10 infectious units per cell, the optimal ratio depending on the particular virus and cell line. The virus is then grown from about 1 to 7 days, the length of time being primarily determined by the residual survival of the cell line. For NDV, the optimal time of harvest is 1 to 5 days after
30 virus inoculation.

The virus can then be harvested by either removing the supernatant and replacing it with fresh medium or fresh medium with fresh cells at 12 to 48 hour intervals or freeze-thawing the cells to release virus in the supernatant. The supernatant can then be centrifuged

and ultracentrifuged to recover the virus in relatively pure form or by chromatography methods. The purity of the viral preparation may be tested by protein determination and/or by electrophoresis. The virus can then be added to a pharmaceutically-acceptable carrier, described further below.

5

Therapy

Therapy may be provided wherever cancer therapy is performed: at home, the doctor's office, a clinic, a hospital's outpatient department, or a hospital. In one embodiment, the invention provides for the use of an Newcastle disease virus (NDV) (*e.g.*, r73T-R116).

10 Treatment generally begins at a hospital so that the doctor can observe the therapy's effects closely and make any adjustments that are needed. The duration of the therapy depends on the kind of cancer being treated, the age and condition of the patient, the stage and type of the patient's disease, and how the patient's body responds to the treatment. Drug administration may be performed at different intervals (*e.g.*, daily, weekly, or monthly).

15 Therapy may be given in on-and-off cycles that include rest periods so that the patient's body has a chance to build healthy new cells and regain its strength.

Depending on the type of cancer and its stage of development, the therapy can be used to slow the spreading of the cancer, to slow the cancer's growth, to kill or arrest cancer cells that may have spread to other parts of the body from the original tumor, to relieve symptoms
20 caused by the cancer, or to prevent cancer in the first place. Cancer growth is uncontrolled and progressive, and occurs under conditions that would not elicit, or would cause cessation of, multiplication of normal cells.

As described above, if desired, treatment with a composition of the invention may be combined with therapies for the treatment of proliferative disease (*e.g.*, radiotherapy, surgery,
25 or chemotherapy).

Formulation of Pharmaceutical Compositions

The administration of a virus of the invention (*e.g.*, NDV r73T-R116) for the treatment of tumors may be by any suitable means that results in a concentration of the
30 therapeutic that, combined with other components, is effective in preventing, ameliorating, or reducing tumors. The agent may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The composition may be provided in a dosage form that is

suitable for parenteral (*e.g.*, subcutaneously, intravenously, intramuscularly, or intraperitoneally) administration route. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see, *e.g.*, Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

Pharmaceutical compositions according to the invention may be formulated to release the active compound substantially immediately upon administration or at any predetermined time or time period after administration. The latter types of compositions are generally known as controlled release formulations, which include (i) formulations that create a substantially constant concentration of the drug within the body over an extended period of time; (ii) formulations that after a predetermined lag time create a substantially constant concentration of the drug within the body over an extended period of time; (iii) formulations that sustain action during a predetermined time period by maintaining a relatively, constant, effective level in the body with concomitant minimization of undesirable side effects associated with fluctuations in the plasma level of the active substance (sawtooth kinetic pattern); (iv) formulations that localize action by, *e.g.*, spatial placement of a controlled release composition adjacent to or in a sarcoma (v) formulations that allow for convenient dosing, such that doses are administered, for example, once every one or two weeks; and (vi) formulations that target proliferating neoplastic cells by using carriers or chemical derivatives to deliver the therapeutic agent to a sarcoma cell. For some applications, controlled release formulations obviate the need for frequent dosing during the day to sustain the plasma level at a therapeutic level.

Any of a number of strategies can be pursued in order to obtain controlled release in which the rate of release outweighs the rate of metabolism of the compound in question. In one example, controlled release is obtained by appropriate selection of various formulation parameters and ingredients, including, *e.g.*, various types of controlled release compositions and coatings. Thus, the therapeutic is formulated with appropriate excipients into a pharmaceutical composition that, upon administration, releases the therapeutic in a controlled manner. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, microspheres, molecular complexes, nanoparticles, patches, and liposomes.

A composition of the invention, may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the compounds to patients suffering from a disease that is caused by excessive cell proliferation.

5 Administration may begin before the patient is symptomatic.

Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intratumoral, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intrahepatic, intracapsular, intrathecal, intracisternal, intraperitoneal, intranasal, aerosol, suppository, or
10 oral administration. For example, therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols. For any of the methods of application described above, a composition of the invention is desirably administered intravenously or is applied to the site of the needed apoptosis event
15 (*e.g.*, by injection).

Methods well known in the art for making formulations are found, for example, in "Remington: The Science and Practice of Pharmacy" Ed. A. R. Gennaro, Lippincott Williams & Wilkins, Philadelphia, Pa., 2000. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as
20 polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for delivering agents include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes.
25 Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

The formulations can be administered to human patients in therapeutically effective
30 amounts (*e.g.*, amounts which prevent, eliminate, or reduce a pathological condition) to provide therapy for a disease or condition. The preferred dosage of a composition of the invention is likely to depend on such variables as the type and extent of the disorder, the

overall health status of the particular patient, the formulation of the compound excipients, and its route of administration.

Human dosage amounts for any therapy described herein can initially be determined by extrapolating from the amount of compound used in mice, as a skilled artisan recognizes it is routine in the art to modify the dosage for humans compared to animal models. In certain embodiments it is envisioned that the dosage may vary from between about 10^7 pfu to about 10^{11} pfu; or from about 10^8 pfu to about 10^{10} pfu or from about 10^9 pfu to about 10^{11} pfu. In other embodiments this dose may be about 10^7 pfu, 10^8 pfu, 10^9 pfu, 10^{10} pfu, 10^{11} pfu. . Of course, a dosage amount may be adjusted upward or downward, as is routinely done in such treatment protocols, depending on the results of the initial clinical trials and the needs of a particular patient.

Selection of a treatment method

After a subject is diagnosed as having neoplasia a method of treatment is selected. In neoplasia, for example, a number of standard treatment regimens are available. The marker profile of the neoplasia is used in selecting a treatment method. In one embodiment, neoplasia cells that are responsive to cell killing by NDV (*e.g.*, r73T-R116).

Less aggressive neoplasia are likely to be susceptible to conservative treatment methods. More aggressive neoplasia (*e.g.*, metastatic neoplasia) are less susceptible to conservative treatment methods and are likely to recur. When methods of the invention indicate that a neoplasia is very aggressive, an aggressive method of treatment should be selected. Aggressive therapeutic regimens typically include one or more of the following therapies: surgical resection, radiation therapy, or chemotherapy.

Assays for measuring cell viability

Agents (*e.g.*, NDV) useful in the methods of the invention include those that induce neoplastic cell death and/or reduce neoplastic cell survival, *i.e.*, viability.

Assays for measuring cell viability are known in the art, and are described, for example, by Crouch *et al.* (J. Immunol. Meth. 160, 81–8); Kangas *et al.* (Med. Biol. 62, 338–43, 1984); Lundin *et al.*, (Meth. Enzymol. 133, 27–42, 1986); Petty *et al.* (Comparison of J. Biolum. Chemilum. 10, 29–34, 1995); and Cree *et al.* (AntiCancer Drugs 6: 398–404, 1995). Cell viability can be assayed using a variety of methods, including MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) (Barltrop, Bioorg. & Med. Chem.

Lett.1: 611, 1991; Cory *et al.*, Cancer Comm. 3, 207–12, 1991; Paull J. Heterocyclic Chem. 25, 911, 1988). Assays for cell viability are also available commercially. These assays include but are not limited to CELLTITER-GLO® Luminescent Cell Viability Assay (Promega), which uses luciferase technology to detect ATP and quantify the health or number
5 of cells in culture, and the CellTiter-Glo® Luminescent Cell Viability Assay, which is a lactate dehydrogenase (LDH) cytotoxicity assay (Promega).

Candidate compounds that induce or increase neoplastic cell death (*e.g.*, increase apoptosis, reduce cell survival) are also useful as anti-neoplasm therapeutics. Assays for measuring cell apoptosis are known to the skilled artisan. Apoptotic cells are characterized
10 by characteristic morphological changes, including chromatin condensation, cell shrinkage and membrane blebbing, which can be clearly observed using light microscopy. The biochemical features of apoptosis include DNA fragmentation, protein cleavage at specific locations, increased mitochondrial membrane permeability, and the appearance of phosphatidylserine on the cell membrane surface. Assays for apoptosis are known in the art.
15 Exemplary assays include TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assays, caspase activity (specifically caspase-3) assays, and assays for fas-ligand and annexin V. Commercially available products for detecting apoptosis include, for example, Apo-ONE® Homogeneous Caspase-3/7 Assay, FragEL TUNEL kit (ONCOGENE RESEARCH PRODUCTS, San Diego, CA), the ApoBrdU DNA
20 Fragmentation Assay (BIOVISION, Mountain View, CA), and the Quick Apoptotic DNA Ladder Detection Kit (BIOVISION, Mountain View, CA).

Neoplastic cells have a propensity to metastasize, or spread, from their locus of origination to distant points throughout the body. Assays for metastatic potential or invasiveness are known to the skilled artisan. Such assays include *in vitro* assays for loss of
25 contact inhibition (Kim *et al.*, Proc Natl Acad Sci U S A. 101:16251-6, 2004), increased soft agar colony formation *in vitro* (Zhong *et al.*, Int J Oncol. 24(6):1573-9, 2004), pulmonary metastasis models (Datta *et al.*, *In vivo*, 16:451-7, 2002) and Matrigel-based cell invasion assays (Hagemann *et al.* *Carcinogenesis*. 25: 1543-1549, 2004). *In vivo* screening methods for cell invasiveness are also known in the art, and include, for example, tumorigenicity
30 screening in athymic nude mice. A commonly used *in vitro* assay to evaluate metastasis is the Matrigel-Based Cell Invasion Assay (BD Bioscience, Franklin Lakes, NJ).

If desired, candidate compounds selected using any of the screening methods described herein are tested for their efficacy using animal models of neoplasia. In one

embodiment, mice are injected with neoplastic human cells. The mice containing the neoplastic cells are then injected (*e.g.*, intraperitoneally) with vehicle (PBS) or candidate compound daily for a period of time to be empirically determined. Mice are then euthanized and the neoplastic tissues are collected and analyzed for levels of NDV, NDV polypeptides, and/or NDV markers (*e.g.*, a transgene encoding a detectable moiety) using methods described herein. Compounds that decrease NDV, NDV polypeptides, or NDV marker levels mRNA or protein expression relative to control levels are expected to be efficacious for the treatment of a neoplasm in a subject (*e.g.*, a human patient). In another embodiment, the effect of a candidate compound on tumor load is analyzed in mice injected with a human neoplastic cell. The neoplastic cell is allowed to grow to form a mass. The mice are then treated with a candidate compound or vehicle (PBS) daily for a period of time to be empirically determined. Mice are euthanized and the neoplastic tissue is collected. The mass of the neoplastic tissue in mice treated with the selected candidate compounds is compared to the mass of neoplastic tissue present in corresponding control mice.

Kits

The invention provides kits for the treatment or prevention of sarcoma. In one embodiment, the kit includes a therapeutic or prophylactic composition containing an effective amount of an NDV (*e.g.*, r73T-R116) in unit dosage form. In a further embodiment, the kit includes a therapeutic or prophylactic composition containing an effective amount of NDV (*e.g.*, r73T-R116) in unit dosage form.

In some embodiments, the kit comprises a sterile container which contains a therapeutic or prophylactic composition; such containers can be boxes, ampoules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

If desired an antibody of the invention is provided together with instructions for administering an NDV (*e.g.*, r73T-R116) to a subject having or at risk of developing neoplasia. The instructions will generally include information about the use of the composition for the treatment or prevention of neoplasia. In other embodiments, the instructions include at least one of the following: description of the therapeutic agent; dosage schedule and administration for treatment or prevention of neoplasia or symptoms thereof; precautions; warnings; indications; counter-indications; overdosage information; adverse

reactions; animal pharmacology; clinical studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

The practice of the present invention employs, unless otherwise indicated,
 5 conventional techniques of molecular biology (including recombinant techniques),
 microbiology, cell biology, biochemistry and immunology, which are well within the purview
 of the skilled artisan. Such techniques are explained fully in the literature, such as,
 “Molecular Cloning: A Laboratory Manual”, second edition (Sambrook, 1989);
 “Oligonucleotide Synthesis” (Gait, 1984); “Animal Cell Culture” (Freshney, 1987);
 10 “Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1996); “Gene
 Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Current Protocols in
 Molecular Biology” (Ausubel, 1987); “PCR: The Polymerase Chain Reaction”, (Mullis,
 1994); “Current Protocols in Immunology” (Coligan, 1991). These techniques are applicable
 to the production of the polynucleotides and polypeptides of the invention, and, as such, may
 15 be considered in making and practicing the invention. Particularly useful techniques for
 particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art
 with a complete disclosure and description of how to make and use the assay, screening, and
 therapeutic methods of the invention, and are not intended to limit the scope of what the
 20 inventors regard as their invention.

EXAMPLES

Example 1. Assembly of antigenome cDNA of NDV strain 73T.

Six subgenomic cDNA fragments generated by high-fidelity RT-PCR were
 25 assembled in the pUC19 vector. The full length cDNA of NDV 73T was designated as p73T.
 The nucleotide and deduced amino acid sequences of the F protein cleavage site (FPCS) in
 73T were modified to that of the NDV LaSota strain (lentogenic, lento) or glycoprotein B
 (gB) of cytomegalovirus (CMV) (S116) (Figure 1; double slash indicates the site of cleavage
 of the F protein). The cDNA was completely sequenced to confirm the viral sequence. F
 30 protein cleavage site: Wt: ggg agg aga cag aaa cgc tt; Lento: ggg ggg aga cag gaa cgc
 ctt; S116: cat aat aga acg aaa tcc tt; S116KM: cat aat aaa atg aaa tcc tt; R116:
 cat aat aga acg aaa cgc tt.

Example 2. Transgene insertion into NDV 73T genome.

Transgenes were inserted into p73T at two locations: at the intergenic sequences between P and M or at the intergenic sequences between the HN and L junctions (Figure 2A).

- 5 To insert a single transgene cassette at P-M or HN-L junctions, construction of p73T cDNA containing a transgene at the P-M or HN-L junctions was performed by inserting the transgene cassette into AfeI sites created between the P and M genes (nt 3148) or between the HN and L genes (nt 8231). The inserted gene cassette contains the gene end (GE; 5'-TTAAGAAAAAA -3'), intergenic nucleotide (T), gene start sequence (GS; 5'-ACGGGTAGA -3'), and open reading frame (ORF) of the transgene. In addition, ten
- 10 nucleotides (5'-cgccgccacc-3') were inserted upstream of the initiation site to introduce a Kozak sequence. The full length (FL) 73T cDNA containing the transgene at P-M or HN-L junction was designated as p73T-P1 or p73T-HN1, respectively. Full-length cDNA containing two separate transgenes at P-M and HN-L junctions in a single genome were
- 15 constructed and designated as p73T-P1-HN1 (Figure 2B). To insert two transgene cassettes at the same junction (*e.g.*, P-M), an AfeI site was introduced at the end of ORF the first transgene (#1) (nt 3169). The 2nd transgene ORF was PCR amplified with primers containing GE and GS sequences and inserted at the AfeI site. The antigenomic cDNA containing two transgene cassettes the P-M junction was designated as p73T-P2.

20

Example 3. Recovery of infectious recombinant NDV strain 73T (r73T) with modified FPCS.

- The NDV 73T NP, P, L proteins and antigenic cDNA (p73T-lento or p73T-S116) were cloned under the control of the T7 RNA polymerase promoter and terminator. The four
- 25 plasmids were co-transfected into an RNA polymerase expressing cell line (Figure 3A). The recovered viruses were designated as r73T-lento or r73T-S116. The r73T-lento and r73T-S116 were passaged in Vero cells with media with and without trypsin supplement. The growth of r73T-lento is trypsin dependent whereas r73T-S116 can grow in medium without trypsin supplement. F protein cleavage sequences (FPCS) in r73T-S116, with and without
- 30 hGM-CSF at the P-M junction, were assessed. The 73T-S116 strains were further passaged for 10 passages in Vero and human fibrosarcoma HT1080 cells at MOI 0.01 in media without supplement of trypsin. Mutations in the FPCS (R113K and/or Q114M) appeared at passage 7.

The S116R mutation was detected at passage 9. At passage 10, the F, HN and transgene were sequenced and no additional mutations were found.

Example 4. Characterization of the recombinant 73T strain with different F protein cleavage sequences.

Recombinant 73T strain with novel modified F protein cleavage sequences (FPCS) included the following sequences:

- Lento: $^{111}\text{G}-\text{G}-\text{R}-\text{Q}-\text{E}-\text{R}/\text{L}-\text{I}^{118}$
- S116: $^{111}\text{H}-\text{N}-\text{R}-\text{T}-\text{K}-\text{S}/\text{F}^{117}$
- S116K: $^{111}\text{H}-\text{N}-\text{K}-\text{T}-\text{K}-\text{S}/\text{F}^{117}$
- S116M: $^{111}\text{H}-\text{N}-\text{R}-\text{M}-\text{K}-\text{S}/\text{F}^{117}$
- S116KM: $^{111}\text{H}-\text{N}-\text{K}-\text{M}-\text{K}-\text{S}/\text{F}-\text{I}^{118}$
- R116: $^{111}\text{H}-\text{N}-\text{R}-\text{T}-\text{K}-\text{R}/\text{F}-\text{I}^{118}$

The recombinant 73T strains with different FPCS were characterized with regard to MDT, ICPI, relative HT1080 cell killing, replication in Vero cells, and replication in eggs (Figure 4).

Avian virulence of NDV is mainly determined by the F protein cleavage sequences (FPCS). r73T-lento was engineered to contain the FPCS of the non-virulent strain LaSota. Replication of LaSota virus in the tissue cultures is trypsin dependent, as F protein cannot be cleaved. r73T-lento forms tiny plaques in Vero cells without trypsin supplement, indicating that the F protein is not cleaved and virus cannot spread efficiently from cell-to-cell. r73-lento replicated at a low level in Vero cells (7.5×10^3 pfu/ml), but efficiently in eggs with endogenous trypsin-like enzyme (5.7×10^8 pfu/ml). r73-lento is not virulent in chickens as demonstrated by the mean death time (MDT) of embryos inoculated with the virus (MDT >156hr) and by an intracerebral pathogenicity index (ICPI; ICPI=0.00), and has low cytotoxicity in HT1080 cells (13% cell killing).

r73T-S116 can form relatively large plaques, and reaches a titer of 4.4×10^6 pfu/ml in Vero cells. This was comparable to the titers obtained when r73T-S116 was grown in Vero cells supplemented with trypsin. This data indicated that the fusion protein cleavage site (FPCS) of r73T-S116 can be cleaved without exogenous trypsin in tissue cultures. It was not

virulent in chickens and showed 31% cell killing in HT1080 cells. r73T-S116 was examined for its genetic stability by *in vitro* cell passage.

After 10 passages in Vero or HT1080 cells, amino acid substitutions were found in the FPCS: R113K, Q114M, and/or S116R. To eliminate the possibility that additional sequence change had occurred in the viral genome, recombinant r73T-S116 mutant viruses were constructed by reverse genetics and evaluated. Except for r73T-R116, r73T-S116 and its derivatives were similar to the parental S116 in that these mutant viruses were not virulent in chickens and were capable of similar levels of HT1080 cell killing. HT1080 cell killing was between 29%-31% for the single mutation and 48% for the double mutations.

The plaque size of M114 and K113M114 were significantly larger than S116. The r73T-R116 mutant acquired one amino acid change at residue 116 (S116R) in the FPCS. The R116 next to the cleavage site is known to be important for efficient cleavage of the F protein. r73T-R116 formed large plaques in Vero cells, grew to similar titers with and without trypsin supplement, and efficiently killed HT1080 cells (80%). R116 increased chicken virulence as shown by the MDT assay (72, 80 hrs). Although the ICPI value (0.65) was <0.7 in one test, it is preferable to further reduce its chicken virulence.

Example 5. r73T-R116 derivatives have reduced chicken virulence.

Virus can be engineered to express a transgene at the P-M junction (1) a 2nd transgene at the HN-L junction (2) and an increased HN-L intergenic region that is extended by insertion of non-coding sequence (3) (Figure 5A). The same design for insertion of a transgene cassette at P-M junction as in Figure 2A is used here. The 2nd transgene cassette contains the L gene start sequence (GS; 5'-ACGGGTAGA -3'), open reading frame (ORF) of the transgene, sequences from 3' untranslated region of the L gene (highlighted in italics) and the L gene end sequence (GE; 5'-TTAAGAAAAAA -3'). The non-coding sequence used to increase the HN-L junction were taken from paramyxoviruses type -1 (APMV-1), respiratory syncytial virus (RSV) or random sequence which does not have homology with any known sequence. The insertion can be in the range of 60-318 nt. Insertion of a 2nd transgene at HN-L allowed the virus to express two transgenes, such as hGM-CSF and GFP. Inserted sequences at the HN-L junction are listed (Figure 5B).

Example 6. Modification of r73T-R116 and characterization of r73T-R116 derivatives.

To reduce r73T-R116 virulence in chickens, r73T-R116 virus was modified to increase the HN and L intergenic sequence by insertion of sequences of various lengths.

r73T-R116 derivatives were evaluated for infectivity by examining plaque formation and replication in cells and eggs; for avian pathogenicity by examining MDT and ICPI, and for tumor cell killing (Figure 6A).

Intergenic insertions of 318nt from APMV, 198nt from RSV and 198 random
 5 sequences indeed reduced virulence in chickens, with MDT >156hr and ICPI of 0.27, 0.0375 and 0, respectively. A long insertion (random 198 nt) had an increased effect on reducing virulence than a short insertion (random 60 nt). The insertion of 144, 102 and 60nt had some virulence in chickens, but MDT times were shorter. ICPI values for the insertion of 144, 102 and 60nt were 0.74, 0.51 and 0.78, respectively. The insertion of non-viral sequence
 10 (random 198nt) more effectively reduced virulence than insertion of viral sequence (RSV-198nt). The insertion of the 2nd transgene cassette (EGFP) at the HN-L junction did not reduce chicken virulence (MDT 86hr and ICPI of 0.82). All insertions slightly reduced viral replication in eggs by up to 4-fold, but did not effect viral replication in Vero cells (~10⁶ pfu/ml). Although mutant viruses were more attenuated in chicken, tumor cell killing
 15 function was not affected. All r73T-R116 derivatives had tumor cell killing efficiency in the range of 75%-86% on day 3 of infection.

Example 7. Growth kinetics of r73T viruses in eggs and Vero cells.

To determine growth conditions for the r73T viruses, growth kinetic studies were
 20 performed in eggs (Figure 7) and Vero cells (Figure 8). Chicken embryonated eggs were infected with 100, 1000, 10,000 or 100,000 FFU/egg of indicated viruses and incubated for 2, 3 or 4 days. The allantoic fluid was harvested and viral titers were determined by FFA. As shown in Figure 7, 100 FFU/egg had low titer on day 1, but reached peak titer on day 2. Overall, most viruses reached peak titers of ~ 8 logs/mL on Day 2 regardless of the inoculate
 25 dose used. R116i-198 had the lowest titer, low inoculum dose generated the highest yield on day 2, indicating that there might be accumulation of defective particles. R116i-318-APMV insertion also had a lower peak titer of ~7 logs. R116i-198-RSV reached a peak titer of 7.5 logs. S116 constructed by reverse genetics had no reduction in virus production in eggs. Therefore, among the R116 derivatives, the virus with RSV-198nt insertion was a top
 30 candidate of oncolytic virus in terms of growth properties in eggs.

The viruses were also evaluated in sero-free Vero cell clone 51D11, a proprietary cell line generated by MedImmune. All the viruses replicated well under both MOI conditions

(0.001 and 0.0001) (Figure 8). The modification of 73T FPCS and intergenic insertion in the HN-L junction of R116 did not affect virus growth in Vero cells.

Example 8. Selective cell killing of r73T and derivatives in cancer cells compared to the normal cells.

r73T and its derivatives were evaluated for their cell killing in human fibrosarcoma HT1080 compared to normal human skin fibroblast CCD1122Sk cells, relative to untreated control cells (Figures 9A and 9B). Data were obtained after infection with r73T derivatives at different doses ranging from 1 to 100,000 PFU for 72 hr. In HT1080 cancer cells, the virus with lentogenic FPCS had the least killing, S116 at the FPCS was intermediate, and virus with R116 at FPCS had cell killing as efficient as r73T wt virus. In normal CCD1122SK cells, cell killing of all viruses was not as efficient as that for cancer cells. The reduction in killing efficiency was ~100-fold. Regardless of the FPCS sequences, all viruses had similar killing efficiency in the normal cells. The cell killing efficiency in cancer and normal cells was expressed as 50% effective concentration (EC₅₀) interpolated from the dose response curve using Prism 6.0 (Figure 9C). R116 derivative had a similar EC₅₀ value as r73T wt with EC₅₀ of 10 PFU, indicating that the modification of FPCS did not affect virus replication and cell killing efficiency in cancer cells. Replication of these viruses in the HT1080 and CCD1122SK cells at MOI 0.01 was determined on day 3 post infection (Figure 9D). All viruses preferentially replicated in the cancer cells over normal cells, with a difference of about 1.5-2.0 logs.

Example 9. r73T derivatives had anti-tumor activity *in vivo* when delivered either systemically or intratumorally to immunodeficient mice carrying human tumor xenografts.

To evaluate oncolytic activity *in vivo*, HT1080 xenograft model was established by injecting the HT1080 cells at a concentration of 5×10^6 cells/0.1ml subcutaneously into Balb/C athymic nude mice at age of 5-6 week old. As hGM-CSF has no cross reactivity in mouse, this study was not geared to assess the transgene effect, but the oncolytic capability of various r73T constructs. R116-318i-hGM-CSF was given intratumorally (it) or intravenously (iv) and tumor growth rate was compared between the treatment and the control groups (Figure 10A).

Mice were randomized into groups (N=10) as indicated when tumor volume reached approximately 65 mm³. Mice received a single dose of either PBS or 2 x 10⁷ Pfu of r73T-hGM-CSF-R116i-198 administered intratumorally (IT) or 1 x 10⁸ PFU administered intravenously (IV) via tail vein injection. Tumor size was measured every 3-4 days. As presented in Figure 10A, r73T-R116i could induce significant tumor regression when it was either administrated by IV or IT. The amount of tumor regression induced by the two routes was comparable, and was significantly different from the control group.

The oncolytic activities of r73T derivatives were compared in the HT1080 xenografts by IT injection of 1 x 10⁸ PFU (Figure 10B). Mice were randomized into groups (n=7) when tumor volume reached approximately 180 mm³. Mice received either PBS or 1 x 10⁸ PFU of r73T-hGM-CSF-lento (lento) or r73T-hGM-CSF-S116K113M114 (S116 KM) or r73T-hGM-CSF-R116i-318 nt APMV-N (R116i) or r73T-hGM-CSF (r73T wt). Tumor size was measured every 3-4 days (* P< 0.05, un-paired student T test). Two doses of r73T derivatives were capable of inducing significant tumor regression albeit differences in effectiveness.

r73T-lento was the least effective whereas r73T wt was the most effective in tumor regression. r73T-lento had similar effect as the S116 virus although S116 virus had 10-fold lower EC₅₀ for *in vitro* cell killing (Figure 9A). r73T-R116i-318 was as potent as 73T wt in inhibition of tumor growth until 9 days post the 2nd dose (day 19 post tumor implantation), the tumor grew back in R116i treated mice, but not in the 73T wt treated group. These data showed that r73T derivatives had anti-tumor activity *in vivo* when delivered either systemically or intratumorally to immunodeficient mice carrying human tumor xenografts. The efficient cleavage of the F protein is important for virus replication *in vitro* and *in vivo*. The viruses with the R116 at the FPCS were more potent in cell killing *in vitro* and *in vivo*.

Example 10. Tissue biodistribution of r73T derivative following intravenous delivery.

To determine if the oncolytic NDV virus selectively replicates in tumor tissues and viral clearance, virus distribution in different organs was determined. Athymic nude mice bearing subcutaneous HT1080 tumors with size of ~ 250 mm³ were treated with R116i (r73T-hGM-CSF-R116i-318 nt APMV-N) at a dose of 1 x 10⁸ PFU intravenously and sacrificed on day 1, 4, or 8 (n=3 per time point). Serum, lungs, spleen, ovaries and tumor were collected.

The presence of virus was quantified by plaque assay in Vero cells, and hGM-CSF transgene expression was measured by ELISA assay. Virus replication in tumor and organs were assessed on day 1, 4 and 8 post infection. Virus was only detected in organs on day 1

(no virus was detected in ovary at all time points) and virus load in tumor tissues was ~100-fold higher than lungs and spleens (Figure 11A). The presence of virus in tumor persisted for at least 8 days, indicating that the virus selectively replicated in tumor tissues. Consistent with the viral replication data, the level of hGM-CSF was the highest and lasted more than 8 days (Figure 11B). These data demonstrated that the NDV virus can effectively replicate in tumor tissue and deliver the transgene to the local tumor tissue effectively.

Example 11. Antigenome cDNA of 73T containing chimeric F and/or HN gene.

Viral surface glycoproteins are important antigens for immunogenicity and virulence in chickens. Strategies were explored to replace the F and HN genes of NDV by the corresponding extracellular (ecto) domains of other paramyxoviruses which are not virulent in chickens individually or in combination. Parainfluenza virus 5 (PIV 5) is a canine paramyxovirus and does not cause diseases in human. Pigeon paramyxovirus type 1 (PPMV-1) has been shown to be nonvirulent in chickens with an ICPI of 0.025, as previously reported, and is antigenically distinct from NDV (Dortmans *et al*, *Veterinary Microbiology*, 2010, vo. 143, pages 139-144.) There exist two genetically closely related pigeon paramyxovirus type 1 (PPMV-1) variants with identical velogenic fusion protein cleavage sites, but with strongly contrasting virulence (*Veterinary Microbiology*, 2010, 143:139-144). Full-length antigenomic cDNAs of NDV 73T in which the F and/or HN glycoprotein ectodomain were swapped with that of PPMV-1 and/or PIV5 were generated (Figure 12A). NDV, PIV5 and PPMV-1 sequences are indicated with colored boxes as blue, purple or green, respectively).

The amino acid lengths of individual proteins or protein domains are indicated. Plaque formation in Vero, relative HT1080 cell killing and MDT were performed as described previously (Figure 12B). Chimeric viruses, except for PIV-5 F-HN, were recovered and were capable of growing in the cells in the absence of exogenous trypsin. All three viruses formed good sized plaques, showing efficient cell-to-cell spread. PPMV-1 F-HN or F chimeric viruses had MDT values of 79 and 84 hr, respectively, indicating potential virulence in chickens. Both PPMV-1 chimera killed HT1080 cells efficiently at levels of 71 and 61%. PIV5-F chimera did not grow in eggs and was not virulent in chickens, and killed HT1080 cells at 47%. Serum cross reactivity between the NDV and PPMV-1 or PIV5 chimeras was examined by neutralization assay using the serum collected from mice that received two intravenous doses of 1×10^8 PFU of r73T-R116i. The r73T virus was

neutralized with the NDV-infected serum (titer 960) whereas the PPMV-1 and PIV5 chimera were not neutralized (titer < 4). This result confirmed that there was no cross reactivity between NDV and PPMV-1 or PIV5. Chimeric viruses with distinct antigenicities have the potential to serve as boosting oncolytic viruses for the patients that have developed anti-NDV immune responses during prior NDV treatment.

A comparison of NDV RNA polymerase activity with other paramyxoviruses by mini-genome assay (Figure 12C). The T7 expressing cells were transfected with the three plasmids expressing the NP, P, L proteins of NDV and the plasmid encoding the NDV anti-minigenomic cDNA encoding the GFP gene using Lipofectamine 2000, or the plasmids encoding N, P, L genes of measles virus (MV) or respiratory syncytial virus (RSV) and respective RSV or MV GFP anti-minigenome cDNA plasmid. Two or three days post transfection, minigenome replication as indicated by the expression of GFP proteins was examined under fluorescent microscope. The NDV had the strongest polymerase activity compared to measles virus and RSV.

Example 12. Cancer cells sensitive to NDV were identified by cell panel screening.

In order to understand what tumor types may be sensitive to NDV oncolysis, 180 cancer cell lines covering a broad range of tumor types and indications were tested for sensitivity to recombinant NDV and variants thereof. Cell lines were obtained from American Type Tissue Collection (Manassas, VA) or the European Collection of cell cultures (ECACC) and were cultured in media and under conditions recommended by the supplier. 10,000 cancer cell lines were seeded in 96 well plates and were infected 6 hours later with a virus. Virus concentrations ranged from MOI 10- 0.0001 (or 1 to 100,000 pfu per well). Cell viability was determined 48-96hrs post infection. Sensitivity was determined using a cut off of >30% cell kill at 72hrs post infection with virus at an MOI of 0.1. Figures 13A and 13B provide an overview of the sensitivity by tumor type. Haematological cancer cell lines (leukemia and lymphoma) were relatively insensitive to NDV oncolysis whereas a majority of the melanoma, ovarian and pancreatic cell lines tested were sensitive to NDV. Approximately 58% of all human cancer cell lines tested were sensitive to NDV R116i at FPCS relative to 4% of cell lines tested were sensitive to the S based virus. This was most likely due to the cleavage of the F protein by proteases. The R116i virus is more likely to be cleaved by ubiquitous furin-like proteases whereas the protease that is likely to cleave the

S116 F protein is undefined and is probably less ubiquitously expressed. The re-derived S116-KM variant yielded larger plaque size and enhanced oncolytic potency relative to the original S116 variant. To test this further a smaller panel of 22 cancer cell lines that had been determined to have a range of sensitivity to NDV R116 virus was infected with GFP

- 5 expressing variants and cell viability was determined at 72hr post infection. Using the same sensitivity cut off as described above, 41% of cell lines tested were sensitive to R116i, 4% sensitive to S116 and the rederived S-KM is more potent than the S116 NDV with 27% of cell lines tested being sensitive.

10 **Table 4. Summary of cancer cell sensitivity to virus killing by R116-hGM-CSF**

Indication	No. cell lines tested	No. cell lines sensitive to S116	No. cell lines sensitive to R116i
Bladder	8	0	6
Bone	4	0	4
Brain	4	0	4
Breast	26	2	14
Colorectal	14	0	3
gastric	4	0	3
HNSCC	9	0	5
Haem	28	0	4
Kidney	4	0	4
Liver	9	2	7
Lung	28	0	20
Melanoma	8	0	8
Ovarian	10	0	8
Pancreatic	14	0	11
Prostate	6	2	3
Other	3	1	1

35

The cells derived from the indicated cancer tissues were examined for cell killing by recombinant NDV 73T with R116 at the FPCS and human GM-CSF. The number of the cells that showed greater than 50% killing by the virus infection at moi of 0.1 and total cell lines screened are indicated.

- 40 **Example 13. r73T derivatives had tumor killing and/or tumor growth inhibiting activity in syngeneic melanoma model.**

Following tumor model refinement, S116-RD NDV encoding human or murine GM-CSF was tested for efficacy in refined B16F10 syngeneic model (Figure 15A and 15B). 1×10^8 pfu was infected intratumorally for 3 doses and there were a minimum of 8 mice per group. Significant tumor growth inhibition is demonstrated with >80% tumor growth inhibition (Figure 15B). Short term tumor regression was achieved with repeat intratumoral (i.t.) dosing, which also led to a significant increase in survival time 18 days for control relative to 42 days in treated group. Cessation of treatment after 3 doses was followed by tumor re-growth. A single animal with no evidence of residual tumor in S116-mGM-CSF group at 50 days was re-challenged by implantation of B16F10 tumor cells on the alternate flank. Tumor growth was delayed but not inhibited, suggesting in this model that a full immune memory response was not achieved within this single animal.

In order to assess oncolytic and immune effects on tumor growth, NDV variants R116i and S116 encoding hGM-CSF or mGM-CSF respectively were tested for efficacy in the mouse syngeneic immune competent CT26 colorectal tumor model. Each virus was dosed with 1×10^8 PFU of virus intra-tumorally for 4 doses. Tumors were a minimum of 100 mm^3 before dosing commenced. All animals treated with virus demonstrated potent anti-tumor activity as a monotherapy. With 11/12 animals tumor free following treatment with R116 encoding human GM-CSF which is a 92% complete response rate. The less lytic re-derived S116-KM virus had a reduced tumor growth inhibition achieving 53% TGI and a complete response rate of 36%. However, in the presence of murine GM-CSF which unlike human GM-CSF will be active in the mouse model this response rate was increased to 54% with a tumor growth inhibition of 75%. Thus, it is likely that arming the S116 virus with GM-CSF may enhance anti-tumor activity.

Virus	% TGI	% Complete response (CR)
R116 hGM-CSF	83	92
R116 mGM-CSF	ND	ND
S116 hGM-CSF	53	36
S116 mGM-CSF	75	54

Tumors that were remaining were taken for histological analysis and stained by Hematoxylin and eosin stain (H and E) and using immunohistochemistry methods for NDV detection (Figure 15C). There is clear evidence of NDV expression and this appears to be

concentrated around the necrotic areas of the tumor, suggesting that NDV is contributing to tumor cell death and necrosis. There is also clear evidence of immune cell infiltration into the tumor and necrotic areas. In areas that have strong staining for NDV multinucleated cell syncytia formation can also be noted. Additionally, there appears to be very minimal
5 remaining viable tumor, indicating the tumor growth inhibition data may underestimate the activity of NDV within this model.

Figure 15C shows NDV has potent anti-tumor activity in immune-competent mouse CT26 colorectal tumor model. In order to assess oncolytic and immune effects on tumor growth, NDV variants (R116i and S116 encoding hGM-CSF or mGM-CSF respectively were
10 tested for efficacy in the mouse syngeneic immune competent CT26 colorectal tumor model. Each virus was dosed with 1×10^8 PFU of virus intra-tumorally for 4 doses. Tumors were a minimum of 100mm^3 before dosing commenced. All animals treated with virus demonstrated potent anti-tumor activity as a monotherapy. With 11/12 animals tumor free following treatment with R116 encoding human GM-CSF which is a 92% complete response
15 rate. The less lytic re-derived S116-KM virus had a reduced tumor growth inhibition achieving 53% TGI and a complete response rate of 36%. However, in the presence of murine GM-CSF which unlike human GM-CSF will be active in the mouse model this response rate was increased to 54% with a tumor growth inhibition of 75%. Thus, arming the S116 virus with GM-CSF enhances anti-tumor activity.

Figures 15D-F show that multiple dosing with rNDV R116i caused tumor growth inhibition and drove immune cell recruitment into ovarian cancer (OVCAR4) xenograft model. To further evaluate rNDV oncolytic activity *in vivo* a human ovarian cancer xenograft model (OVCAR4) was utilized. This model is slow growing and has gross pathology reminiscent of human ovarian tumors with a poorly differentiated cell morphology
25 and large ascetic like fluid filled areas. Once tumors had reached 100mm^3 mice were randomized to receive 8 doses of R116i NDV encoding either mouse or human GM-CSF or PBS as a control. Only the product from the murine gene sequence would be bioactive in the mouse model. 2.5×10^7 pfu were injected intra-tumorally in a volume of less than $50\text{ }\mu\text{l}$ once weekly. Tumor growth curves are shown in Figure 15D. Long term tumor inhibition
30 achieved in tumor-bearing animals treated with either NDV variant. There was also evidence of viral genome using RT-PCR within the tumor at the end of the study (24 hrs post last dose). Following histological analysis there is clear evidence of immune infiltrate as well as other histological changes in residual tumor (Figures 15E and F). Treated tumors are far less

de-differentiated than control treated animals and appear to have less ascites fluid filled areas. A high level of inflammatory infiltrate found in number of treated tumors adjacent to remaining tumor and immunohistochemistry (IHC) analysis reveals these immune infiltrates are positive for NDV protein. There is a strong degree of innate immune cells recruitment
 5 into the tumors in NDV treated groups. These data demonstrate potent anti-tumor efficacy in a morphologically relevant ovarian cancer model which may be caused via direct oncolysis and innate immune recruitment and activation.

Example 14: NDV viruses induced tumor regression.

10 73T-R116i-hGM-CSF and 73T-R116i-mGM-CSF were evaluated for oncolytic effect in the B16 melanoma model. The study evaluated virus tolerability in the B16 mouse. Each virus was dosed at 2×10^7 pfu twice on days 11 and 14 intravenously (i.v) or intraperitoneally (i.p), or once on Day 11 at 1.1×10^7 pfu intratumorally (i.t). The groups treated with R116-hGM-SCF or mGM-SCF by three different routes of administration had slower rate of tumor
 15 growth compared to the untreated group (Figure 15A). Each group included 3 mice. The tumor inhibition rates were statistically significant from the control group. Thus, r73T derivatives of the invention had advantageously low avian pathogenicity, high oncolytic activity, and replicated to high titers in chicken eggs. Based on these results, viruses of the invention are useful to induce tumor regression and enhance cancer patient treatment
 20 outcomes (Figure 16).

In addition to GM-CSF, a number of transgenes (Table 2) may be inserted into NDV 73T strain to enhance tumor killing. These transgenes include the following:

(1) Cytokines or engineered variants of cytokines, such as GM-CSF, IL-2, IL-21, IL-15, IL-12 and IL-12p70

25 (2) Cell surface ligands and chemokines, including OX40L, CD40L, ICOSL, Flt3:, B.1 (CD80), CD137L, CXCL10 (IP-10), CCL5, CXCL9. (3) Myc

inhibitor: Omomyc. (4) Transgenes for *in vivo* imaging purposes, such as Sodium iodide symporter (NIS)-mediated radiovirotherapy for

radiovirotherapy. (5) Additional modulators of tumor cell survival to

30 enhance tumor killing including, but not limiting to, inhibitors of cell cycle progression, inhibition of anti-apoptotic proteins, enhancement of pro-apoptotic proteins, inhibition of key oncogenic drivers of malignant transformation. These may include transgenic delivery of proteins

following selective NDV replication in tumor cells, the production of selective or broad activity siRNA, the delivery of miRNA or the inhibition of selected miRNA (6) Tumor antigens such as E6, E7, cancer testis antigens, oncofetal antigens, artificial or overexpressed proteins as novel tumor antigens either alone or in combination with other transgenes. (7) Antibodies or recombinant fusion proteins that target immunomodulatory proteins to either block negative regulation or provide an agonistic signal to enhance T-cell function. Example of such antibodies may include but are not limited to; PD-L1, CTLA4, CD-137 (4-1BB), OX40, GITR, TIM-3, CD73, PD-1, HVEM, and LIGHT. (8) Augmentation of recNDV's pharmacodynamic/pharmacokinetic activity by engineering or expressing recNDV in cells that transfer proteins onto recNDV to reduce clearance by complement or to diminish the adaptive immune response to NDV.

Table 5. Transgenes for potential insertion into NDV 73T and their biological activities

Transgene	Gene Size	MOA (functions relating to immunotherapy)
Cytokines		
<u>GM-CSF</u>	0.47kB	• Hematopoietic cell growth factor • Stimulates stem cells to differentiate to granulocytes and monocytes (and ultimately APCs)
<u>IL-2</u>	0.48kB	• Stimulates CD8 T, CD4 T, NK, and B-cells • Augments cytokine production • Induces T-reg expansion
<u>IL-21</u>	0.6kB	• Stimulates CD8 T-cells, NK, and B cells • Does not induce T-reg expansion
<u>IL-15</u>	0.5kB	• Similar to IL-2 function • Supports survival of CD8 CTLs (in contrast to IL-2 which promotes memory CD8 CTLs)
<u>IL-12p70</u>		• Stimulates CD8 T-cell and NK cells
<u>IL-12A</u>	0.76 kB	• Drives Th1 CD-4 T-cells differentiation
<u>IL-12B</u>	0.99 kB	• Reduces T-regs • Re-polarizes M2 macrophages to M1 macrophages
Cell Surface Ligands and Chemokines		
<u>OX40L</u>	0.55kB	• Binds receptor on activated (not naïve) T-cells delivering activation signal • Downstream effects of activation on T-cell: - Proliferative and anti-apoptotic - Induces cytokines, Ag-spec Ab, memory T-cells
<u>CD40L or CD40</u>	0.79kB	• Binding to receptor on DCs is critical for T-cell priming (MHC-II, CD86, CD80 upreg) • Stimulates IL-12, MIP-1a from DCs driving Th1-diff and migration to inflammatory sites • Important for CTL (cross-priming)
<u>ICOSL</u>	0.9kB	• ICOS Receptor expressed on naïve T-cells and upreg. after TCR and CD28 stim. • Literature shows evidence of both Th1 and Th2 differentiation through ICOS • Provides signal for high affinity Ab production
<u>Flt3L</u>	0.7kB	• Key driver of hematopoietic cell (DCs, NK, and B cells) development and differentiation • Ubiquitously expressed (receptor limited to immune cells)
<u>B7.1 (CD80)</u>	0.866 kB	• Typically up-regulated on maturing DCs • Co-stimulatory molecule for CD4-Naïve T-cell differentiation into T-helper subsets (Th1; IL-
<u>CD137L</u>	0.767 kB	• Expressed on DCs, NK cells, activated CD8-T cells • Triggers T-cell proliferation, IL-2 secretion, and cytolytic abilities
<u>CXCL10 (IP-10)</u>	0.296 kB	• ELR-negative molecule with demonstrated anti-tumor activity • Chemoattractant for T-cells, NK cells, monocytes, DCs • Attenuates angiogenesis
<u>CCL5 (RANTES)</u>	0.275 kB	• Chemoattractant for Eosinophils, Basophils, Mast cells, monocytes, CTLs, CD4-T-cells, • Anti-tumor functions thought to be the result of immune cell infiltration
<u>CXCL9 (MIG)</u>	0.367 kB	• Chemoattractant for Leukocytes • IFN-gamma dependent expression; NOT induced by IFN-alpha/beta
myc inhibitor		
<u>Omomyc</u>	1.3 kB	• Demonstrated efficacy in mouse lung cancer model
Image		
<u>NIS</u>	2 kb	human sodium iodide symporter to concentrate ¹³¹ I in cancer cells for radiotherapy

5 Example 15. Cancer therapy involving administration of oncolytic NDV in combination with immune modulatory mAb.

NDV oncolytic virus can be administered concurrently or sequentially with therapeutic antibodies or agonistic fusion proteins where appropriate (*e.g.* anti-PD-L1, anti-CTLA4, anti-OX40, anti-GITR, anti-TIM-3, anti-PD-1 and anti-ICOS). Preclinical data are generated that establish the most effective dose and schedule of molecules that enhance the activity of NDV in tumor models in combination with the novel NDV constructs described herein. Transgenes may be inserted into recombinant NDV for expression either singly or in combination to deliver multiple modes of activity, *e.g.*, to enhance the tumor cell death induced by the novel variants of NDV. Increasing the release of tumor cell antigens combined with an immunomodulatory approach has the potential to increase the adaptive immune response to these liberated tumor antigens.

Example 16. F protein cleavage efficiency and fusion activity were reduced in F protein with R, S or S-KM mutation at the F protein cleavage site.

In order to understand whether the difference in the F protein cleavage site affects F protein cleavage in the infected cells and its impact on the fusion activity, the F protein plasmid was transfected into 293 cells to examine F protein cleavage. In addition, the F and HN plasmids were cotransfected to examine fusion activity in the transient assay as both the F and HN proteins are required for fusion formation (Figures 3B and 3C). Wt F protein was cleaved by host protease more efficiently than the F protein with R, S or S-KM mutation at the F protein cleavage site, very little F protein cleavage product (F1) was detected in F with the S cleavage site and no cleavage product was detected in lentogenic F plasmid transfected cells. The F protein of R and S construct has an N-linked glycosylation site (NXT) at the cleavage site resulting in the slower migrating mobility on the SDS-PAGE than lentogenic F and S-KM as the KM mutation abolished the glycosylation site. These data demonstrated that the R and S mutations at the F protein cleavage site affected F protein cleavage and S-KN is more efficient than S. Since the F and HN gene each was cloned in the pViro2-neo-MCS vector encoding an EGFP gene, syncytial formation can be visualized by the fused green cells. Large syncytial formation was observed in wt F and HN plasmid transfected 293 cells. All the F mutants with R, S or S-KM residues in the F protein cleavage sites resulted in greatly reduced syncytial formation.

Example 17. r73T-R116i virus with 198nt insertion exhibited slower growth and differential RNA and protein synthesis profile in DF-1 cells compared to Vero cells.

R116i virus with 198nt inserted between the HN-L junction exhibited slower growth kinetics in chicken DF-1 cells under high moi condition as shown in Figures 6B and 6D. The growth difference of R116i with 198nt insertion was reduced compared to the wt and the R virus that does not have the inergenic insertion at the earlier time points of infection (10-20h).

5 In Vero cells, the growth difference among these viruses was not very apparent (Figures 6C and 6E). To understand if the inergenic insertion in the R116i viruses affected viral RNA transcription and replication, viral RNA and protein synthesis in infected DF-1 cells were examined by Northern and Western blotting analyses, respectively (Figures 6F and 6G). The RNA and protein synthesis of R116i with 198nt insertion were greatly reduced in infected DF-1
10 cells. In contrast, in infected Vero cells, the upstream gene transcript such as the NP mRNA was greatly increased in R116i-198 or 198RSV infected cells while the genomic RNAs of these two viruses were reduced. The level of the L protein of these two viruses was reduced, but the other viral protein products were increased such as NP, F and HN as shown in Figures 6H and I. The data obtained in Figures 6F-I were conducted at the high moi condition (moi=5). RNA
15 and protein synthesis were further evaluated under the low moi condition (moi=0.001). Again, R116i-198 or 198RSV had differential RNA and protein synthesis profile in DF-1 and Vero cells. Both RNA and protein synthesis of R116i-198 or 198RSV were reduced in chicken DF-1 cells (Figures 6J). In contrast, the upper stream RNA and protein were increased in Vero cells but the level of L mRNA and L protein was reduced due to the intergenic insertion of 198nt
20 sequence (Figure 6K). Viral RNA and protein synthesis were further compared in human cell lines, human fibrosarcoma HT1080 and Hela cells, and DF-1 cells (Figure 6L). These data confirmed that R116i-198 or 198RSV had differential expression, the upstream RNA and protein synthesis increased while the L protein expression was down regulated. These data explained why R116i-198 or 198RSV viruses replicated well in the mammalian cell lines such
25 as Vero, human HT1080 and Hela cells but did not grow well in embryonated chicken eggs and chicken DF-1 cells. It also provided basis of reduced chicken pathogenicity of these R116i viruses as demonstrated by their low intra-cerebral pathogenicity index (ICPI) value.

Example 18. Mouse GM-CSF transgene expression had lower tumor growth inhibition efficacy than human GM-CSF transgene expression in R116i-198RSV, but not in S116-KM.

Figure 11C compared contribution of the mGM-CSF with hGM-CSF transgene expression on oncolytic activity of R116i-198RSV and S116-KM in HT1080 xenograft mouse

tumor model. hGM-CSF transgene does not cross react with mGM-CSF and it was therefore used as the control. The mGM-CSF transgene in R116i-198 administered intra-tumorally had lower efficacy in tumor growth inhibition compared to hGM-CSF. Similar tumor growth inhibition of mGM-CSF and hGM-CSF was observed for S116 virus. Viral titers in viral treated tumors were determined by plaque assay (Figure 11D). On day 4 post tumor injection, similar titer was detected for the R116i-198RSV and S116-KM with mGM-CSF or hGM-CSF pairs. However, on day 7 post injection, R116i-198RSV with mGM-CSF was not detected while R116i-198RSV with hGM-CSF still had viral titer of approximately 4.5 logs/g tissue. There was no difference in viral titers of S116 with hGM-CSF or mGM-CSF. These data indicate that the expression of mGM-CSF by R116i facilitated viral clearance.

Immune cell infiltration in virus infected tumor tissues was examined (Figure 11E). R116i-198RSV with mGM-CSF had the greatest number of neutrophil, NK and macrophage cells in the treated tumors compared to R116i-198RSV with hGM-CSF and the S116 hGM-CSF and mGM-CSF pair. The cytokines and chemokines in virus treated tumor were determined by luminex assay (Figure 11F). All four viruses stimulated cytokine and chemokine production by many folds compared to the untreated tumor tissues. The expression of mGM-CSF by R116i is about 10-fold higher than S116 (106.7 vs 9.9-fold).

Figure 11G showed similar tumor growth inhibition activity of R116i with 198 or 318 nt inserted between the HN-L junction containing the same hGM-CSF transgene in the HT1080 xenograft mouse tumor model. The 318 nucleotide insertion in the HN-L junction did not reduce viral oncolytic virus activity.

Example 19. Evaluation of complement-mediated NDV inactivation and role of regulatory proteins in complement evasion.

The complement (C') system is a major defense system against microbial invasion in the host. There are about 30 different glycoproteins in the human complement system, of which 20 act in plasma and 10 are regulators or receptors on cell membranes. Membrane bound C' regulators (RCA) include 4 well characterized molecules: hCD46, hCD55, hCD59 and hCD35. Their main function is to protect human cells against autologous complement attack without affecting the role of C' in eliminating foreign agents. These RCA proteins are host species-specific. NDV used for viral therapy in the past was generally produced in embryonated chicken eggs. It is expected that NDV oncolytic virus administered by intravenous injection to cancer patients might be cleared rapidly, therefore reducing effective

viral dosing. Since enveloped viruses produced from human cells incorporate RCA proteins during their egress from the infected cells, it is therefore desirable to produce NDV in human cell culture to reduce C' mediated viral lysis or inactivation.

Sensitivity of NDV to C' mediated inactivation was evaluated by examining NDV
5 produced in embryonated chicken eggs, human 293 and Hela S3 suspension cell lines (Figure 17). Virus grown in eggs was sensitive to C' inactivation, serum inhibition was abolished by heat treatment (56°C for 30min) of the serum. Guinea pig complement had the similar level of viral inactivation effect (data not shown), confirming that viral inactivation was due to C'. In addition, the viruses produced from Hela cells were more resistant to human C' mediated viral
10 inactivation than 293 cells and eggs. Thus, the data suggest that Hela cells are better than 293 cells for NDV oncolytic virus production, which likely resulted in slower viral clearance and thus increased the therapeutic index.

To explain why the NDV produced from the Hela S3 cells is more resistant to C', 293 and Hela S suspension cell lines were evaluated for the levels of the 4 well characterized
15 human RCA proteins, hCD46, hCD55, hCD59 and hCD35. hCD35 was not detected in the 293 and Hela cells by Western analysis and the data are therefore not shown in Figure 18. hCD46, hCD55, and hCD59 were detected in higher abundance in Hela S3 cells than in 293 cells. Thus, the levels of RCA proteins inversely correlate with viral sensitivity to C'.

In order to determine if all three RCA proteins regulate C' function, hCD55, hCD59 or
20 hCD46 transgene was inserted into NDV genome by reverse genetics and recombinant viruses expressing each of the three RCA proteins were produced. Western blot analysis showed that each of these RCA proteins was expressed by virus and incorporated into virions (Figure 19). hCD55 was determined to be the major RCA protein conferring to C' inactivation function (Figure 20). The virus expressing hCD55 produced in eggs with hCD55 incorporated into
25 virions was most resistant to C' mediated inactivation, which is very close to the viruses produced in Hela cells. In comparison, hCD46 had marginal improvement in terms of viral resistant to C' inactivation and hCD59 did not have detectable role in C' regulation.

In conclusion, to reduce viral clearance for oncolytic viral therapy and to improve NDV therapeutic index, Hela cells are considered the cell line of choice for viral production.

30 The results described herein were obtained using the following materials and methods.

Cells and viruses.

The following cell lines and corresponding media were used: African green monkey kidney Vero cell line (ATCC) and human fibrosarcoma (HT1080, ATCC), Eagle's minimal essential medium (EMEM, Hyclone) with 10% fetal bovine serum (FBS); Vero clone 51D11 line (MedImmune), serum free media (SFMMegaVir, Hyclone) with 1% glutamine; normal
 5 human skin fibroblast cells (CCD1122Sk, ATCC), ATCC formulated Iscove's Modified Dulbecco's medium (IMEM) with 10% FBS. Recombinant Newcastle disease viruses (NDV) were grown in the allantoic cavities of 10-11-day-old specific-pathogen free (SPF) embryonated chicken eggs, Vero, or Vero clone 51D11 cells.

10 *Construction of NDV antigenomic cDNA and supporting plasmids NP, P and L.*

Viral RNA of NDV strain 73T was obtained from Dr. Mark Peebles (Nationwide Children's Hospital). NDV sequences (GenBank) were aligned to obtain consensus sequences to design DNA oligonucleotides for RT-PCR of the viral RNA. Six subgenomic cDNA overlapping fragments spanning the entire NDV genome were generated by high-
 15 fidelity RT-PCR (Figure 1). The pUC19 vector was modified to include an 88 nt oligonucleotide linker containing restriction sites introduced between the *EcoRI* and *HindIII* sites for sequential assembly of full-length antigenomic cDNA of the NDV 73T strain. In addition, the 73T strain cDNA plasmid (p73T) contains a 27 nucleotide (nt) T7 RNA polymerase promoter at the 5' end and a 189 nt containing HDV antigenome ribozyme
 20 sequence and a T7 RNA polymerase transcription-termination signal at the 3' end. To generate non-virulent NDV, the sequence encoding the protease cleavage site of the fusion protein was modified by site-directed mutagenesis to those of the non-virulent NDV LaSota strain (lentogenic, lento) or glycoprotein B (gB) of cytomegalovirus (S116). For construction of NP, P and L expression plasmids, the protein open reading frames (ORF) were amplified
 25 by RT-PCR and cloned into plasmid pCITE2a under the control of the T7 RNA polymerase.

Insertion of the transgene into the NDV.

For insertion of a transgene at the P-M junction, an AfeI restriction site was introduced at nt 3148 in the subclone plasmid containing SacII-PmlI fragment (Fig 2A). The
 30 cDNA encoding human or mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin 2 (IL-2) was codon optimized and synthesized by DNA 2.0. A gene cassette contains the gene end (GE) of N, the gene start (GS) of P and the open reading frame

(ORF) of the transgene, which was inserted into the AfeI site. The SacII-PmlI fragment from the resulting plasmid was shuffled into plasmid r73T and named as p73T-P1.

To insert a transgene into the HN-L junction between the HN ORF and the gene end signal (GE) sequence of HN, an AfeI restriction site was introduced at nt 8231 in the plasmid containing the AgeI-XbaI fragment (Figure 2A). The gene cassette was generated by PCR using a pair of phosphate sense and antisense primers (Table 3) and inserted into AfeI site.

Table 3. Oligonucleotide primer sequences for insertion of transgene transcriptional cassette.

Transgene	Sense	Antisense
hGM-CSF	5' <u>TTAAGAAAAAATACGGGTAGAA</u> cgccgaccATGTGGCTGCAGAGCCTGCTG 3'	5' TCATTCCTGCACGGGCTCCCAGCAGTC 3'
mGM-CSF	5' <u>TTAAGAAAAAATACGGGTAGAA</u> cgccgaccATGTGGCTGCAGAACCTGCTG TTCCTGG 3'	5'GTATCACTTCTGGCCGGGTTTCTTGCACTC 3'
hIL-2	5' <u>TTAAGAAAAAATACGGGTAGAA</u> cgccgaccATGTATAGGATGCAACTTCTG TC 3'	5' GTCAAGTCAGGGTAGAGATAATGCTCTGGC 3'
mIL-2	5' <u>TTAAGAAAAAATACGGGTAGAA</u> cgccgaccATGTATTCAATGCAGCTGGCA TC 3'	5' GAGTTACTGAGGGGAAGTTGAAATG 3'
EGFP	5' <u>TTAAGAAAAAATACGGGTAGAA</u> cgccgaccATGGTGAGCAAGGGCGAGGA GCTG 3'	5'AATTACTTGTACAGCTCGTCCATGC 3'
EGFP (HN-L)	5' <u>ACGGGTAGGACATGGTGAGCAAGG</u> GCGAGG 3'	5' TTTTCTAACATAGTATAATTAAATCACCAAGGATACAATTGGCCAGAAAAGAGCCTATTAATATGTGATTTTCGCGTTACTTGTACAGCTCGTCCAT 3'

The gene end (GE) and gene start (GS) sequences are underlined. Kozak sequence is shown in lower case. The sequences correspond to 5' or 3' sequences of the transgene are shown in italics.

Except for the EGFP (H-N), all other primer pairs can be used for inserting the transgene between G-M or HN-L.

hGM-CSF: human granulocyte-macrophage colony-stimulating factor; mGM-CSF: mouse GM-CSF; hIL-2 and mIL-2 correspond to human and mouse interleukin 2 (IL-2), respectively.

The AgI-XbaI fragment from the resulting plasmid was shuffled into plasmid p73T, yielding p73T-HN1. Another strategy to insert sequence at the HN-L junction was to insert a

transgene cassette or sequences from other paramyxoviruses between the gene end signal (GE) of the HN and the gene start signal (GS) of the L (Figure 4) at AfeI site that was introduced at nt 8359. The FL cDNA plasmid was designated p73T-R116i. Since the NDV genome length has to be in a multiple of 6 nucleotides (rule of 6), the antigenomic cDNA of various constructs were made to follow the rule of 6.

To insert two transcriptional cassettes into the P-M junction, an AfeI site was introduced at the end of the ORF of GM-CSF (nt 3619) (Figure 2B). The IL-2 ORF was amplified using a pair of phosphate sense and antisense primers containing the GE and GS sequences and inserted at the AfeI site. The SacII-PmlI fragment from the resulting plasmid including GM-CSF and IL-2 transcriptional cassettes was swapped back into plasmid r73T, yielding p73T-P2.

Generation of r73T chimeric viruses containing ectodomain of other paramyxovirus.

The chimeric NDV genomic DNA was produced by replacing the F and HN of NDV with those of pigeon paramyxovirus 1 (PPMV-1). The C-terminal coding sequence for the cytoplasmic tail and transmembrane portion of NDV 73T F (amino acid residues 503 to 553) was joined with the ectodomain F protein coding sequence of PPMV-1 (residues 1 to 502), the N-terminal coding sequences of the NDV HN (amino acid sequence residues 1 to 45) was fused with the HN (residues 46 to 577) by overlapping PCR reactions using GeneArt kit (Invitrogen). The amplified fragment was digested and cloned into PmlI-AgeI digested NDV cDNA. The parainfluenza virus 5 (PIV-5) F or HN were introduced into the NDV 73T antigenomic cDNA by a similar cloning strategy. The PIV5 F (residues 1 to 486) ectodomain was fused with the transmembrane and the cytoplasmic tail of NDV 73T F (residues 503 to 553). The NDV HN (residues 1 to 45) was joined with the PIV5 HN ectodomain (residues 36 to 565). The cDNA fragment was cloned into PmlI-AgeI digested NDV antigenomic cDNA.

Recovery of recombinant NDV from transfected cDNA plasmids.

The mammalian cell line expressing the T7 RNA polymerase such as the BHK-T7 cells were transfected with the three plasmids expressing the NDV NP, P, and L proteins (0.4 µg, 0.4 µg, and 0.2 µg per well of a 6-well dish, respectively) and a plasmid encoding the NDV antigenomic cDNA (1.6 µg) using Lipofectamine 2000. Three days after transfection, the cell culture supernatant was injected into the allantoic cavities of 10 to 11-day-old SPF embryonated chicken eggs or passaged in Vero cells to amplify the rescued virus. Recovery

of the virus was confirmed by hemagglutination assay using 1% chicken red blood cells (RBCs). Rescue of viruses can also be performed by electroporation of the NP, P, L, antigenomic cDNA plasmids together with a plasmid expressing the T7 RNA polymerase into Vero cells as previously described (Kaur *et al.*, Optimization of plasmid-only rescue of highly attenuated and temperature-sensitive respiratory syncytial virus (RSV) vaccine candidates for human trials. 2008 J. Virol. Methods 153:196-202). The recovered virus was confirmed by sequencing of RT-PCR amplified cDNA.

In vitro passage to select virus with stable F protein cleavage site.

To examine if the F protein cleave sequence (FPCS) was stable and if any stabilizing mutations could be selected after passaging in tissue culture, r73T-S116 were serially passaged for 10 times in Vero and human fibrosarcoma HT1080 cells at MOI of 0.01. After every 2-3 passages, viral RNA was isolated from the culture media, cDNA was amplified by RT-PCR and the F and/or HN genes were sequenced.

Virus plaque morphology in Vero cells and titer quantitation by plaque assay.

Vero cells on a 6-well plate were infected with serial diluted virus and incubated under 1% methylcellulose overlay at 37° C for 3 days or 6 days for plaque morphology in the presence of trypsin (TrypLE™, Invitrogen) for viral titer quantitation. The cell monolayers were fixed with methanol and stained with chicken anti-NDV polyclonal antibody against whole inactivated NDV virus followed by exposure to horseradish peroxidase (HRP)-conjugated anti-chicken antibody (Dako).

Virus chicken pathogenicity test by egg mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays.

The pathogenicity of the r73T viruses was determined by the mean death time (MDT) test in 10-day-old SPF embryonated chicken eggs. The ICPI test in 1-day-old SPF chicks was conducted at the USDA's National Veterinary Service Laboratory (NVSL, Ames, Iowa). For the MDT test, 0.1 ml of a series of 10-fold dilution between 10⁻⁶ and 10⁻⁹ was inoculated into the allantoic cavities of 8-10 of 9-10-day-old eggs per dilution and incubated at 37°C. The eggs were examined twice a day for 7 days to record the time of embryo death. The MDT was calculated as the mean time (hr) for the minimum lethal dose of virus to kill all the inoculated embryos. The MDT assay provides a reasonable prediction of virus pathogenicity.

The viruses with MDT <60 hr are normally verogenic (virulent) strains; with MDT = 60 to 90 hr, mesogenic (intermediate) strains; >90 h as letogenic (avirulent) strains. For the ICPI test, 0.05 ml of a 1:10 dilution of fresh infective allantoic fluid for each virus was inoculated into group of 10 1-day-old SPF chicks via the intracerebral route. The birds were observed for clinical symptoms and mortality once every 8 hr for a period of 8 days. At each observation, the birds were scored as follows: 0 if normal, 1 if sick, and 2 if dead. The ICPI is the mean of the score per bird per observation over the 8-day period. The ICPI values ranges from 0.0 to 2.0. The low virulent (LoND): ICPI<0.7; virulent (vND): ICPI≥ 0.7.

10 *Virus cell killing assessed by cell viability assay.*

The cells were plated in 96-well plates at 5×10^3 cells/well overnight infected with r73T at various MOI. Cell viability was determined by CellTiter Glo kit (Promega) per manufacture's manual. The relative percent of surviving cells is determined by comparing the ATP level of each testing sample to the untreated sample control of 100% viable. The data presented in the table is relative percent of the killed cells.

The effect of NDV tumor killing assessed in the subcutaneous HT1080 xenograft model.

Athymic NCR homogenous nude mice (Taconic) were implanted subcutaneously (s.c.) with 5×10^6 HT1080 cells (in 100 μ L PBS) into one flank. Viral treatment started when tumors reached a volume of 65–300 mm³. Recombinant 73T in 100 μ L was administered at different dose levels either locally by intratumor (i.t) injection or systemically by intratumor (i.t) injection into the tail vein, respectively. The control animals were injected with 100 μ L PBS only. Tumor growth was measured using a digital caliper, and tumor volume was calculated as $0.5 \times (\text{height}) \times \text{width} \times \text{length} (\text{mm}^3)$. Mice were sacrificed when the body weight dropped by 20% of the original body weight or the tumor volume exceeded 2000 mm³.

Viral biodistribution in the HT1080 xenograft mice.

Nine Nude mice bearing HT1080 human fibrosarcoma xenograft subcutaneous tumors were i.v injected with 10^8 pfu of r73T- R116i-hGM-CSF. Three mice were terminated at 1, 4, and 8 day(s) post-injection. One mouse injected with PBS was terminated on day 8. The tumors, lungs, spleen, ovaries and serum samples were collected. The infectious virus titer in the tissue homogenates was quantified by plaque assay.

Quantitation of the GM-CSF protein level by ELISA.

Tumors from NDV infected and PBS injected mice were homogenized in PBS using gentle MACS Dissociator (Miltenyi Biotec) per manufacturer's instruction. The supernatant
5 from homogenized tissues or serum collected from mice were tested for the level of GM-CSF by a DuoSet ELISA kit (R&D).

Statistical analysis

All statistical analyses were performed using the GraphPad Prism 6.0 software. The
10 unpaired t-test was used to assess differences in tumor regression between groups. GraphPad Prism software was also used to calculate the IC50 of rNDV 73T for *in vitro* cell killing in normal and tumor cells.

Other Embodiments

15 From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of
20 listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

All patents, publications, CAS, and accession numbers mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent, publication, and accession number was specifically and individually indicated to be
25 incorporated by reference.

WHAT IS CLAIMED IS:

1. An attenuated Newcastle disease virus (NDV) comprising an F protein cleavage site derived from glycoprotein B (gB) of cytomegalovirus (CMV) (S116).

5 2. The attenuated Newcastle disease virus of claim 1, wherein the modified F protein cleavage sequence (FPCS) comprises a sequence selected from the group consisting of:

- S116: ¹¹¹H-N-R-T-K-**S**/F¹¹⁷

- S116K: ¹¹¹H-N-**K**-T-K-S/F¹¹⁷

- S116M: ¹¹¹H-N-R-**M**-K-S/F¹¹⁷

10 - S116KM: ¹¹¹H-N-**K-M**-K-S/F-I¹¹⁸

- R116: ¹¹¹H-N-R-T-K-**R**/F-I¹¹⁸

3. The attenuated Newcastle disease virus of claim 1 or 2, wherein the attenuated virus strain is a modified 73T strain.

15

4. The attenuated Newcastle disease virus of claim 1 or 2, wherein the attenuated NDV virus is r73T-R116 virus.

5. The attenuated Newcastle disease virus of claim 1 or 2, wherein the virus comprises an
20 increased HN-L intergenic region.

6. The attenuated Newcastle disease virus of any of claims 1-5, wherein the HN-L intergenic region comprises a non-coding sequence between at least about 50-300 amino nucleotides in length.

25

7. The attenuated Newcastle disease virus of claim 6, wherein the non-coding sequence is derived from a paramyxoviruses type -1 (APMV-1), a respiratory syncytial virus (RSV) or a random sequence.

30 8. The attenuated Newcastle disease virus of claim 6, wherein the HN and L intergenic non-coding sequence is 60, 102, 144, 198, or 318 nt in length.

9. The attenuated Newcastle disease virus of any of claims 1-8, wherein the virus comprises one or more heterologous polynucleotide sequences inserted at the P-M junction and/or the HN-L junction.
- 5 10. The attenuated Newcastle disease virus of claim 9, wherein the virus comprises two or more heterologous polynucleotide sequences, wherein at least one heterologous polynucleotide sequence is inserted at the P-M junction and at least one is inserted at the HN-L junction.
- 10 11. The attenuated Newcastle disease virus of claim 9, wherein the heterologous polynucleotide sequence is a transgene encoding a polypeptide that enhances the oncolytic properties of the virus.
12. The attenuated Newcastle disease virus of claim 10, wherein the transgene encodes a
15 cytokine, cell surface ligand, and/or chemokine.
13. The attenuated Newcastle disease virus of claim 10, wherein the cytokine is selected from the group consisting of GM-CSF, IL-2, IL-21, IL-15, IL-12, and IL-12p70.
- 20 14. The attenuated Newcastle disease virus of claim 13, wherein the cytokine is human GM-CSF.
15. The attenuated Newcastle disease virus of claim 9, wherein the heterologous polynucleotide sequence is a transgene encoding a detectable moiety.
25
16. The attenuated Newcastle disease virus of claim 15, wherein the expression level of the detectable moiety correlates with virus replication.
17. The attenuated Newcastle disease virus of any of claims 1-16, wherein the F and HN
30 genes of NDV are replaced by corresponding extracellular domains of canine Parainfluenza virus 5 (PIV 5) or pigeon paramyxovirus type 1 (PPMV-1).

18. The attenuated Newcastle disease virus of claim 1 or 2, wherein the virus is 73T-R116i-hGM-CSF.

5 19. The attenuated Newcastle disease virus of claim 1 or 2, wherein the attenuated virus has a Mean death time in eggs (MDT) of greater than 90hr or about 90-156 hours.

20. The attenuated Newcastle disease virus of claim 1 or 2, wherein the attenuated virus has an intracerebral pathogenicity index between about 0-0.7.

10

21. The attenuated Newcastle disease virus of claim 1 or 2, wherein the attenuated virus has an intracerebral pathogenicity index of about 0.

15 22. The attenuated Newcastle disease virus of claim 1 or 2, wherein the attenuated virus has less than about 15% cytotoxicity in HT1080 cells.

23. The attenuated Newcastle disease virus of claim 1 or 2, wherein the attenuated virus selectively kills tumor cells with killing efficiency at least 10 or 15%.

20 24. The attenuated Newcastle disease virus of claim 23, wherein the tumor cell killing efficiency is in between about 75%-100%.

25. A method of selectively killing tumor cells, the method comprising contacting a tumor cell with the attenuated Newcastle disease virus of any of claims 1-22.

25

26. A method of inducing tumor regression in a subject, the method comprising contacting a tumor cell with the attenuated Newcastle disease virus of any of claims 1-22.

27. A method of reducing tumor cell survival or proliferation, the method comprising
30 contacting a tumor cell with the attenuated Newcastle disease virus of any of claims 1-22.

28. The method of any of claims 25-27, wherein the cell is a cancer cell selected from the group consisting of bladder, ovarian, brain, pancreas, prostate, sarcoma, lung, breast, cervical,

bone, liver, head and neck, gastric, kidney, melanoma, lymphoma, leukemia, thyroid, colon, colorectal, and melanoma cancer cells.

29. A method of treating a neoplasia in a subject, the method comprising administering to the
5 subject an effective amount of an attenuated Newcastle disease virus of any of claims 1-22.

30. The method of any of claims 25-29, wherein the attenuated Newcastle disease virus is r73T-S116.

10 31. The method of claim 26, wherein the attenuated Newcastle disease virus is delivered systemically, intraperitoneally, or intratumorally.

32. The method of claim 26, wherein virus is administered at a dose of about 10^7 pfu to about 10^9 pfu

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33. The method of claim 26, wherein the virus is administered intravenously at a dose of about 10^9 pfu to about 10^{11} pfu.

34. The method of claim 29, wherein the subject has a neoplasia selected from the group
20 consisting of bladder, ovarian, brain, pancreas, prostate, sarcoma, lung, breast, cervical, bone, liver, head and neck, gastric, kidney, melanoma, lymphoma, leukemia, thyroid, colon, colorectal, and melanoma cancer.

35. A method of treating a neoplasia in a subject that has developed an anti-NDV immune
25 response, the method comprising administering to the subject an effective amount of an attenuated chimeric Newcastle disease virus of any of claims 1-22, wherein the virus is a chimeric virus comprising a F and/or HN gene of a canine Parainfluenza virus 5 (PIV 5) or Pigeon paramyxovirus type 1 (PPMV-1), wherein said chimeric Newcastle disease virus is antigenically distinct from NDV.

30

36. The method of claim 29, wherein the method increases the level of oncolytic viruses present in the subject relative to the level of oncolytic viruses present in a control subject that

has developed an anti-NDV immune response, but that is not receiving a chimeric Newcastle disease virus.

37. A nucleic acid comprising the full length cDNA of 73T, wherein the nucleic acid

5 encodes a modified F protein cleavage sequence selected from the group consisting of:

- S116: $^{111}\text{H-N-R-T-K-S}/\text{F}^{117}$
- S116K: $^{111}\text{H-N-K-T-K-S}/\text{F}^{117}$
- S116M: $^{111}\text{H-N-R-M-K-S}/\text{F}^{117}$
- S116KM: $^{111}\text{H-N-K-M-K-S}/\text{F-I}^{118}$
- 10 - R116: $^{111}\text{H-N-R-T-K-R}/\text{F-I}^{118}$

38. A vector comprising full length cDNA of 73T, wherein the vector encodes a modified F protein cleavage sequence selected from the group consisting of:

- S116: $^{111}\text{H-N-R-T-K-S}/\text{F}^{117}$
- 15 - S116K: $^{111}\text{H-N-K-T-K-S}/\text{F}^{117}$
- S116M: $^{111}\text{H-N-R-M-K-S}/\text{F}^{117}$
- S116KM: $^{111}\text{H-N-K-M-K-S}/\text{F-I}^{118}$
- R116: $^{111}\text{H-N-R-T-K-R}/\text{F-I}^{118}$

20 39. A virulent particle comprising the nucleic acid of claim 37.

40. A host cell infected with the attenuated Newcastle disease virus of any one of claims 1-24.

41. A host cell comprising the nucleic acid of claim 37.

25

42. The attenuated Newcastle disease virus of any one of claims 1-24, wherein the Newcastle disease virus strain is 73T.

43. The attenuated Newcastle disease virus of claim of claim 10, wherein the transgene is
30 selected from a transgene listed in Table 5.

An attenuated Newcastle disease virus (NDV) comprising an F protein cleavage site derived from glycoprotein B (gB) of cytomegalovirus (CMV) (S116).

2. The attenuated Newcastle disease virus of claim 1, wherein the modified F protein

5 cleavage sequence (FPCS) comprises a sequence selected from the group consisting of:

- S116: $^{111}\text{H-N-R-T-K-S}/\text{F}^{117}$
- S116K: $^{111}\text{H-N-}\underline{\text{K}}\text{-T-K-S}/\text{F}^{117}$
- S116M: $^{111}\text{H-N-R-}\underline{\text{M}}\text{-K-S}/\text{F}^{117}$
- S116KM: $^{111}\text{H-N-}\underline{\text{K}}\text{-}\underline{\text{M}}\text{-K-S}/\text{F-I}^{118}$
- 10 - R116: $^{111}\text{H-N-R-T-K-}\underline{\text{R}}\text{-I}^{118}$

Figure 1. Construction of NDV 73T antigenomic cDNA

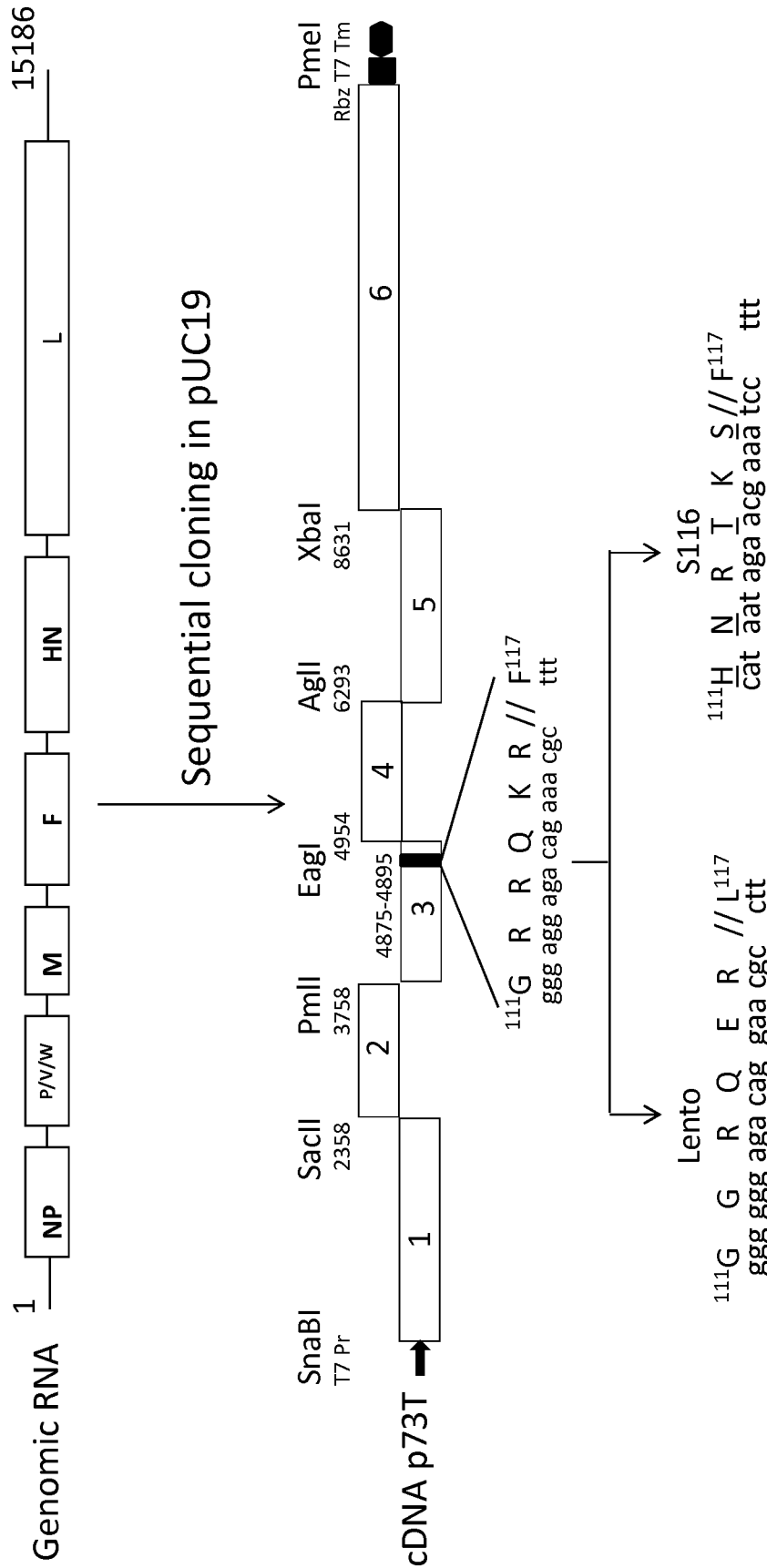


Figure 2. Insertion of transgene cassette(s) into NDV 73T genome

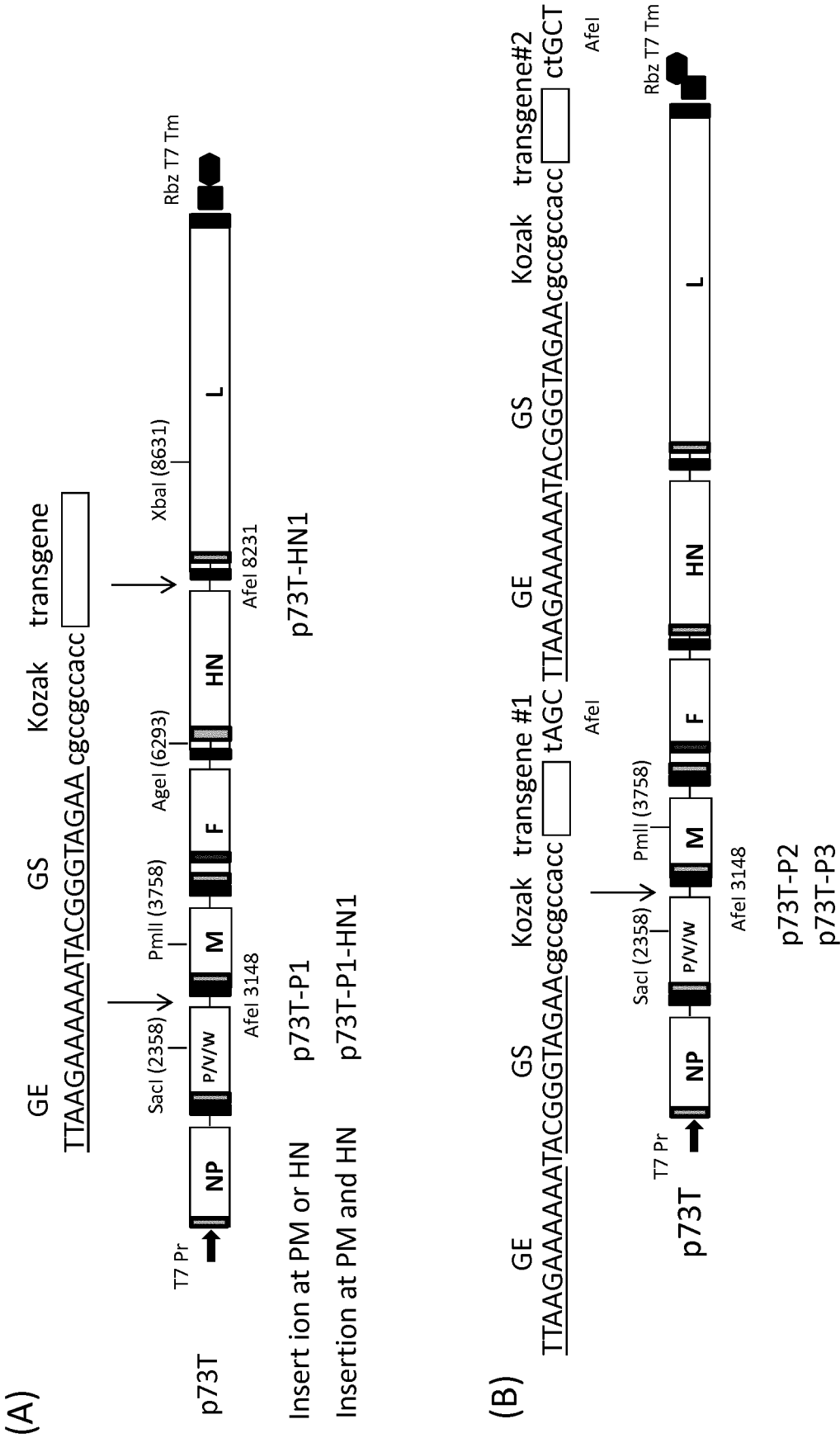
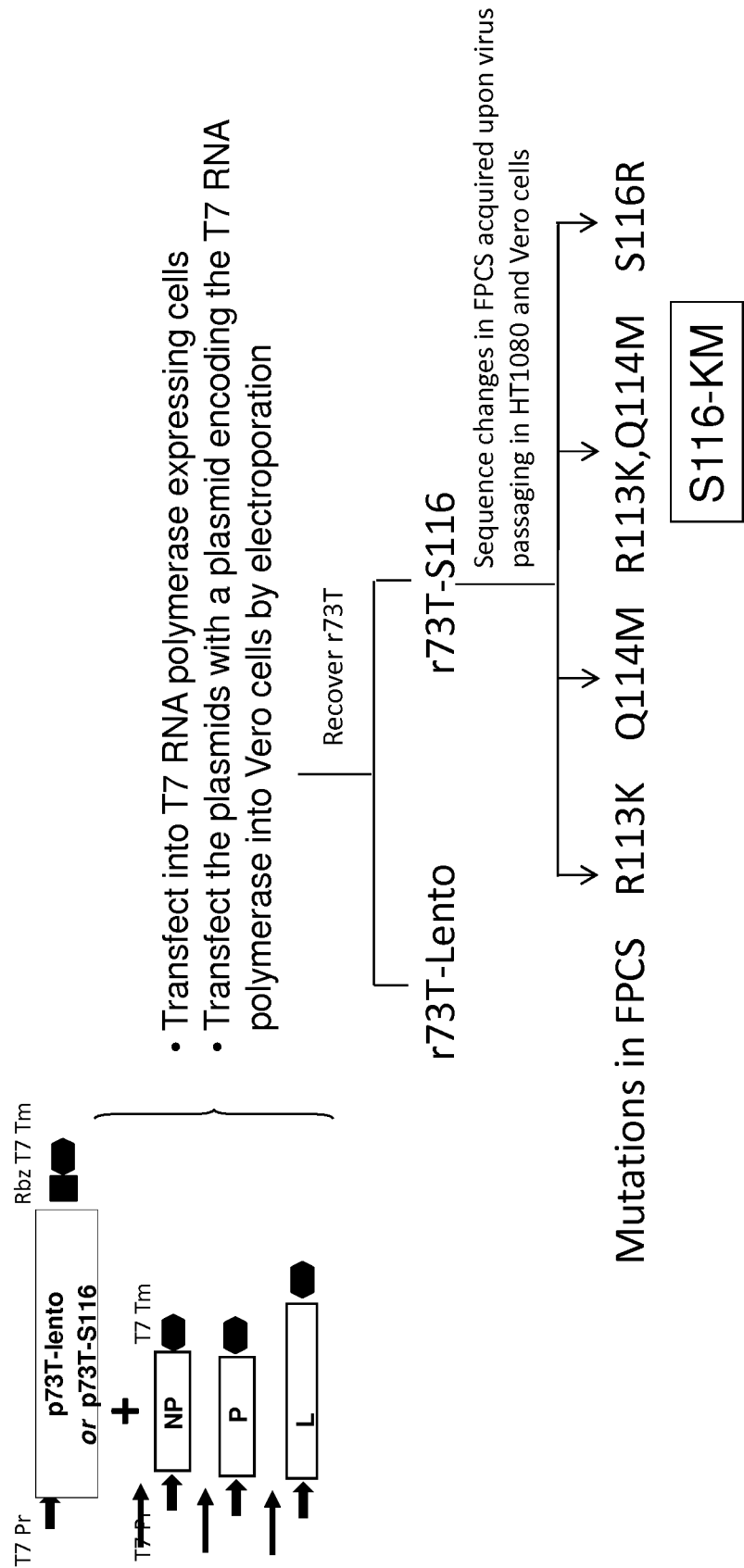


Figure 3A. Method for recovering infectious recombinant NDV strain 73T (r73T) with modified FPCS



Figures 3B and C. Evaluation of F protein cleavage and fusion activity in vitro

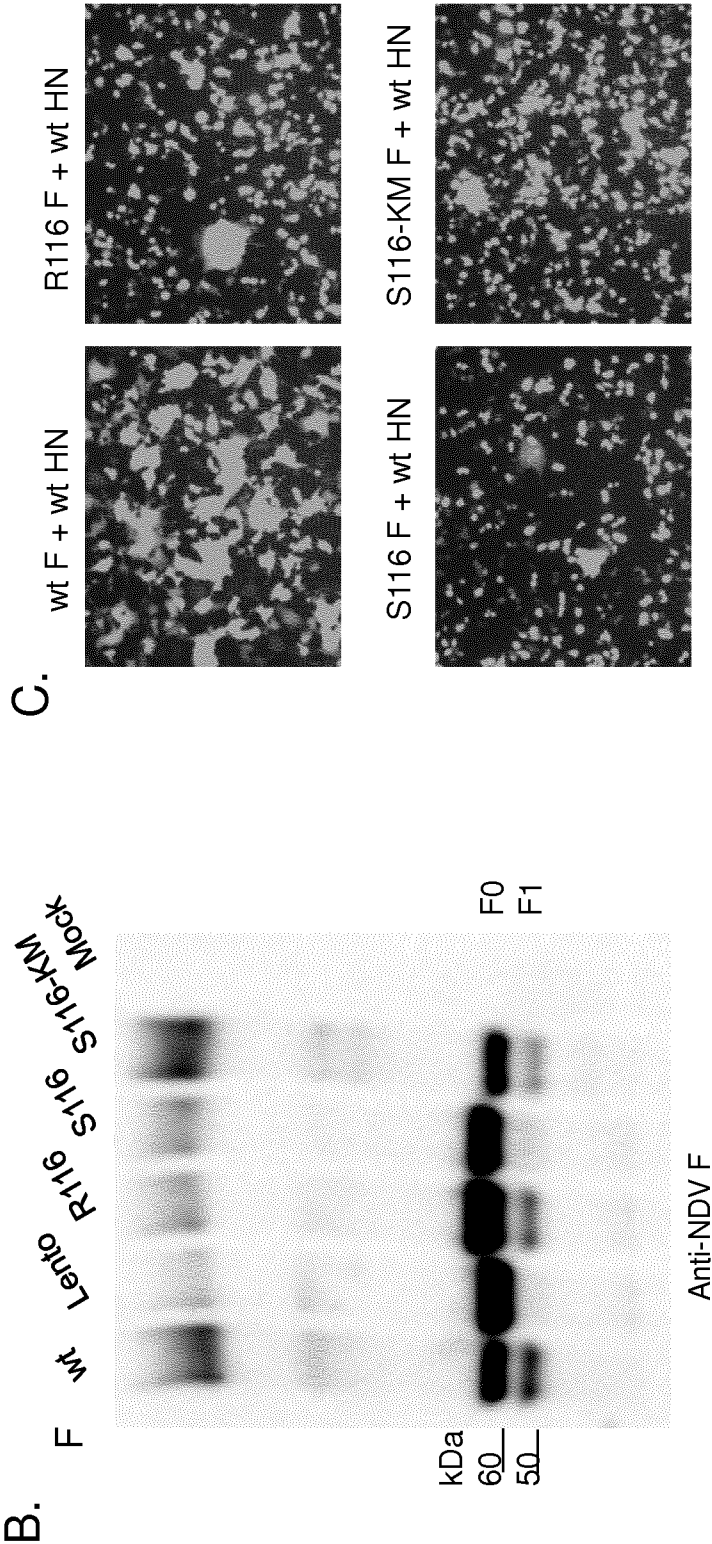


Figure 4. Summary of characteristics of r73T-lento and r73T-S116 derivatives

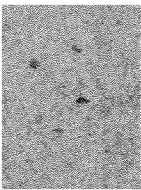
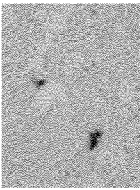
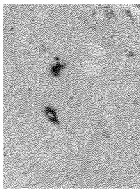
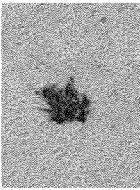
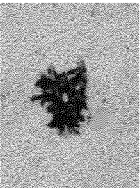
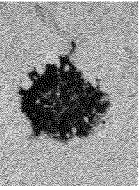
Virus name (73T) ^a	FPCS sequence ^b	Plaque formation ^c	MDT (hrs) ^d	ICPI ^e	Relative HT1080 cell killing ^e	Vero (pfu/ml) ^g	Eggs (pfu/ml) ^h
-lento	¹¹¹ G-G-R-Q-E-R/L- I ¹¹⁸		> 156	0.00	13	7.5E+03	5.7E+08
-S116	¹¹¹ H-N-R-T-K-S/F-I ¹¹⁸		> 156	0.00	31	4.4E+06	4.6E+08
-S116K113	¹¹¹ H-N-K-T-K-S/F-I ¹¹⁸		> 156	ND	29	3.4E+06	3.8E+08
-S116M114	¹¹¹ H-N-R-M-K-S/F-I ¹¹⁸		> 156	ND	31	3.6E+06	3.4E+08
-S116K113M114 (S116-KM)	¹¹¹ H-N-K-M-K-S/F-I ¹¹⁸		> 156	0.00	48	2.4E+06	4.2E+08
-R116	¹¹¹ H-N-R-T-K-R/F-I ¹¹⁸		66	0.65	80	6.2E+06	1.7E+08

Figure 5. Strategies to attenuate r73T-R116 virus virulence in chicken

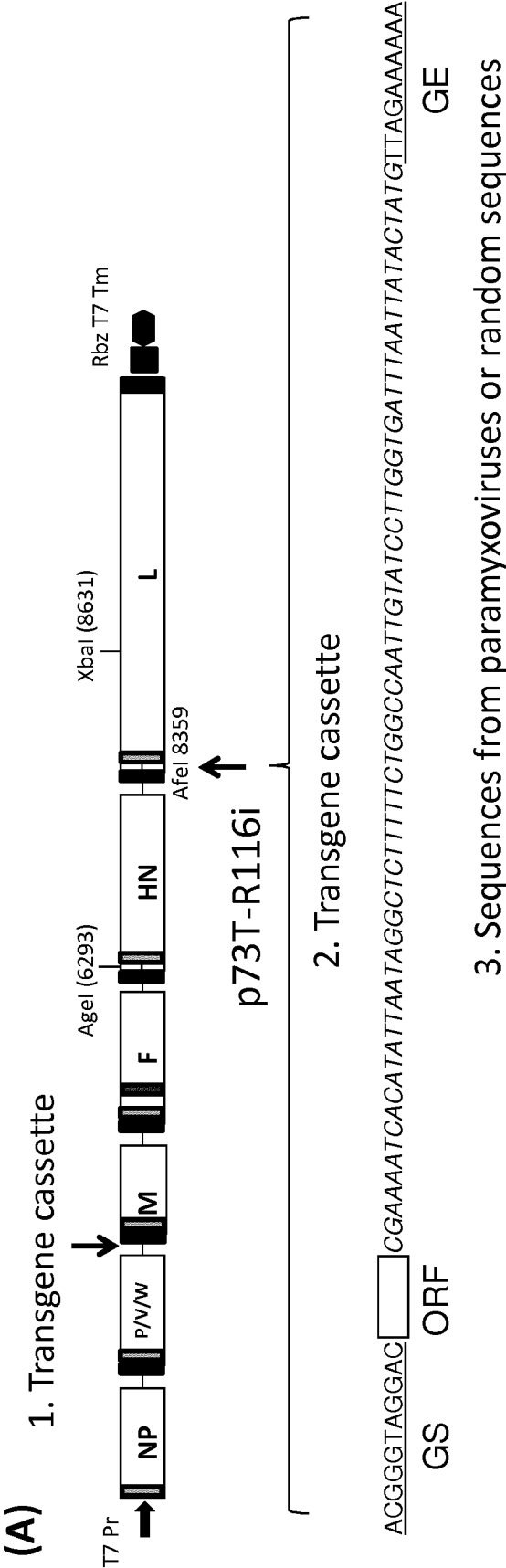


Figure 5. continued

(B) Non-coding sequences inserted at HN-L junction

APMV-1 N 318 nt

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 attactcacatgcagctgaagtaggaatgcagtacgtgagcaccacactgggagcagagcgtaacacagcagataactaaagaactcaggtagtgaggcaggtattgacccaagacatact
 ctcttgggaagggaacaaacagcaaaaggaggaggttgcaaatgttagacatacatgggggttgaaagaag

RSV-NP 198 nt

acttctgtcatccagcaaaatacggccatccaaacgggagcacaggagatagtagtattgatactcctaattatgatgtgcagaaacacatcaataaagttatgtggcatgttttaatcacagaagatgctaata
 cataaattcactgggtaataaggtagttatatgcgattgtctaggtaggaagagaagacaccataaaaaat

Random sequence 198 nt

gctgtgaaagcaaaaaacatcgtctctcgttggcccaatactcatgatccgccaatgatataaaaaacggcgcaaggctcgtcttcgcatcgacacatcgttgatataggcggtgaagtctca
 acatgtggcactcctggaccagtgaggacaacgaaacgcgcctccttagaaggagatacaaatgacccggctgagg

Random sequence 144 nt

gctgtgaaagcaaaaaacatcgtctctcgttggcccaatactcatgatccgccaatgatataaaaaacggcgcaaggctcgtcttcgcatcgacacatcgttgatataggcggtgaagtctca
 acatgtggcactcctcgga

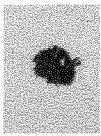
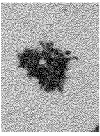
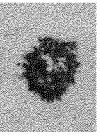
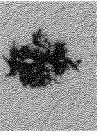
Random sequence 102 nt

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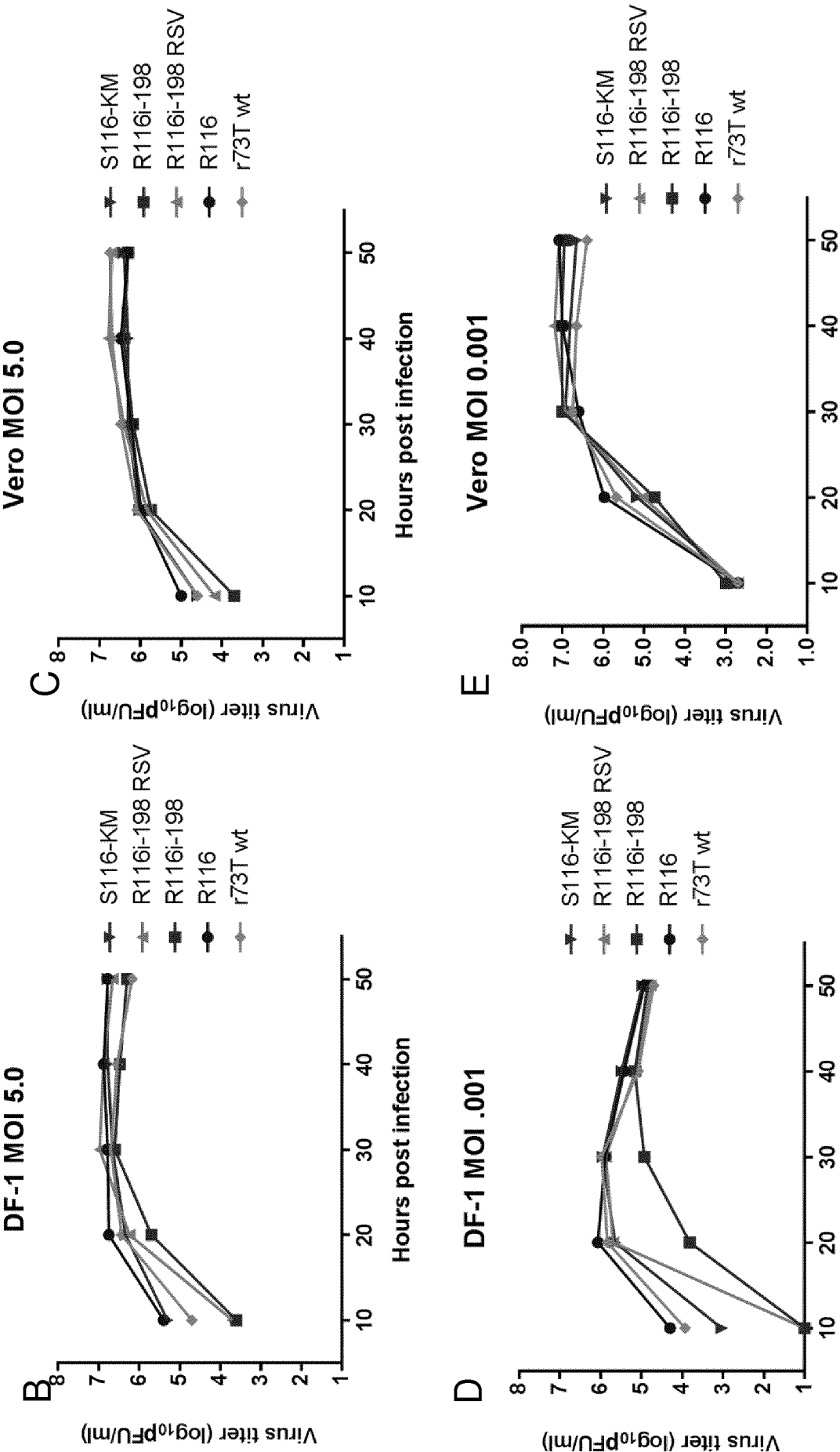
Random sequence 60 nt

gctgtgaaagcaaaaaacatcgtctcgcctcctccttagaaggagatacaaatgacccggctgag

Figure 6A. Summary of characteristics of r73T-R116 derivatives.

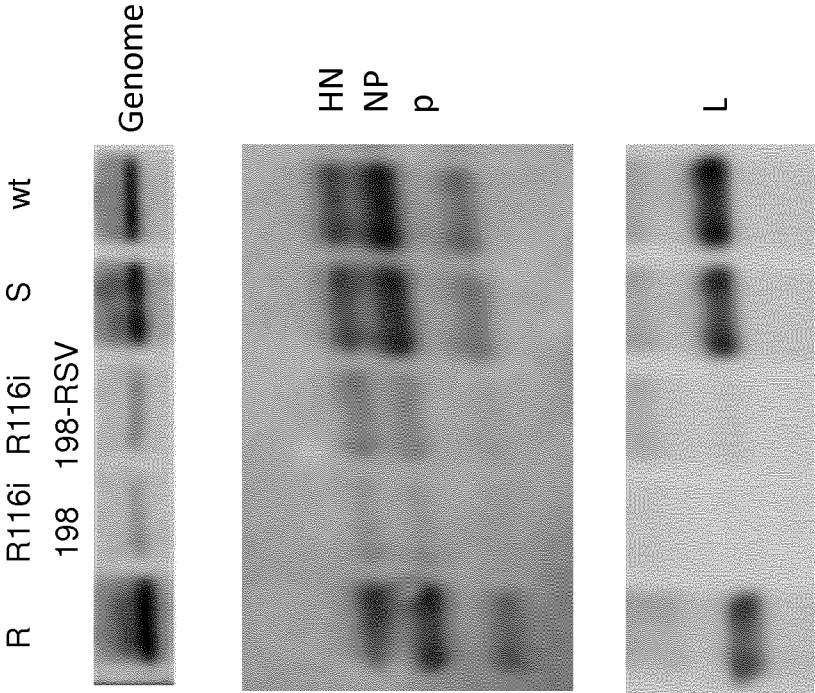
Virus Name (r73T-R116i-) ^a	Insertion at HN-L ^b	Plaque formation ^c	MDT (hrs) ^d	ICPI ^e	Relative cell killing ^f	Vero (pfu/ml) ^g	Eggs (pfu/ml) ^h
R116i	none		62	0.65	80	6.3E+06	2.0E+08
318 APMV	APMV-N 318nt		>156	0.27	80	9.9E+06	5.0E+07
198 RSV	RSV-NP 198 nt		>156	0.0375	86	6.4E+06	6.0E+07
198	Random 198 nt		>156	0	85	7.7E+06	5.0E+07
144	Random 144 nt		79	0.74	82	7.3E+06	2.4E+08
102	Random 102 nt		64	0.51	83	6.4E+06	1.1E+08
60	Random 60 nt		64	0.78	75	6.5E+06	1.3E+08
GFP	EGFP gene cassette		86	0.82	87	5.7E+06	1.5E+08

Figures 6B-E. r73T-R116i virus exhibits slower growth kinetics in DF-1 cells compared to Vero cells

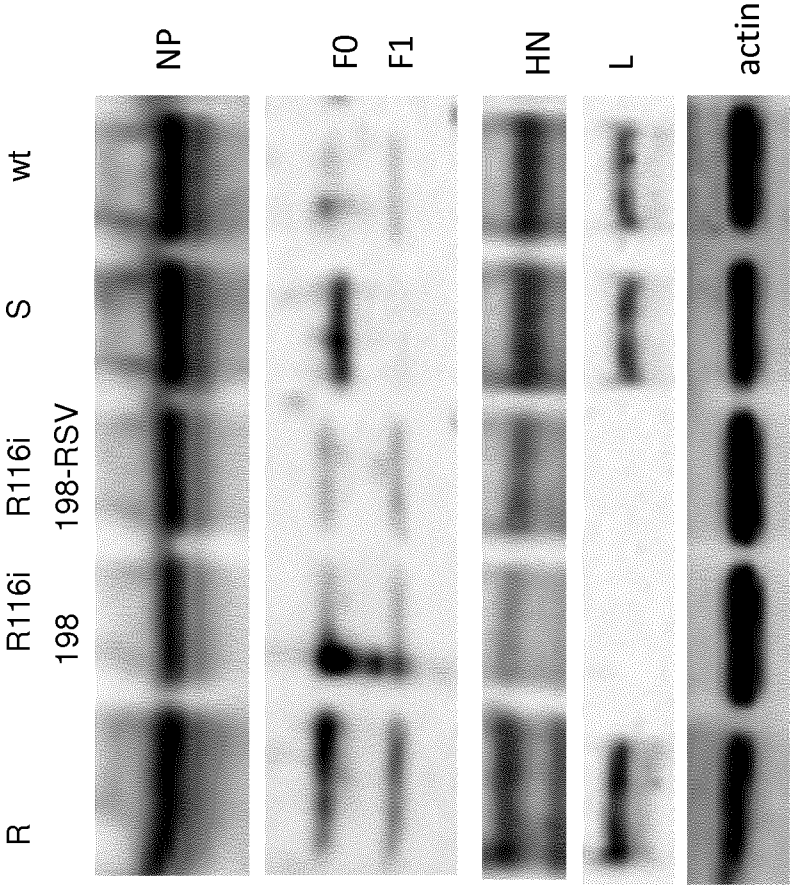


Figures 6F and G. Gene expression of r73T-R116i is reduced in DF-1 chicken fibroblast cells

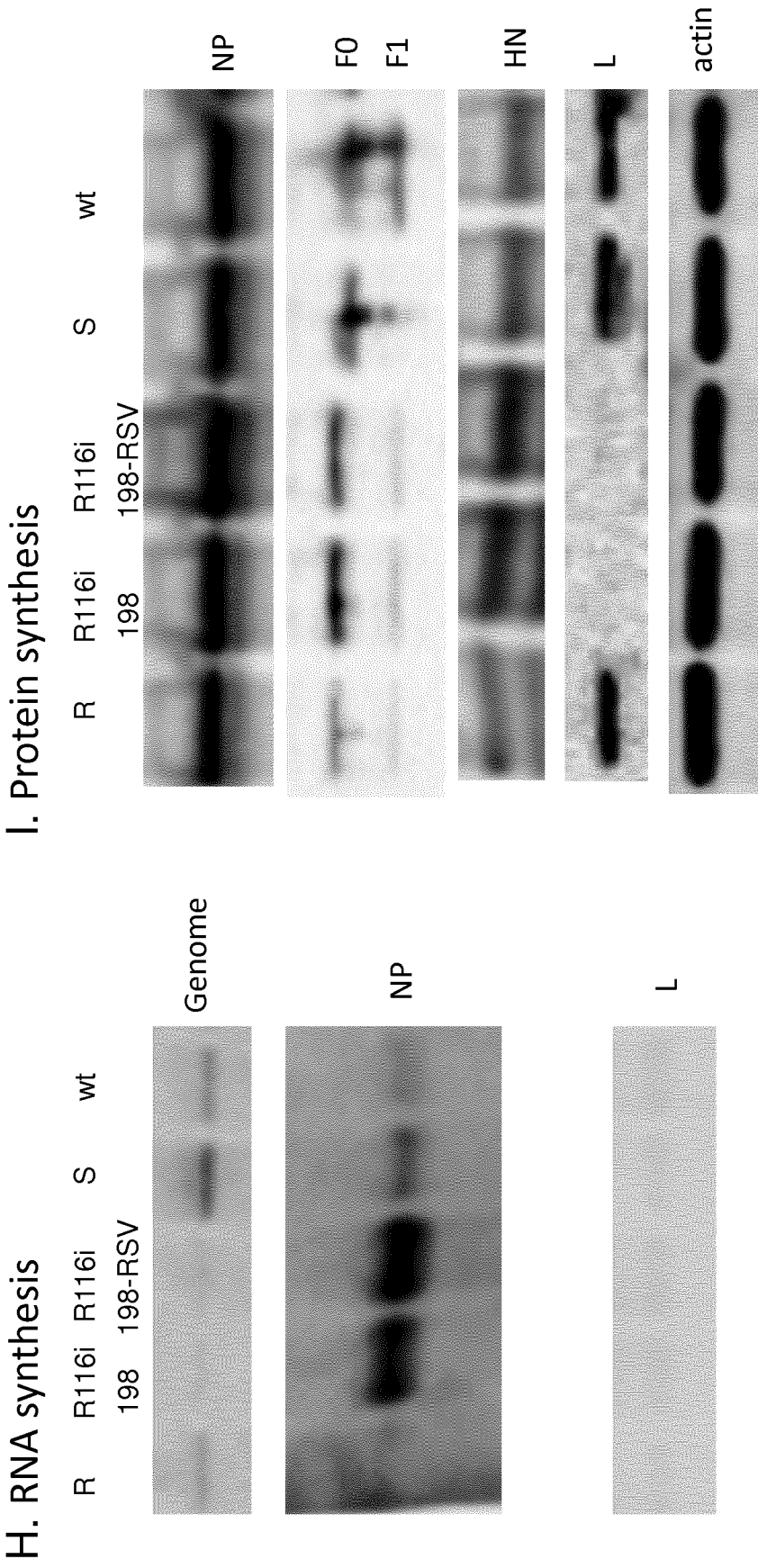
F. RNA synthesis



G. Protein synthesis



Figures 6H and I. Gene expression in the upstream of HN-L insertion is up-regulated whereas gene expression in the downstream is down-regulated in Vero cells infected by r73T-R116i virus



Figures 6J and K. Gene expression in DF-1 and Vero cells is affected by insertion at HN-L junction

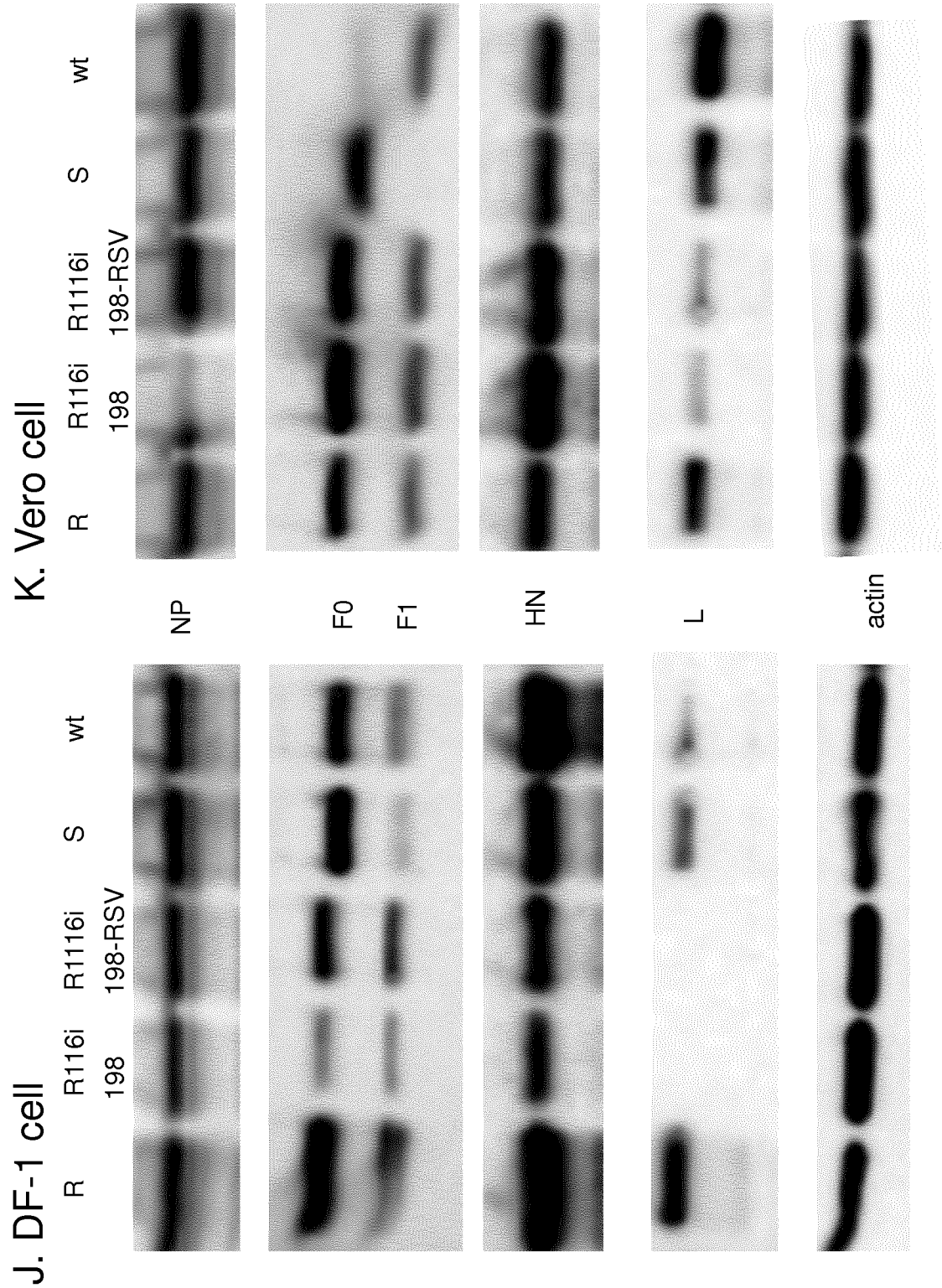


Figure 6L Expression of the upstream genes is up-regulated in r73T-R116i infected human cells

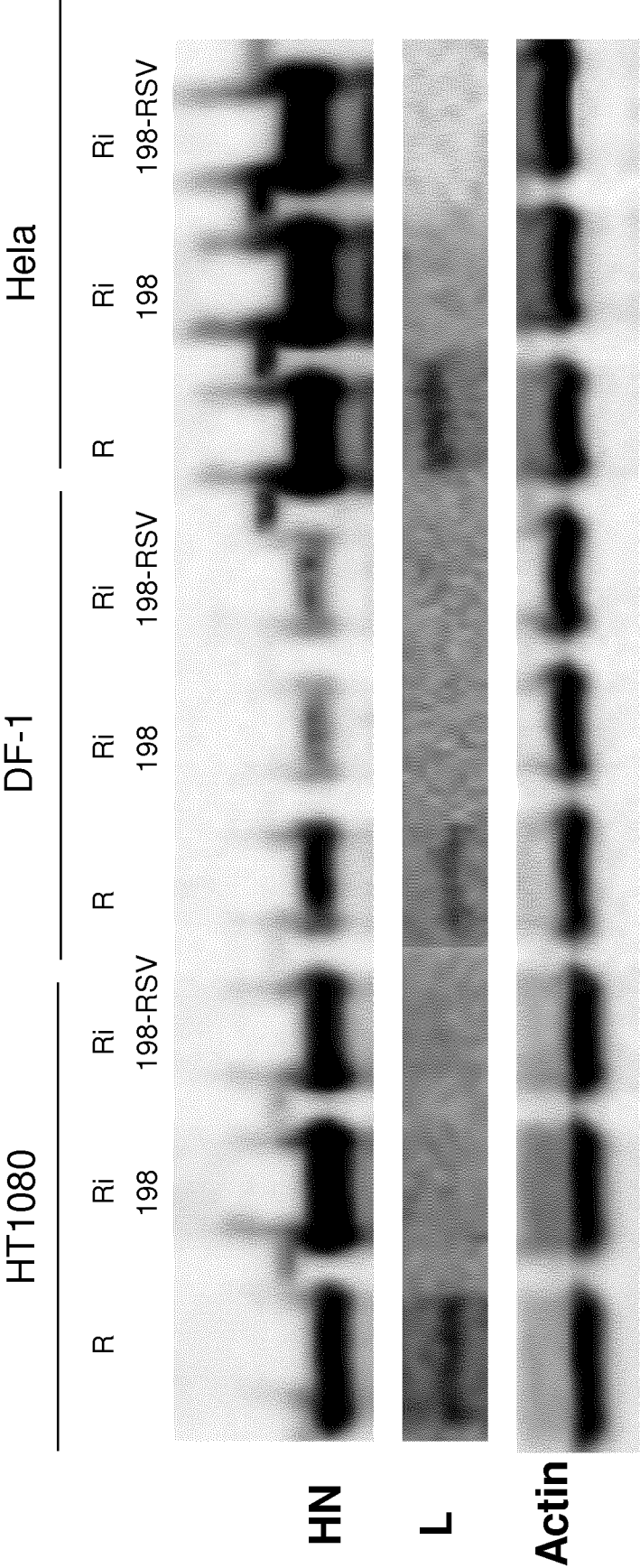


Figure 7. Growth kinetics of r73T viruses in embryonated chicken eggs

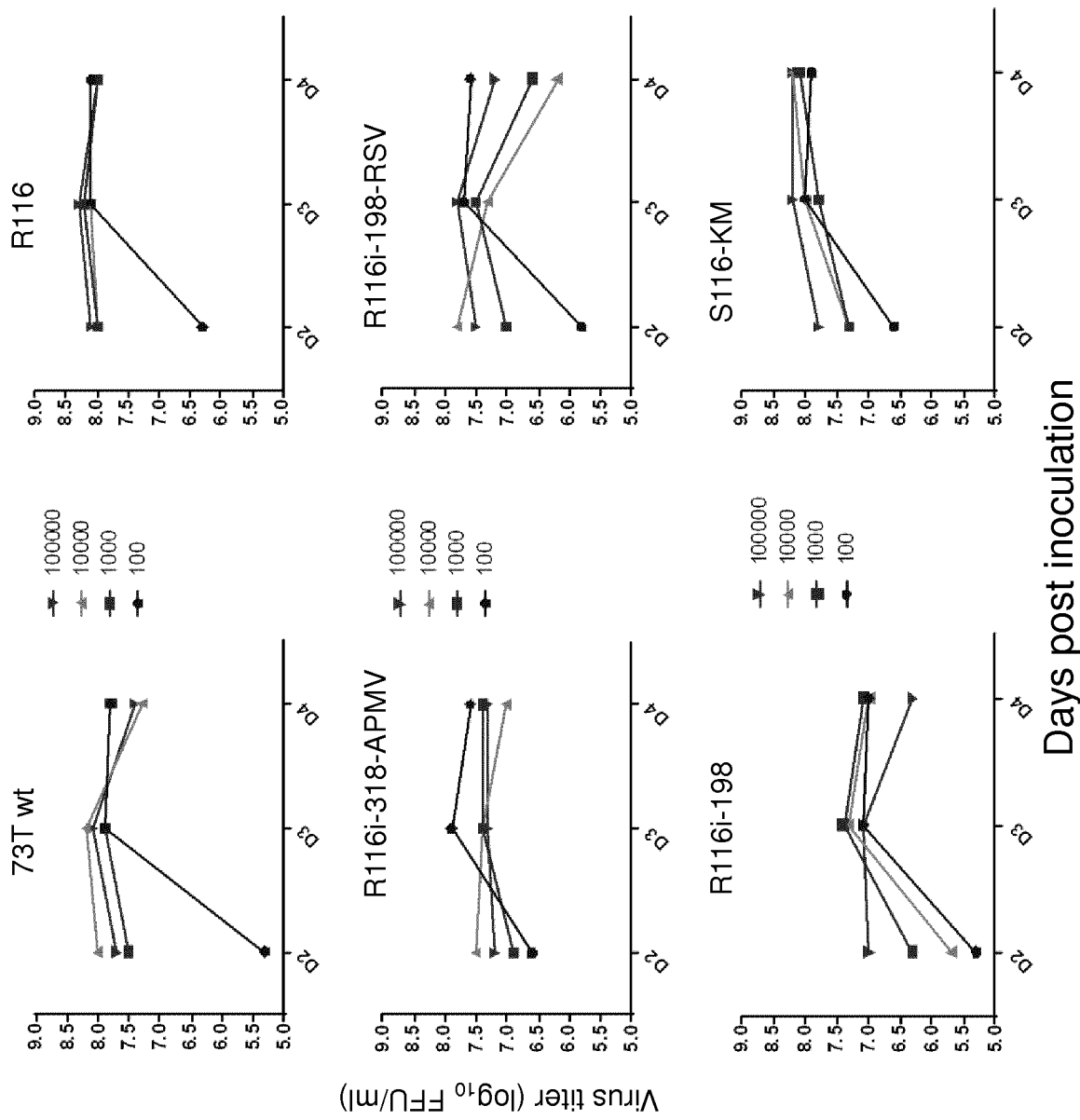


Figure 8. Growth kinetics of r73T viruses in Vero cells

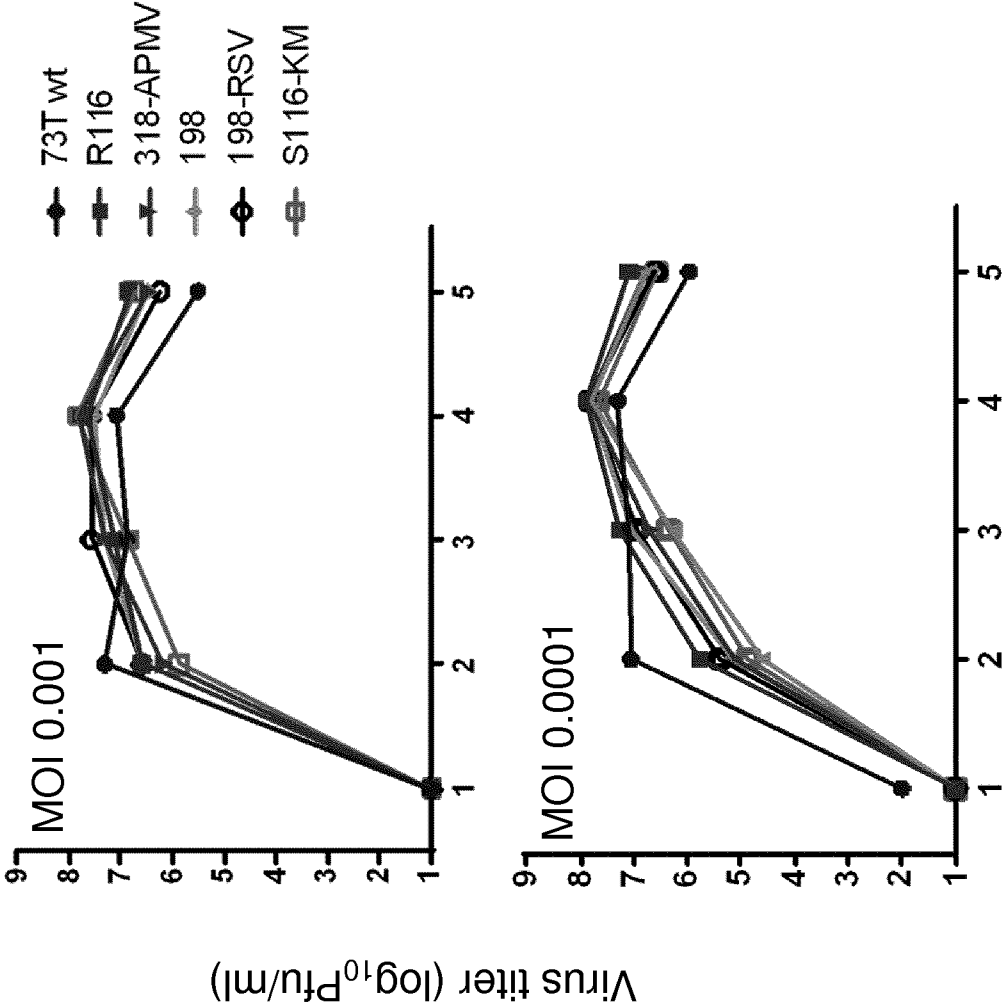


Figure 9. Selective replication and cytotoxicity of r73T virus in tumor cells

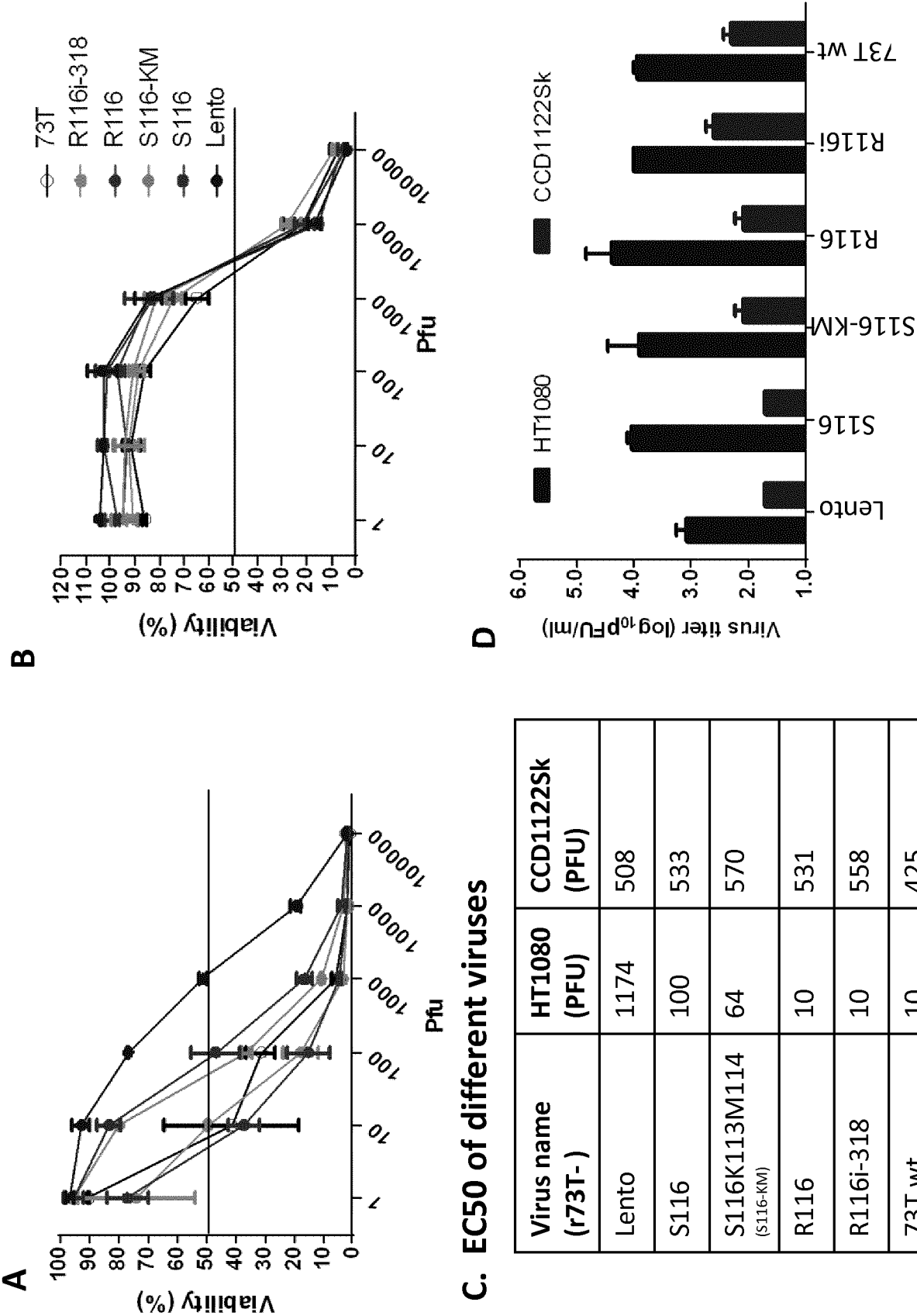
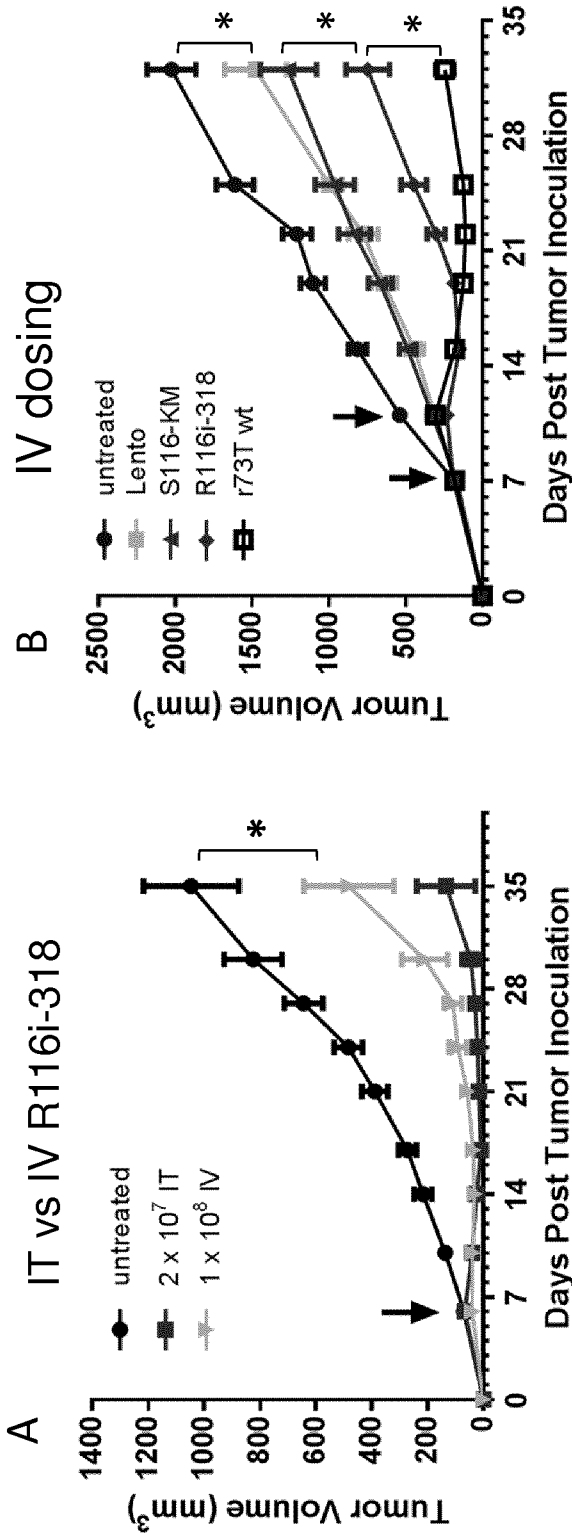


Figure 10. r73T derivatives are effective in tumor regression upon local and systemic administration



Figures 11A and B. r73T-R116i-hGM-CSF selectively replicates in Tumors following a single IV administration

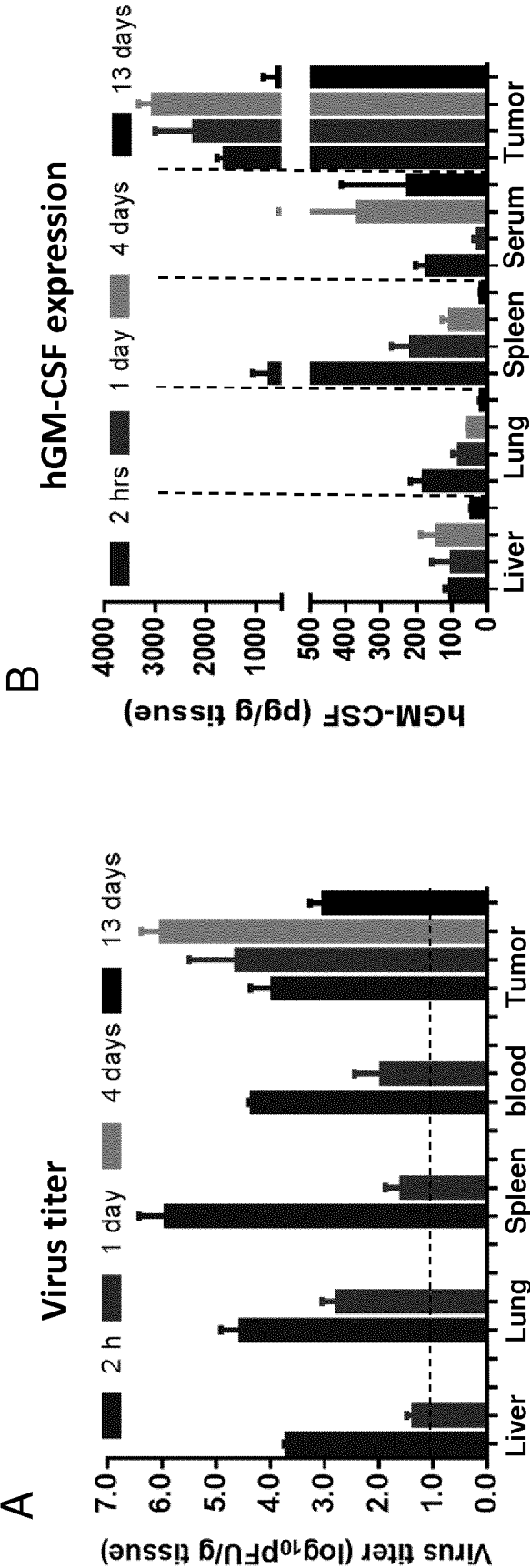


Figure 11C. Effect of mouse GM-CSF on tumor growth inhibition by IV and IT in HT1080 model

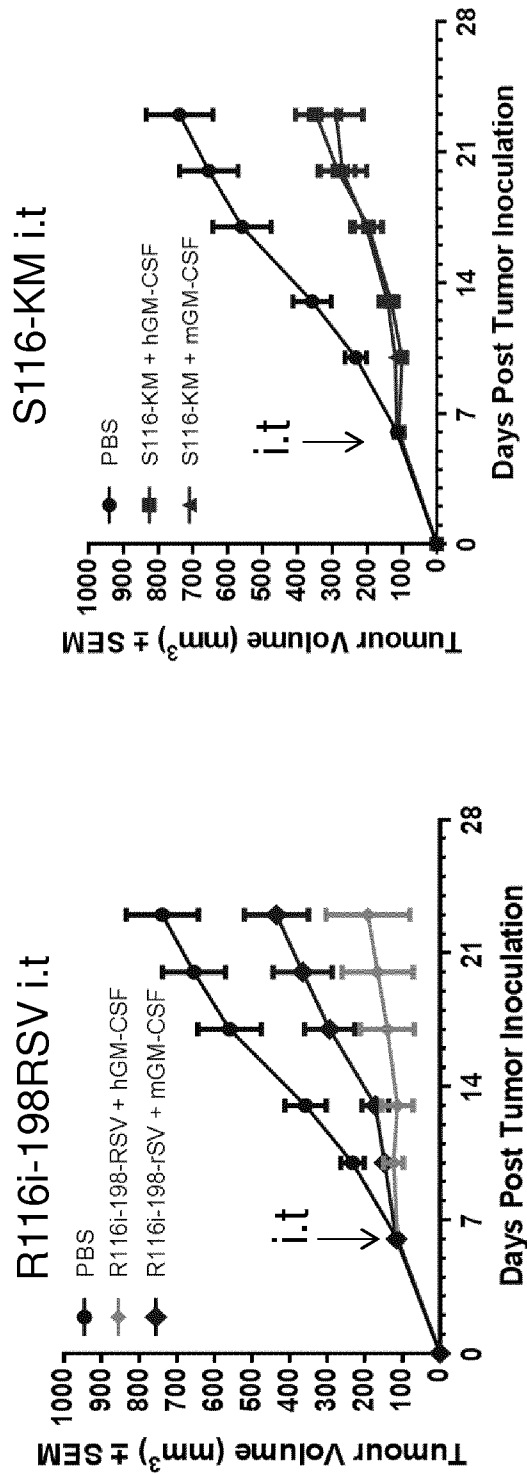


Figure 11D. Expression of mouse GM-CSF accelerated virus clearance from tumors

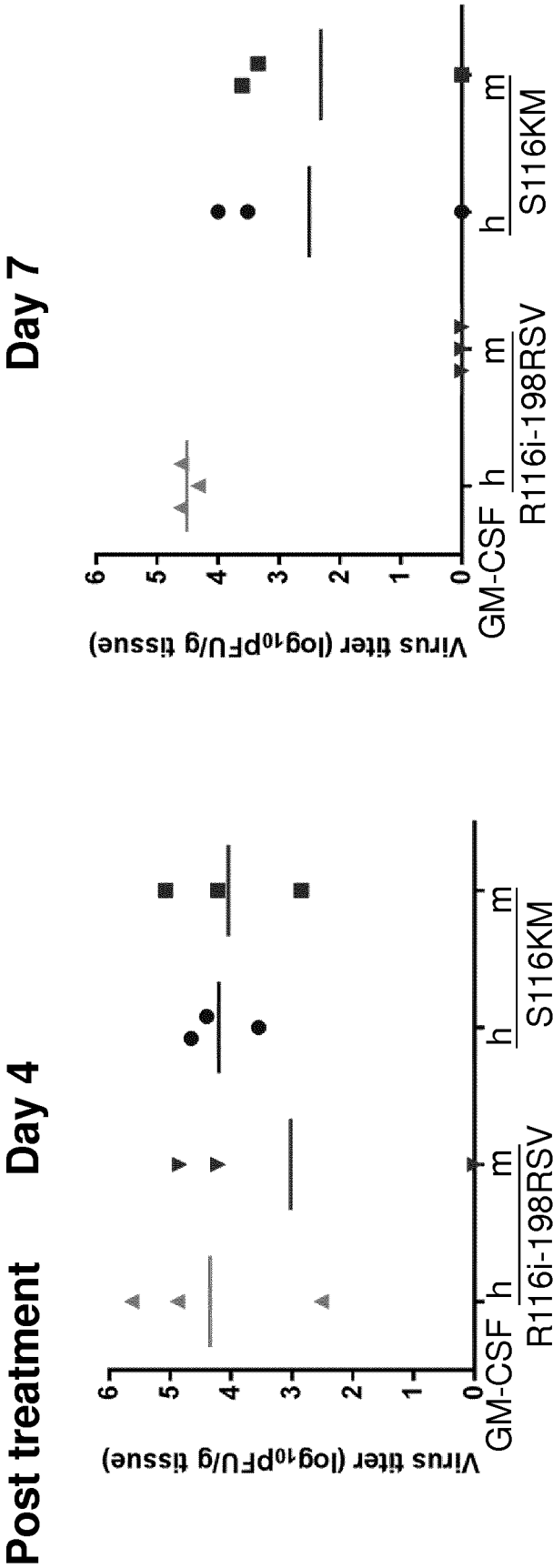


Figure 11E. Mouse GM-CSF expression induces more immune cells infiltration into tumors

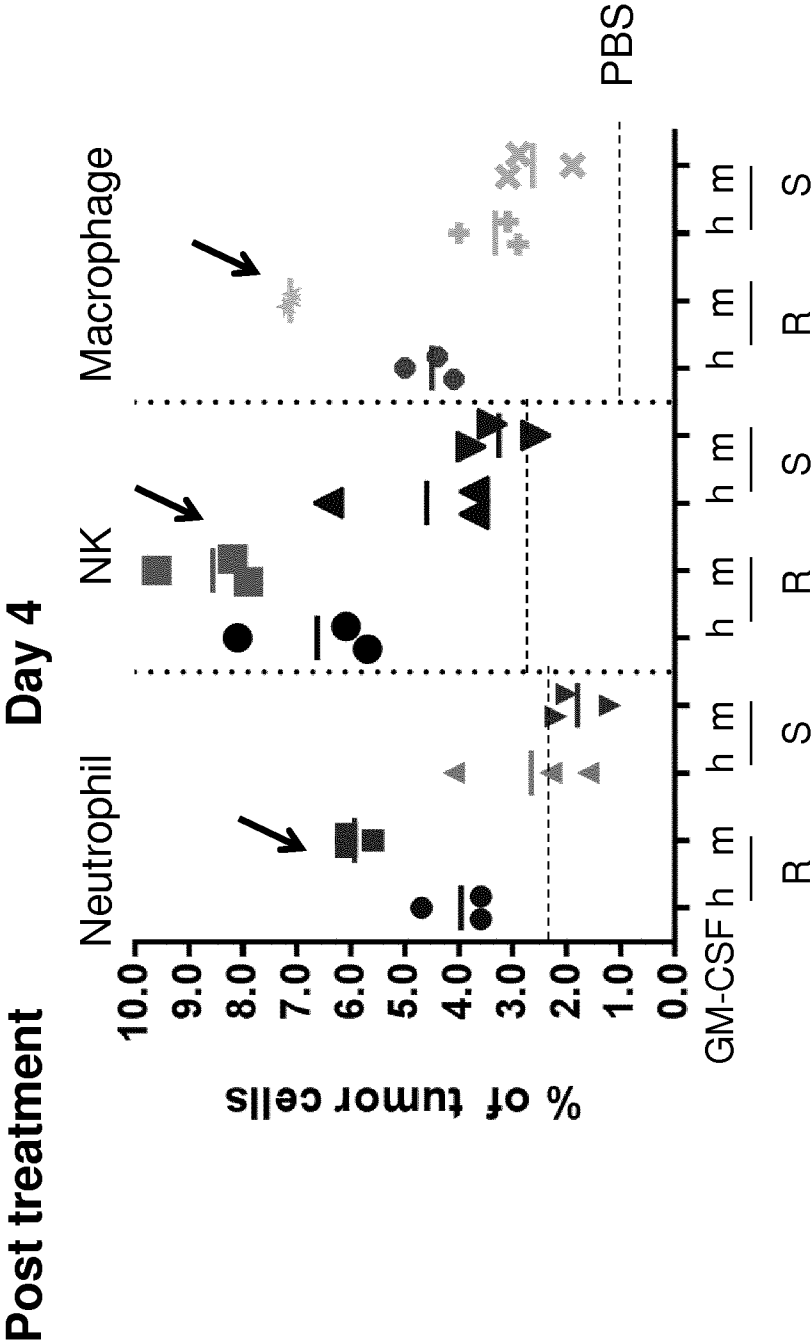


Figure 11F. rNDV treatment elicits up-regulation of cytokines and chemokines in tumor tissues

Cytokine/chemokine on day 4 post treatment	Fold of change relative to PBS group			
	R		S	
	hGM-CSF	mGM-CSF	hGM-CSF	mGM-CSF
mGM-CSF	1.3	106.7	2.4	9.9
MIG (CXCL9)	3.9	3.1	5.3	4.9
IFN- β	51	28	8	12
IFN- γ	6.9	2.7	6	2.4
IL-6	13.2	5.5	6.6	2.7
IL-10	5.7	2.8	0.5	2
MIP-1a (CCL3)	37.9	13.4	15.6	4.4
IL-12	2.9	2.5	2.4	2.3
MCP-1(CCL2)	8.4	9.5	8.9	5
TNF- α	5.2	4.7	3.6	3.7
IP-10 (CXCL10)	8.5	6.6	12.5	6.5
RANTES (CCL5)	24.5	21.2	35.9	10.1

Figure 11G. R116i-318 and -198 is comparable in tumor growth inhibition

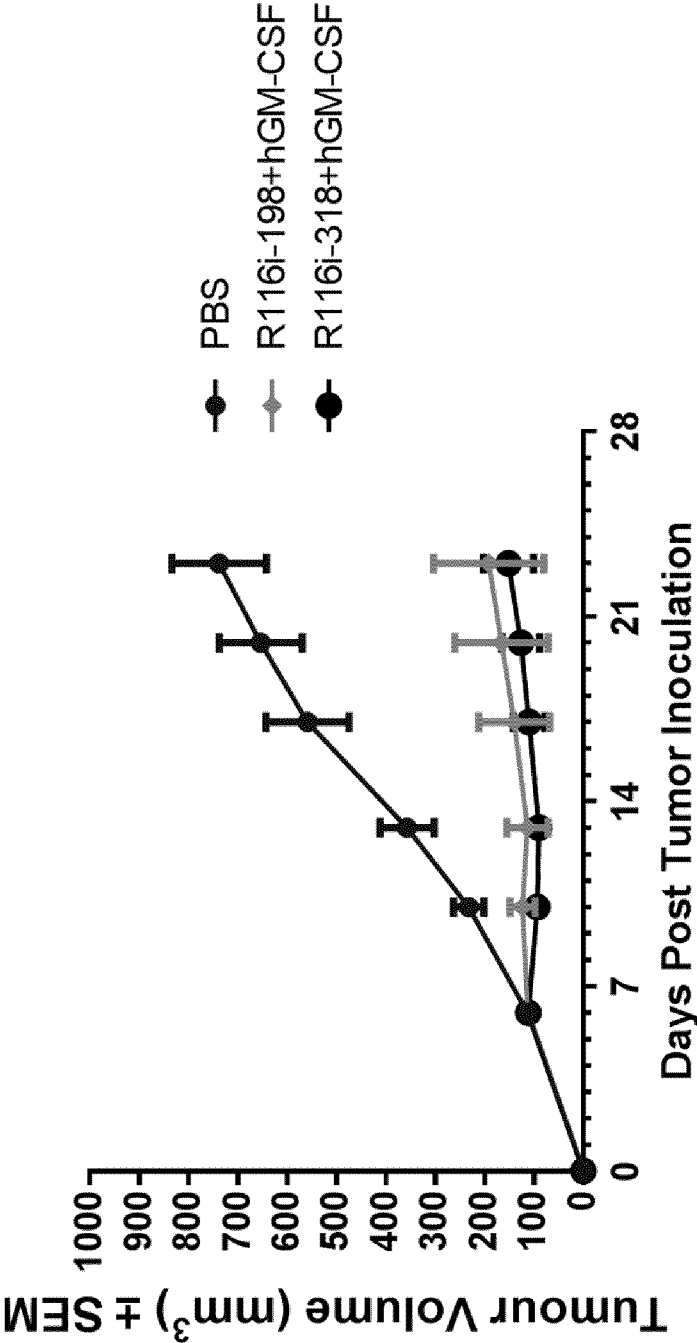


Figure 12A. Construction of antigenome cDNA of 73T containing chimeric F and/or HN gene

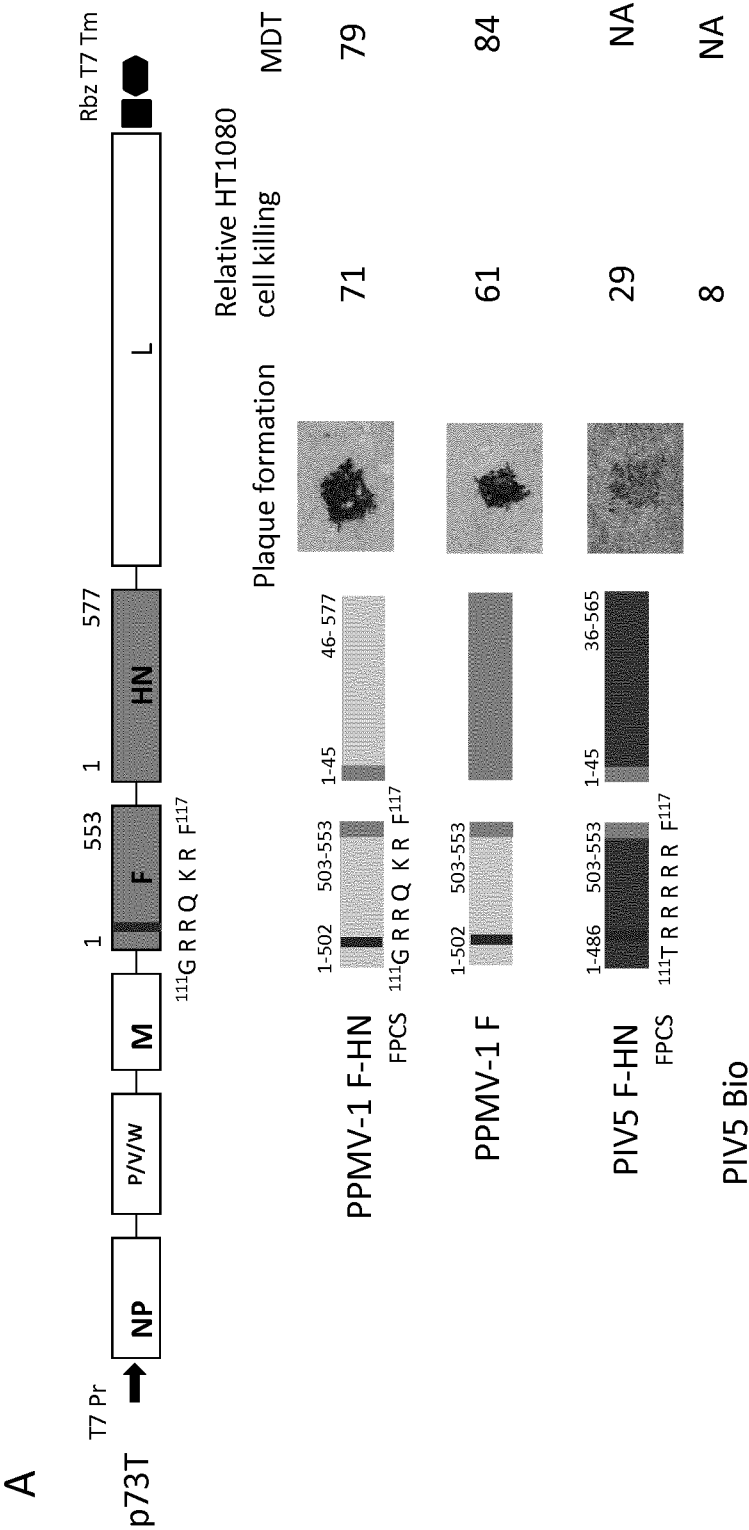


Figure 12B.

B

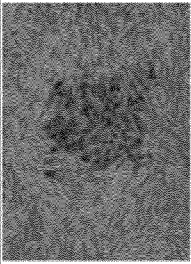
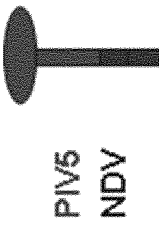
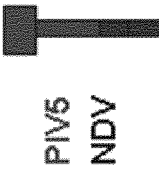
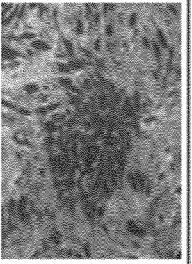
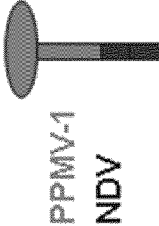
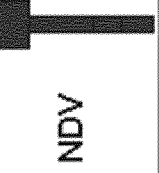
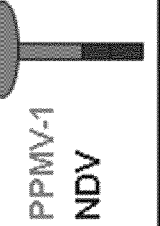
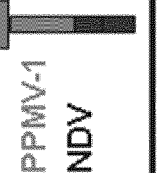
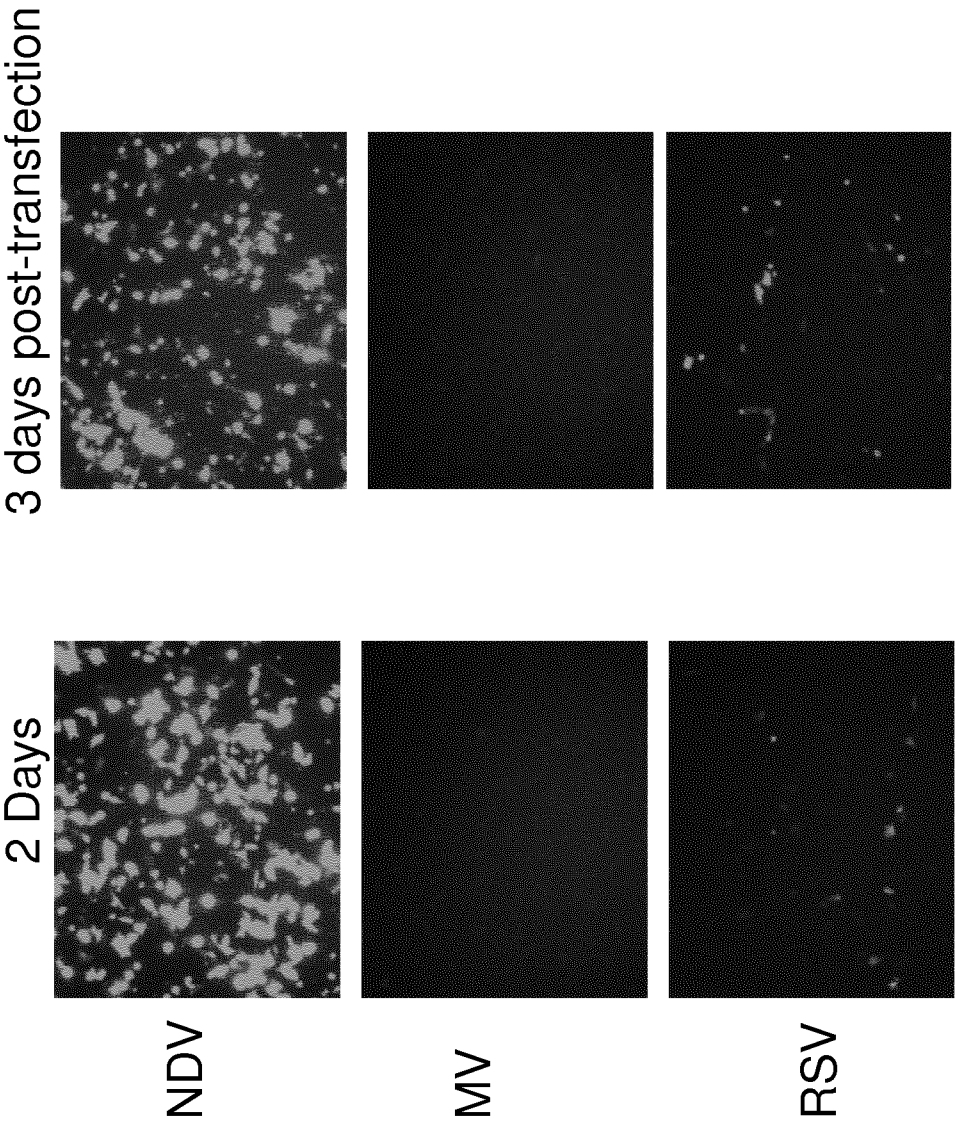
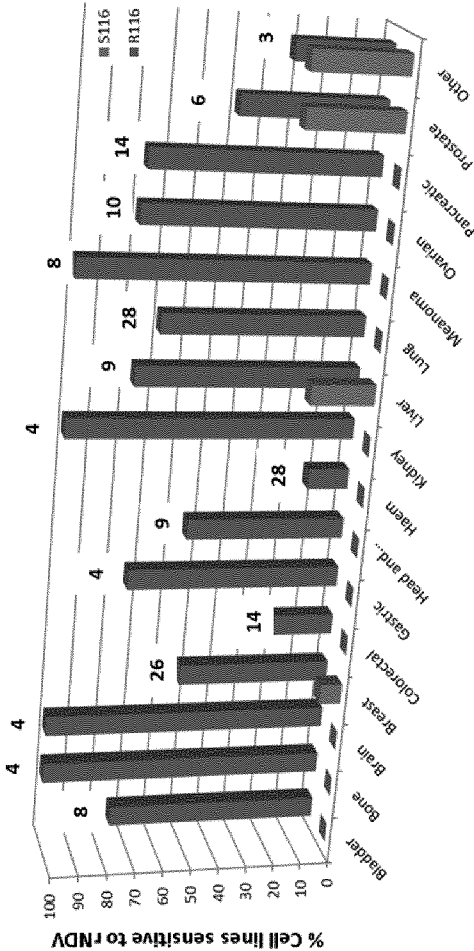
Vero w/o trypsin	Spreading in cells	F gene	HN gene	Grow in eggs (pfu/ml)	Grown in Vero w/o trypsin (pfu/ml)
	+++	 PIV5 NDV	 PIV5 NDV	No	6.0×10^6
	++++	 PPMV-1 NDV	 NDV	2.4×10^8	2.1×10^6
	+++	 PPMV-1 NDV	 PPMV-1 NDV	2.7×10^8	2.5×10^6

Figure 12C. Compare function of RNA polymerase complex by mini-genome assay



Figures 13A and B. Cancer cell line sensitivity to rNDV variants

A.



B.

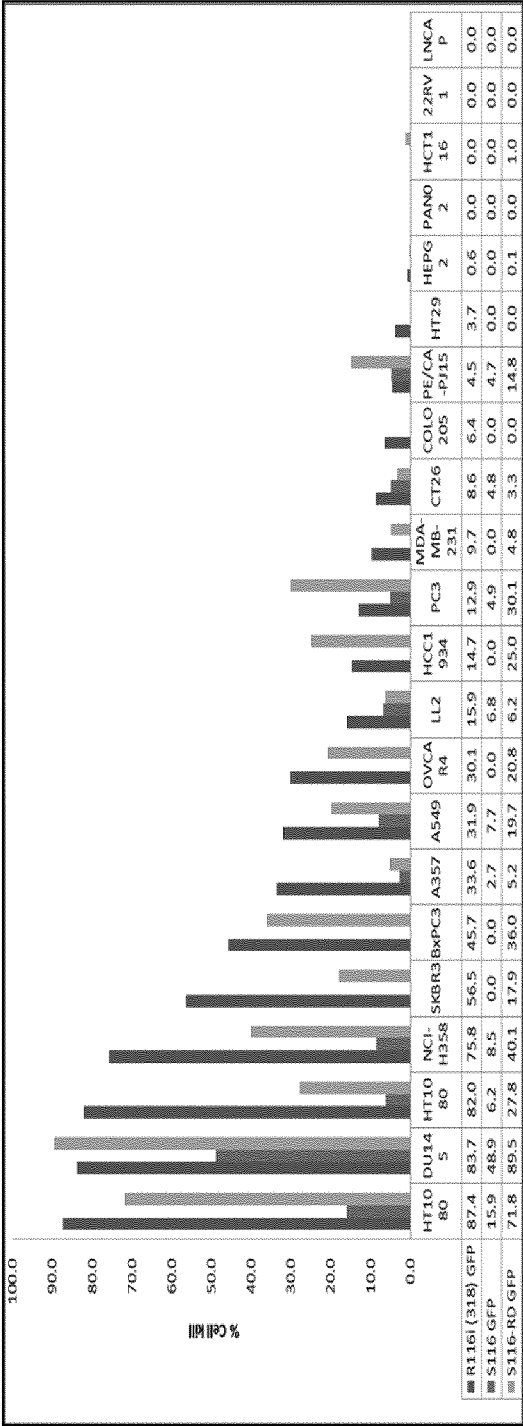


Figure 14. Permissivity of the cancer cell lines to recNDV^{GM-CSF}

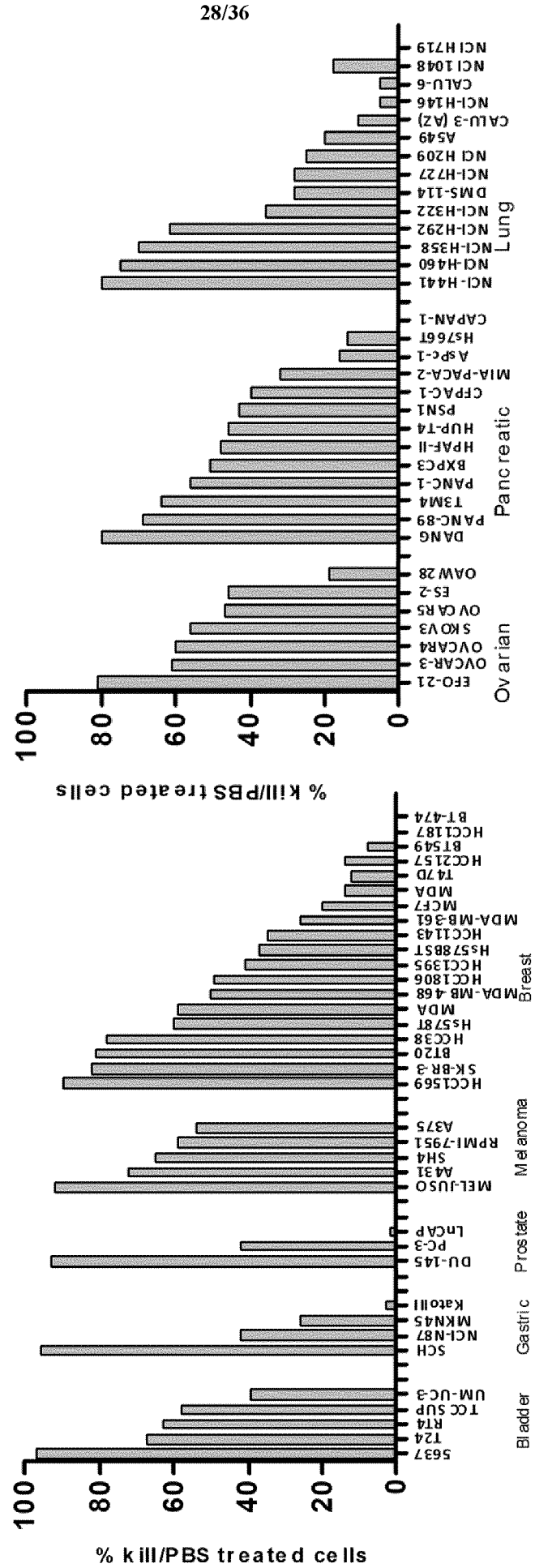
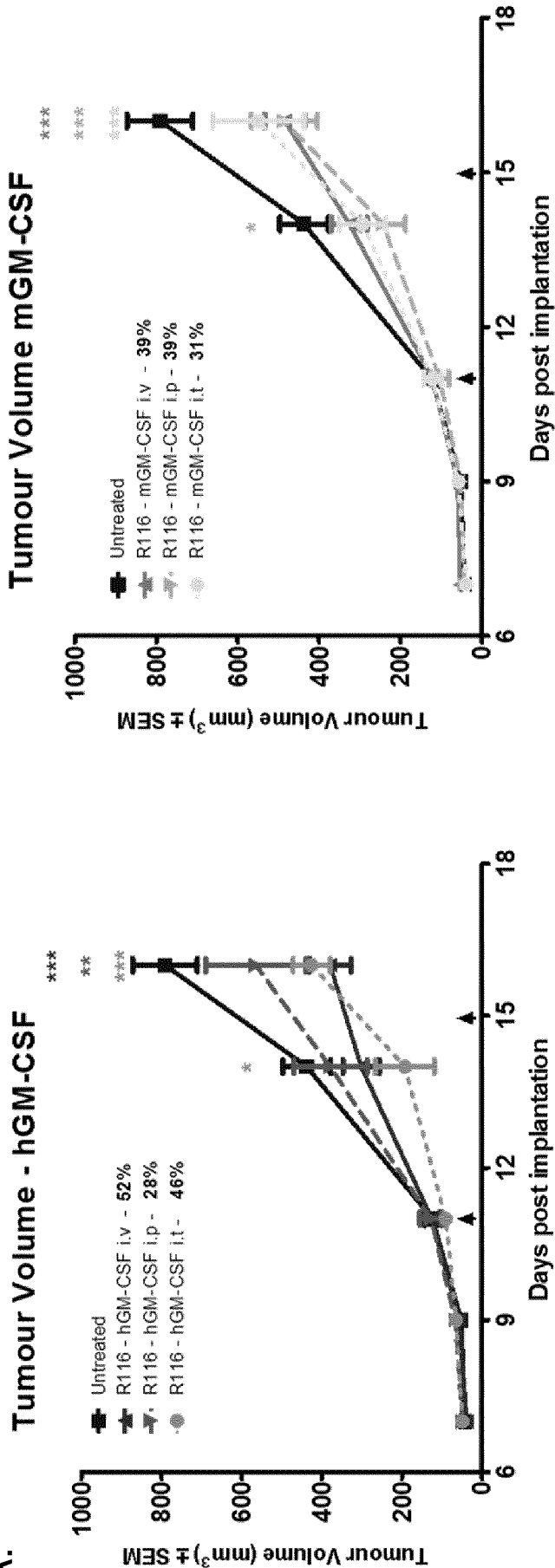


Fig legend: Cytopathic effect of rNDV variants R116i (expressing GM-CSF). Maximum % kill was determine 3 days following infection of tumour cell lines relative to non-infected control .

Figures 15A and B. Tumour growth inhibition in syngeneic mouse melanoma model (B16F10 AP3) by recNDV^{GM-CSF}

A.



B.

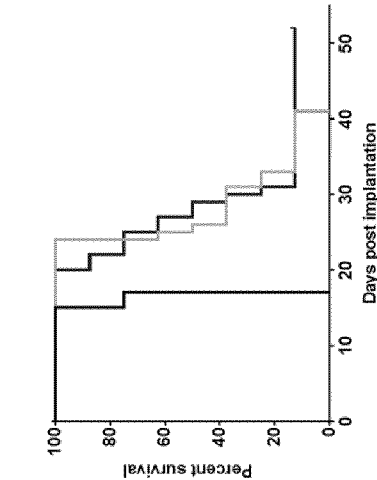
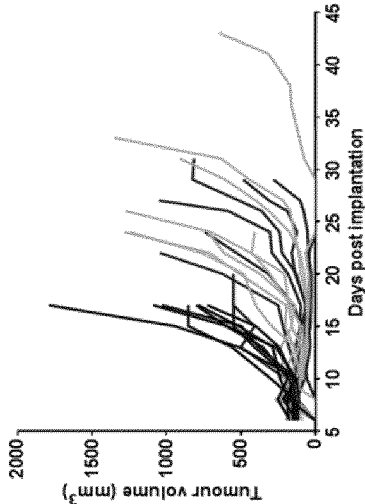
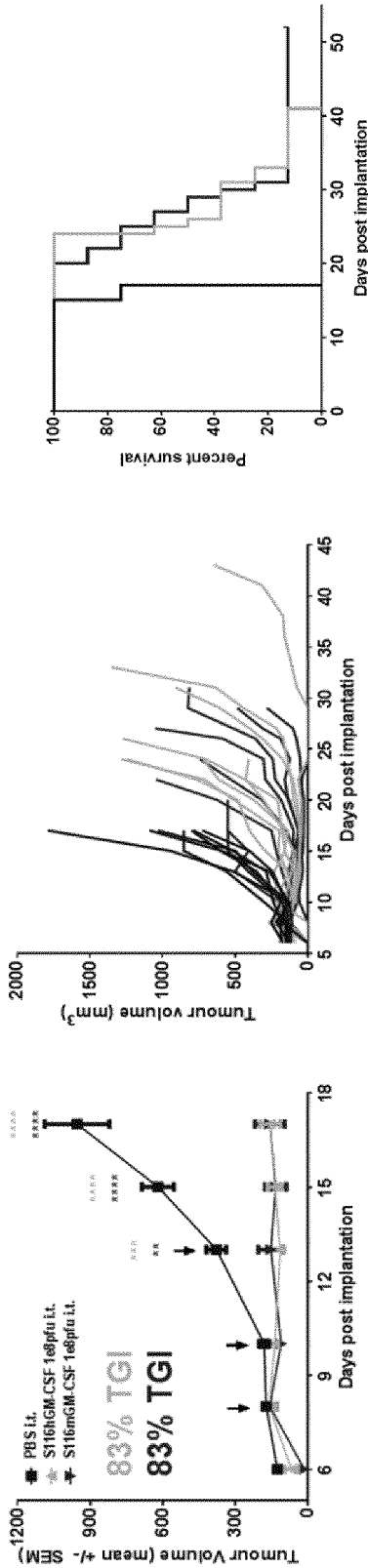
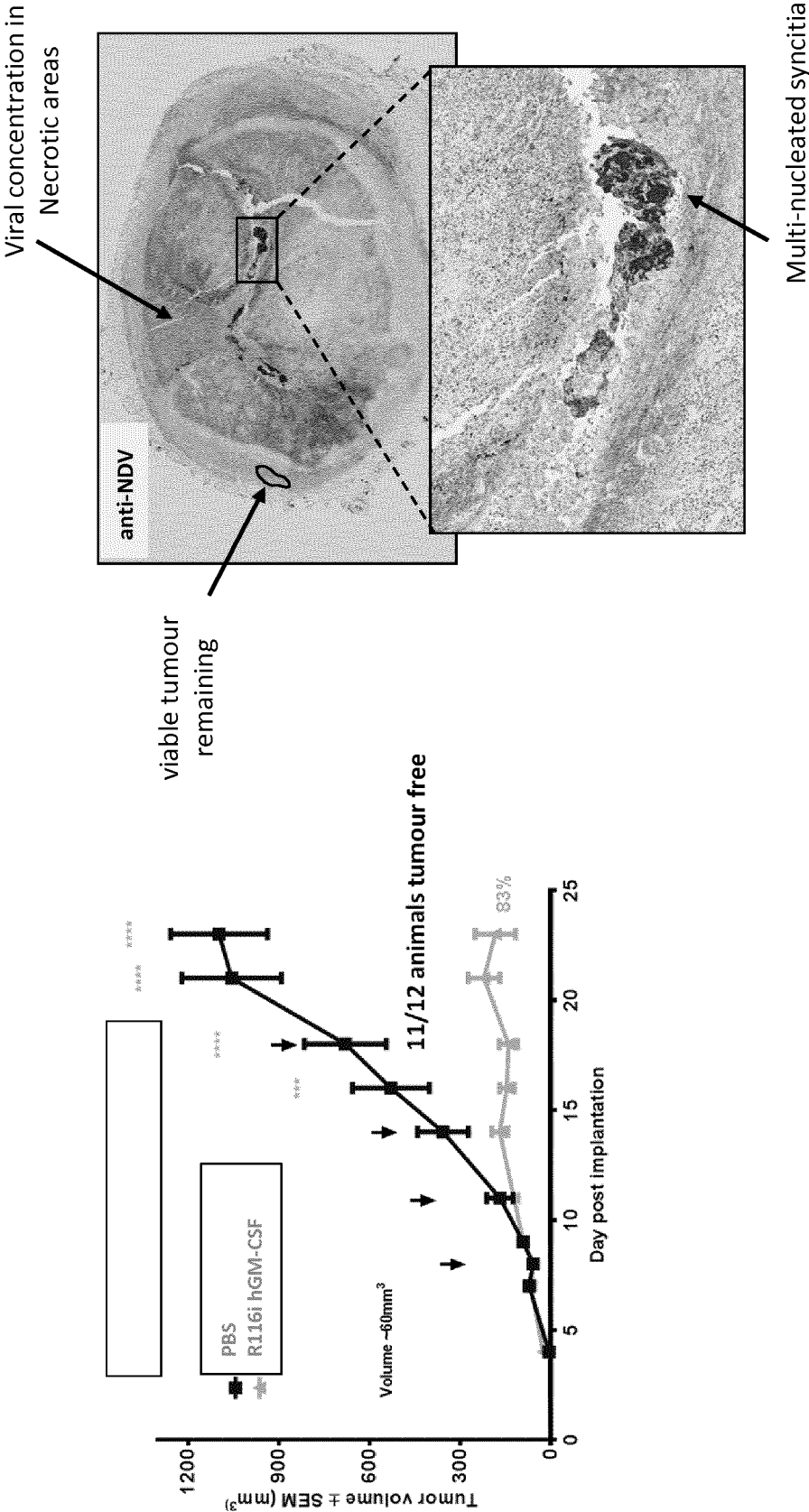


Figure 15C: NDV has potent anti-tumour activity in immune-competent mouse colorectal tumour model



Figures 15D-F Multiple dosing with rNDV R116i causes Tumour growth inhibition and drives immune cell recruitment into ovarian cancer (OVCAR4) xenograft model

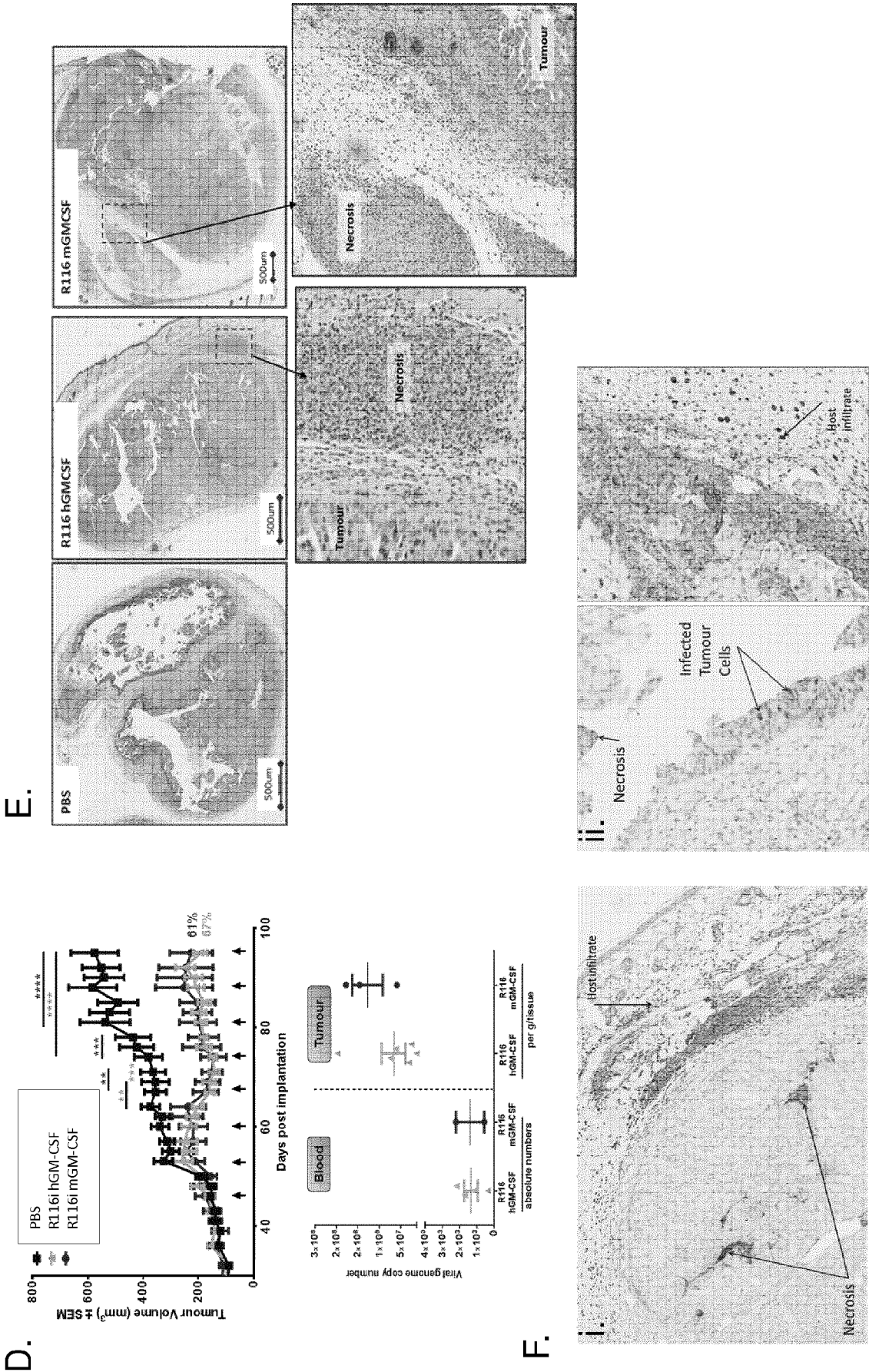
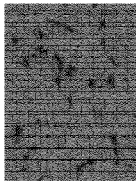
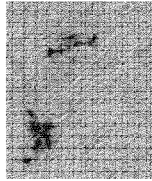
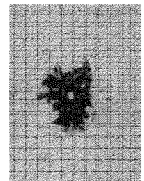
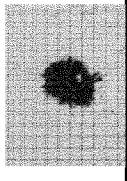
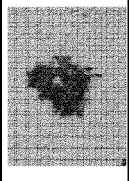


Figure 16. Summary of NDV Constructs

FPCS (R-R-Q-K-R ¹¹⁶ /F)	Plaques in Vero (no trypsin)	Transgene	HT1080 killing (%) at 72h	ICPI	comments
Lento (G-R-Q-E-R/L)		hGM-CSF	20	0.0	Replication depends on trypsin
S116 (N-R-T-K-S/F)		none	60	0.0	Do not need trypsin for intro replications
		hGM-CSF	40	0.075	Larger plaques
R116 (N-R-T-K-R/F)		none	80	tbd	Big plaques, more efficient cell killing
		hGM-CSF	80	0.65	
S116K113M114 (N-K-M-K-S/F)		hGM-CSF	48	0.00	Big plaques, intermediate efficient cell killing
R116-HN/L(318nt) (N-R-T-K-R/F)		hGM-CSF	80	0.05 - 0.27	2 loci control virus att F cleavage and intergenic stretch
R116-HN/L(198nt) (N-R-T-K-R/F)		hGM-CSF	86	0.0375	

All strains have titers of 8-9 log₁₀ PFU/ml in eggs and ~7.0 log₁₀ PFU/ml in Vero cells, lento cleavage site: 3 log₁₀ PFU/ml in Vero.

Figure 17. NDV produced from HeLa cells is less sensitive to C' mediated inactivation

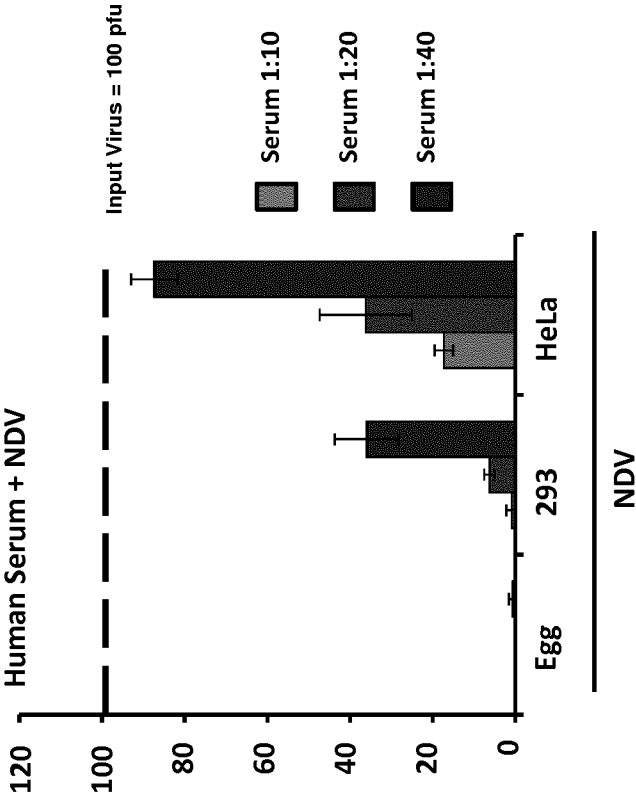


Figure 18. HeLa-3S cells express higher levels of RCA proteins than 293 cells

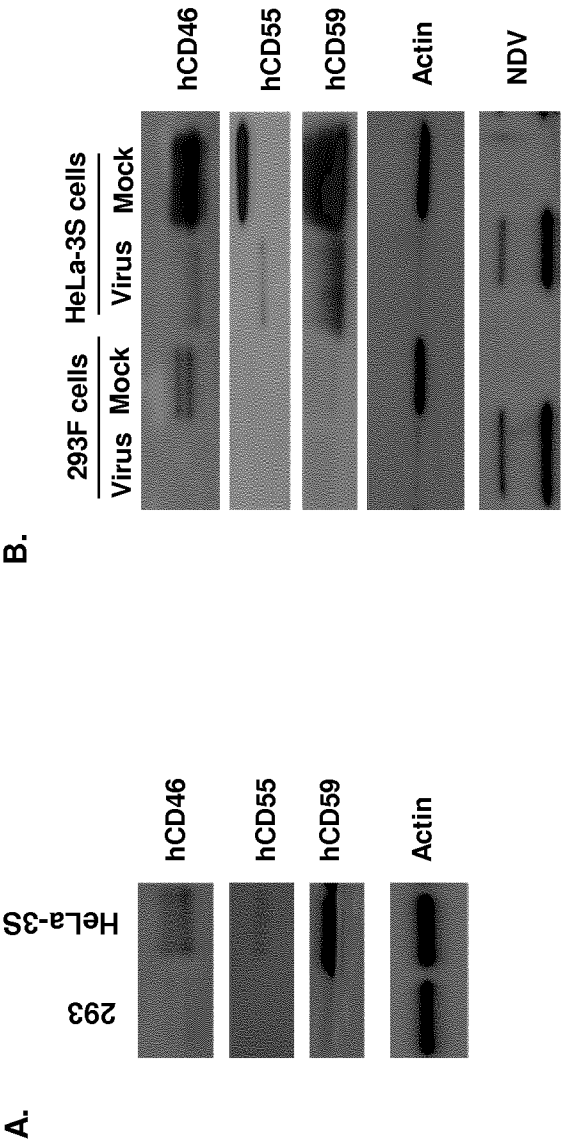
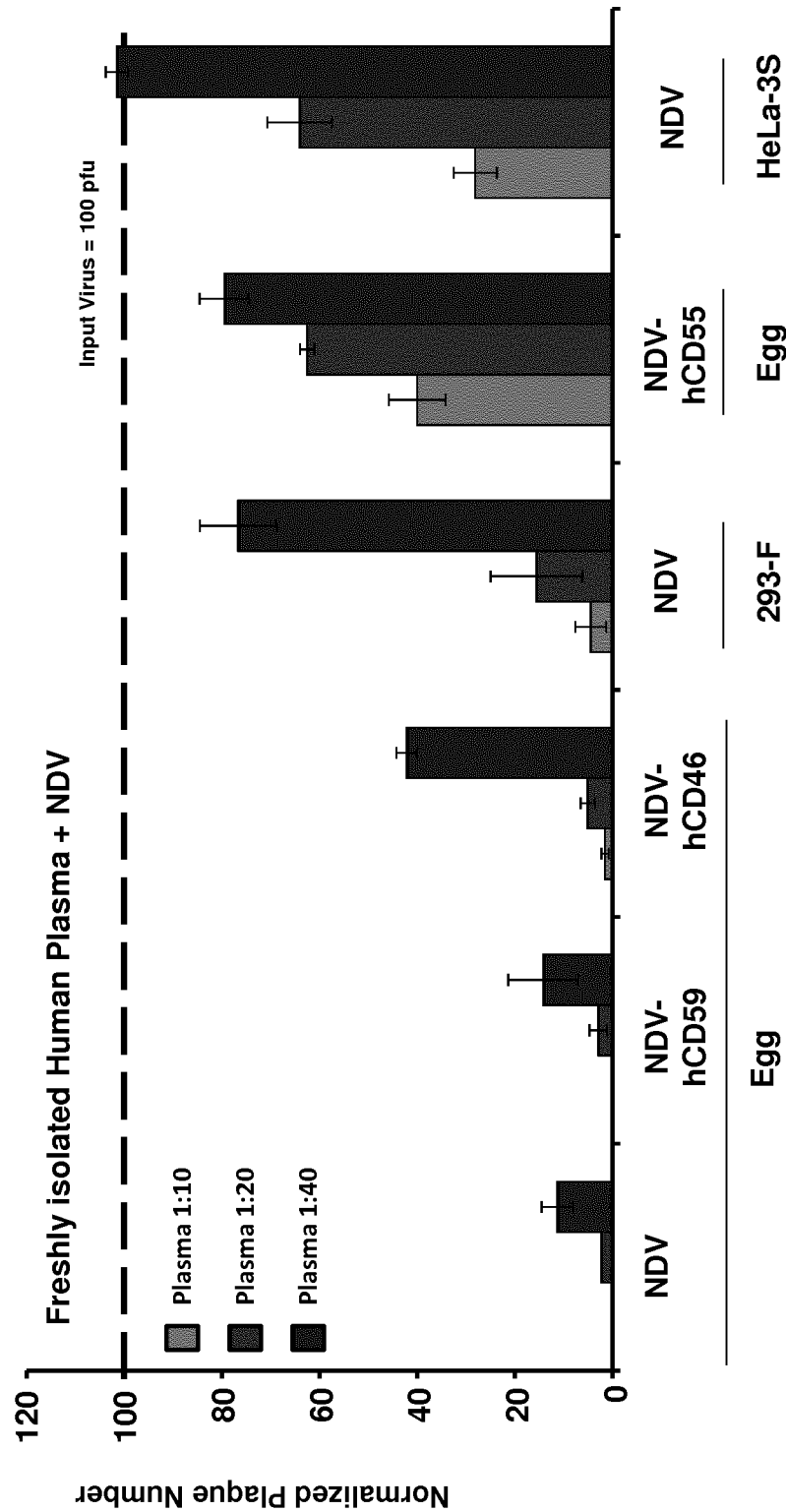


Figure 20. CD55 is a critical RCA protein for preventing C' inactivation of NDV



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/068619

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/76 C07K14/535 C12N15/86
ADD.

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)
A61K C07K C12N

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

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

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/091262 A1 (SINAI SCHOOL MEDICINE [US]; SLOAN KETTERING INST CANCER [US]; ZAMARIN) 12 August 2010 (2010-08-12)	1,3,9, 11, 23-29, 31-34, 36,40, 42,43
Y	page 4 page 13 - page 17; claims 1-22 page 26 - page 39 ----- -/--	5,10, 12-16



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

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Authorized officer

Deleu, Laurent

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/068619

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JANKE M ET AL: "Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy", GENE THERAPY, NATURE PUBLISHING GROUP, GB, vol. 14, no. 23, December 2007 (2007-12), pages 1639-1649, XP002606261, ISSN: 0969-7128, DOI: 10.1038/SJ.GT.3303026 [retrieved on 2007-10-04] page 1640 - page 1644; figure 1 -----	5,10, 12-14
Y	WO 01/20989 A1 (MAYO FOUNDATION [US]; RUSSELL JAMES [US]; CATTANEO ROBERTO [US]; PENG) 29 March 2001 (2001-03-29) claims 1-19,22-26; examples 3,4 page 34 - page 37 -----	6,8, 15-17,35
Y	WO 2009/095167 A1 (BAYER SCHERING PHARMA AG [DE]; BEIER RUDOLF [DE]; PUEHLER FLORIAN [DE]) 6 August 2009 (2009-08-06) page 25 - page 36; claims 1-10 -----	6-8,17, 35
Y	JOS CFM DORTMANS ET AL: "Virulence of newcastle disease virus: what is known so far?", VETERINARY RESEARCH, BIOMED CENTRAL LTD, LONDON, UK, vol. 42, no. 1, 23 December 2011 (2011-12-23), page 122, XP021130364, ISSN: 1297-9716, DOI: 10.1186/1297-9716-42-122 page 124 page 128 - page 129 -----	6-8
Y	J. C. F. M. DORTMANS ET AL: "Passaging of a Newcastle disease virus pigeon variant in chickens results in selection of viruses with mutations in the polymerase complex enhancing virus replication and virulence", JOURNAL OF GENERAL VIROLOGY, vol. 92, no. 2, 21 October 2010 (2010-10-21), pages 336-345, XP055155911, ISSN: 0022-1317, DOI: 10.1099/vir.0.026344-0 page 340 - page 342 -----	17,35
A	WO 2009/101149 A2 (BAYER SCHERING PHARMA AG [DE]; BEIER RUDOLF [DE]; PUEHLER FLORIAN [DE]) 20 August 2009 (2009-08-20) page 11 - page 12; claims 1-10 ----- -/--	1-43

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/068619

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PHILIPPE FOURNIER ET AL: "Oncolytic Newcastle Disease Virus as Cutting Edge between Tumor and Host", BIOLOGY, vol. 2, no. 3, 2 July 2013 (2013-07-02), pages 936-975, XP055155919, DOI: 10.3390/biology2030936 page 943 - page 945 -----	25-34
A	S. SAMAL ET AL: "A single amino acid change, Q114R, in the cleavage-site sequence of Newcastle disease virus fusion protein attenuates viral replication and pathogenicity", JOURNAL OF GENERAL VIROLOGY, vol. 92, no. 10, 15 June 2011 (2011-06-15) , pages 2333-2338, XP055156082, ISSN: 0022-1317, DOI: 10.1099/vir.0.033399-0 page 2334 - page 2336 -----	2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/068619

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010091262 A1	12-08-2010	EP 2393921 A1 US 2012058141 A1 US 2014044678 A1 WO 2010091262 A1	14-12-2011 08-03-2012 13-02-2014 12-08-2010
WO 0120989 A1	29-03-2001	AT 451835 T AU 7607900 A CA 2383276 A1 EP 1217891 A1 WO 0120989 A1	15-01-2010 24-04-2001 29-03-2001 03-07-2002 29-03-2001
WO 2009095167 A1	06-08-2009	AR 070272 A1 AU 2009210301 A1 CA 2711571 A1 CN 102099045 A CO 6290693 A2 CR 11616 A DO P2010000233 A EA 201001202 A1 EC SP10010375 A EP 2085092 A1 EP 2237790 A1 JP 2011510638 A KR 20100113096 A PA 8813901 A1 PE 13552009 A1 TW 200936159 A US 2010092430 A1 WO 2009095167 A1	25-03-2010 06-08-2009 06-08-2009 15-06-2011 20-06-2011 09-09-2010 15-08-2010 28-02-2011 31-08-2010 05-08-2009 13-10-2010 07-04-2011 20-10-2010 26-08-2009 04-10-2009 01-09-2009 15-04-2010 06-08-2009
WO 2009101149 A2	20-08-2009	AU 2009214066 A1 CA 2715136 A1 CN 101945660 A CO 6290694 A2 CR 11631 A DO P2010000251 A EA 201001266 A1 EC SP10010401 A EP 2252307 A2 JP 2011512344 A KR 20100122482 A US 2009208495 A1 WO 2009101149 A2	20-08-2009 20-08-2009 12-01-2011 20-06-2011 05-10-2010 31-08-2010 29-04-2011 30-09-2010 24-11-2010 21-04-2011 22-11-2010 20-08-2009 20-08-2009