(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 April 2013 (18.04.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/055888 A2

(51) International Patent Classification:
C12N 9/24 (2006.01) *A61K 45/06* (2006.01)
A61K 38/47 (2006.01)

(21) International Application Number:
PCT/US2012/059708

(22) International Filing Date:
11 October 2012 (11.10.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/546,248 12 October 2011 (12.10.2011) US

(71) Applicant: **SYNAGEVA BIOPHARMA CORP.** [US/US]; 128 Spring Street, Suite 520, Lexington, MA 02421 (US).

(72) Inventors: **QUINN, Anthony**; 107 Manet Road, Chestnut Hill, MA 02467 (US). **LEAVITT, Markley, C.**; 1140 Latham Drive, Watkinsville, GA 30677 (US). **ZHINAN, Xia**; 29 Burke Lane, Wellesley, MA 02481 (US). **RUTKOWSKI, Joseph, Victor**; Synageva Biopharma Corp., 128 Spring St., Suite 520, Lexington, MA 02421 (US).

(74) Agents: **ZACHARAKIS, Maria, Laccotripe** et al.; McCarter & English, LLP, 265 Franklin Street, Boston, MA 02110 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: RECOMBINANT HUMAN NAGLU PROTEIN AND USES THEREOF

Human NaGlu Amino Acid Sequence (signal peptide: 1-23, underlined)

```
MEAVAVAAAV GVLLLAGAGG AAGDEAREAA AVRALVARLL GPGPAADFSV SVERALAAKP   60
GLDTYSLGGG GAARVRVRGS TGVAAAAGLH RYLRDFCGCH VAWSGSQLRL PRPLPAVPGE  120
LTEATPNRYR YYQNVCTQSY SFVWWDWARW EREIDWMALN GINLALAWSG QEAIWQRVYL  180
ALGLTQAEIN EFFTGPAFLA WGRMGNLHTW DGPLPPSWHI KQLYLQHRVL DQMRSFGMTP  240
VLPAFAGHVP EAVTRVFPQV NVTKMGSWGH FNCSYSCSFL LAPEDPIFPI IGSLFLRELI  300
KEFGTDHIYG ADTFNEMQPP SSEPSYLAAA TTAVYEAMTA VDTEAVWLLQ GWLFQHQPQF  360
WGPAQIRAVL GAVPRGRLLV LDLFAESQPV YTRTASFQGQ PFIWCMLHNF GGNHGLFGAL  420
EAVNGGPEAA RLFPNSTMVG TGMAPEGISQ NEVVYSLMAE LGWRKDPVPD LAAWVTSFAA  480
RRYGVSHPDA GAAWRLLLRS VYNCSGEACR GHNRSPLVRR PSLQMNTSIW YNRSDVFEAW  540
RLLLTSAPSL ATSPAFRYDL LDLTRQAVQE LVSLYYEEAR SAYLSKELAS LLRAGGVLAY  600
ELLPALDEVL ASDSRFLLGS WLEQARAAAV SEAEADFYEQ NSRYQLTLWG PEGNILDYAN  660
KQLAGLVANY YTPRWRLFLE ALVDSVAQGI PFQQHQFDKN VFQLEQAFVL SKQRYPSQPR  720
GDTVDLAKKI FLKYYPRWVA GSW                                          743
```

(SEQ ID NO:1)

Fig. 1

(57) Abstract: The present invention provides compositions comprising an isolated mixture of recombinant human NaGlu proteins in which a substantial amount of the NaGlu proteins in the mixture has increased levels of phosphorylated mannose that confer the proteins to be efficiently internalized into human cells. The present invention also provides methods of producing such mixture of NaGlu proteins, vectors used in transgenesis and expression, host cells harboring such vectors, and methods of isolating and purifying the mixture of NaGlu proteins. The invention further provides methods of treating NaGlu associated diseases.

WO 2013/055888 A2

RECOMBINANT HUMAN NAGLU PROTEIN AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is related and claims priority to U.S. Provisional Application Serial No. 61/546,248, filed October 12, 2011, the entire contents of which are expressly incorporated herein by this reference.

BACKGROUND OF THE INVENTION

Sanfilippo Syndrome B is an autosomal recessive lysosomal storage disease (LSD) caused by a deficiency in a lysosomal enzyme known as N-acetyl-alpha-D-glucosaminidase (NaGlu). NaGlu is required for the degradation of heparan sulfate as part of the stepwise breakdown of glycosaminoglycans (GAG) in the lysosome. The deficiency or absence of NaGlu leads to accumulation and urinary excretion of heparan sulfate. With over 70 different mutations identified to date, Sanfilippo Syndrome B exhibits extensive molecular and genetic heterogeneity.

Approximately 1 out of 200,000 births is affected by Sanfilippo Syndrome B and the deficiency mainly manifests in young children. After initial symptom-free interval, patients suffering from Sanfilippo Syndrome B usually present with a slowing of mental development and behavioral problems, followed by progressive intellectual decline resulting in severe mental retardation, dementia and motor disease. Acquisition of speech is slow and incomplete. Profoundly affected patients may present delayed psychomotor and speech development as early as 2 years of age. The disease usually progresses to increasing behavioral disturbance and sleep disturbance. Although the clinical features are mainly neurological, patients often develop diarrhea, carious teeth, an enlarged liver and spleen, stiff joints, hirsteness and/or coarse hair and may exhibit blood-clotting problems. In the final stage of the illness, patients become immobile and unresponsive and develop swallowing difficulties and seizure. The life-span of an affected child typically does not extend beyond late teens to early twenties.

Different approaches have been attempted to provide the missing enzyme in patients. To produce NaGlu for enzyme replacement therapy (ERT), human NaGlu has been expressed in various mammalian cell culture systems. However, in contrast to the naturally occurring NaGlu which trafficks to the lysosome intracellularly, recombinant NaGlu proteins produced and secreted from mammalian cells were found to contain no or only a trace amount of mannose 6-phosphate (M6P). The absence or scarcity of M6P moieties in the secreted NaGlu has been known to prevent its efficient internalization into target cells (*e.g.,* human skin fibroblasts), which have M6P receptors on the surface on its plasma membrane (*see*, Zhao *et al.*, *Protein Expression and Purification*, 19:202-211 (2000); and Weber *et al.*, *Protein Expression and Purification*, 21:251-259 (2001)). The low degree of phosphorylation was seen in secreted mouse NaGlu expressed in CHO cells, secreted human NaGlu expressed in HeLa cells, secreted human NaGlu expressed in human fibroblasts, and secreted human NaGlu expressed in human embryonic kidney (HEK) cell line 293 (*see*, Zhao *et al.*, *Protein Expression and Purification*, 19:202-211 (2000); Yogalingam *et al.*, *Biochim Biophys. Acta* 1502: 415-425; and Weber *et al.*, *Protein Expression and Purification*, 21:251-259 (2001)). No or weak phosphorylation of N-glycans in the NaGlu proteins secreted from the mammalian cells has posed a major obstacle for the development of a recombinant human NaGlu protein suitable for enzyme replacement therapy as all the aforementioned attempts has failed to produce an enzyme which is efficiently taken up by target cells as the concentration of the internalized proteins, if detectable at all, was nearly a thousand times less than wild-type levels (see, Zhao *et al.*, *Protein Expression and Purification*, 19:202-211 (2000)). To date, no approved product is available for the treatment of Sanfilippo Syndrome B.

Direct administration of mammalian cell-produced recombinant human NaGlu protein (rhNaGlu) having the native amino acid sequence into the central nervous system (CNS) (*e.g.*, intrathecal administration into the cerebrospinal fluid (CSF)) of NaGlu deficient mice has been attempted, but failed to demonstrate successful biodistribution of the enzyme to the brain due to excessive accumulation of the protein on the ependymal ling of the ventricles as well as lack of requisite M6P residues for efficient cellular uptake. Similarly, systemic administration (*i.e.*, intravenous (IV)

injection) of mammalian cell-produced rhNaGlu having the native amino acid sequence also failed to demonstrate successful localization of the protein to the brain. In addition to known risks associated with highly invasive intrathecal administration, these obstacles in targeting rhNaGlu to the brain have been too great a challenge to achieve effective therapy for the treatment of Sanfilippo Syndrome B.

Therefore, there is a need to provide a stable NaGlu protein which is enzymatically active and has physical properties that allow for the protein to cross the blood brain barrier (BBB) and for effective internalization of the protein into the lysosomes of target cells. There is also a need for a high expressing and robust protein production platform which can provide a recombinant human NaGlu that effectively crosses the blood brain barrier and is efficiently internalized into human target cells.

SUMMARY OF THE INVENTION

The present invention is drawn to compositions comprising recombinant human NaGlu protein (rhNaGlu) useful for therapy, for example, in the treatment of Sanfilippo Syndrome B. The present invention is based on the surprising and unexpected discovery that the rhNaGlu described herein has one or more glycosylation patterns that allow the rhNaGlu to efficiently cross the blood brain barrier (BBB), and be taken up into cells within the central nervous system (CNS) of animals deficient in the enzyme, resulting in a dramatic increase in α-N-acetylglucosaminidase activity in the brain, as well as a reduction of substrate levels. Moreover, the rhNaGlu described herein is efficiently taken up into a mammalian cell (*e.g.*, human cell), resulting in an increased enzymatic activity as compared to NaGlu proteins produced and secreted from unmodified mammalian cells that are not designed to produce specific glycosylation. The increased cellular uptake of the NaGlu protein also provides benefits for the use in enzyme replacement therapy for a human patient suffering from Sanfilippo Syndrome B by minimizing the need for an increased amount and frequency of dose, and thereby greatly reducing the potential risk of immunogenicity.

The rhNaGlu protein described herein contains sufficient amount of oligosaccharides (*e.g.,* mannose and phosphorylated mannose (*i.e.,* M6P)) to allow efficient cellular uptake via mannose and/or M6P receptor-mediated endocytosis and be

correctly targeted into human cells. In one embodiment, the rhNaGlu contains at least one mole of protein, for example, 1, 2, 3, 4, 5 or 6 moles of M6P per mole of protein. In one embodiment, rhNaGlu can be internalized into a NaGlu deficient human cell such that the internalized protein fully (100% or more) restores normal levels (*i.e.,* wild-type levels) of NaGlu activity in the NaGlu deficient cell.

Also disclosed herein are methods for producing a transgenic avian that expresses rhNaGlu which benefits from phosphorylation of mannose. In particular, a transgenic avian that expresses rhNaGlu protein in oviduct cells, secretes into the lumen of the oviduct and deposits the protein into egg white. Avian eggs that contain such rhNaGlu are also included in the present invention.

The present invention also contemplates vectors and host cells that contain a transgene encoding rhNaGlu as well as pharmaceutical compositions comprising rhNaGlu to be used in the application of such rhNaGlu for the treatment of Sanfilippo Syndrome B.

In one aspect, the invention provides a composition comprising an isolated mixture of recombinant human N-acetyl-alpha-D-glucosaminidase (rhNaGlu) comprising the amino acid sequence 24-743 of SEQ ID NO:1, wherein at least 10 % of the rhNaGlu in the mixture comprises at least one glycan structure having mannose-6-phosphate (M6P). In one embodiment, the rhNaGlu having M6P is capable of being taken up into a mammalian cell deficient in NaGlu such that internalized rhNaGlu restores at least 50%, 60%, 70%, 80%, 90% or 100% of normal NaGlu activity observed in a wild-type mammalian cell of the same type. In another embodiment, the glycan structure is an N-linked glycan.

In one embodiment, the rhNaGlu contains at least 1 mole of M6P per mole of protein. In another embodiment, the rhNaGlu contains between about 1 and about 6 moles of M6P per mole of protein. In another embodiment, the rhNaGlu contains about 2 moles of M6P per mole of protein. In yet another embodiment, the rhNaGlu contains about 3 moles of M6P per mole of protein. In another embodiment, the rhNaGlu contains about 4 moles of M6P per mole of protein. In another embodiment, the rhNaGlu contains about 5 moles of M6P per mole of protein. In yet another embodiment, the rhNaGlu contains about 6 moles of M6P per mole of protein.

In one embodiment, the mammalian cell deficient in NaGlu is a human cell. In another embodiment, the human cell deficient in NaGlu is a skin fibroblast, a hepatocyte or a macrophage. In one embodiment, the human cell deficient in NaGlu is a neuronal cell.

In one embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when systemically administered. In one particular embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when intravenously administered. In one embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intrathecally.

In one embodiment, the rhNaGlu having M6P is internalized by a NaGlu deficient cell and restores at least 100% of normal NaGlu activity *in vivo*. In one embodiment, the rhNaGlu having M6P contains at least 25 moles of mannose per mole of protein.

In one embodiment, at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the rhNaGlu in the mixture contains M6P. In another embodiment, at least 20% of the rhNaGlu in the mixture contains at least one M6P. In another embodiment, at least 30% of the rhNaGlu in the mixture contains at least one M6P. In another embodiment, at least 40% of the rhNaGlu in the mixture contains at least one M6P. In another embodiment, at least 50% of the rhNaGlu in the mixture contains at least one M6P. In another embodiment, at least 60% of the rhNaGlu in the mixture contains at least one M6P.

In another aspect, the invention provides a composition comprising an isolated mixture of recombinant human N-acetyl-alpha-D-glucosaminidase (rhNaGlu) comprising the amino acid sequence 24-743 of SEQ ID NO:1, wherein the mixture comprises a sufficient amount of rhNaGlu containing one or more glycan structures comprising mannose-6-phosphate (M6P) such that the rhNaGlu containing M6P is internalized into a mammalian cell having NaGlu deficiency via M6P receptor-mediated endocytosis and restores at least 50 % of NaGlu activity observed in a wild-type cell of the same type expressing endogenous NaGlu. In one embodiment, the rhNaGlu is N-linked glycosylated. In another embodiment, the rhNaGlu is O-linked glycosylated.

In one embodiment, the rhNaGlu comprises at least 1 moles of M6P per mole of rhNaGlu. In another embodiment, the rhNaGlu comprises about 1, 2, 3, 4, 5 or 6 moles of M6P per mole of rhNaGlu. In another embodiment, the rhNaGlu comprises about 3 moles of M6P per mole of rhNaGlu. In another embodiment, the rhNaGlu comprises about 4 moles of M6P per mole of rhNaGlu.

In one embodiment, the rhNaGlu comprises mannose. In another embodiment, the rhNaGlu comprises N-acetylglucosamine (GlcNAc). In another embodiment, the rhNaGlu comprises galactose. In another embodiment, the rhNaGlu comprises N-acetylgalactosamine (GalNAc). In another embodiment, the rhNaGlu contains no fucose. In another embodiment, the rhNaGlu contains no glucose. In one embodiment, the rhNaGlu restores at least 60, 70, 80, 90, 95 or 100% of normal NaGlu enzymatic activity.

In another embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered systemically. In one embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intravenously. In another embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intrathecally.

In one embodiment, the mammalian cell deficient in NaGlu is a human cell. In another embodiment, the human cell is a skin fibroblast, a hepatocyte or a macrophage. In one embodiment, the human cell deficient in NaGlu is a neuronal cell.

In one embodiment, the rhNaGlu is a fusion protein comprising a second moiety. In one embodiment, the second moiety is a polypeptide. In another embodiment, the polypeptide is selected from the group consisting of transferrin receptor ligand (TfRL), insulin-like growth factor receptor (IGF2R) ligand, low density lipoprotein (LDL) receptor ligand and acidic amino acid (AAA) residues.

In one embodiment, the rhNaGlu is produced from a transgenic avian. In one embodiment, the transgenic avian is a chicken, a turkey, a duck or a quail. In one embodiment, the transgenic avian is a chicken. In one embodiment, the rhNaGlu is produced from an oviduct cell.

In another aspect, the invention provides a composition comprising an isolated recombinant human N-acetyl-alpha-D-glucosaminidase (rhNaGlu) comprising one or more glycan structures having sufficient amount of mannose-6-phosphate (M6P) that allows for internalization of the rhNaGlu into a mammalian cell having NaGlu deficiency via M6P receptor-mediated endocytosis, such that when internalized *in vivo*, the rhNaGlu restores at least 50% of NaGlu activity observed in a wild-type cell of the same type expressing endogenous NaGlu.

In one embodiment, the rhNaGlu protein is N-linked glycosylated. In another embodiment, the rhNaGlu protein is O-linked glycosylated. In one embodiment, the rhNaGlu comprises about 2, 3, 4, 5 or 6 moles of M6P per mole of rhNaGlu.

In one embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered systemically. In another embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intravenously. In another embodiment, the rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intrathecally.

In another aspect, the invention provides a transgenic avian comprising a transgene containing a promoter operably linked to a nucleic acid sequence encoding a recombinant human NaGlu (rhNaGlu), wherein the transgene is contained in the genome of the transgenic avian and expressed in an oviduct cell such that the rhNaGlu is glycosylated in the oviduct cell of the transgenic avian, secreted into lumen of oviduct and deposited in egg white of an egg of the transgenic avian.

In one embodiment, the rhNaGlu comprises about 2, 3, 4 or 6 moles of M6P per mole of rhNaGlu. In another embodiment, the promoter component is an oviduct-specific promoter. In another embodiment, the oviduct-specific promoter is an ovalbumin promoter. In yet another embodiment, the transgenic avian is selected from the group consisting of a chicken, a turkey, a duck and a quail.

In another aspect, the invention provides an egg produced by the transgenic avian of the invention.

In yet another aspect, the invention provides a method of producing a recombinant human NaGlu (rhNaGlu) comprising: a) producing a transgenic avian

comprising a transgene having a promoter component operably linked to a nucleic acid sequence encoding the rhNaGlu set forth in 24-743 of SEQ ID NO:1, wherein the transgene is contained in the genome of the transgenic avian and expressed in an oviduct cell, such that the rhNaGlu is glycosylated in the oviduct cell of the transgenic avian, secreted into lumen of oviduct and deposited in egg white of an egg laid by the transgenic avian; and b) isolating the rhNaGlu from the egg white.

In one embodiment, the promoter component is an oviduct-specific promoter. In another embodiment, the oviduct-specific promoter is an ovalbumin promoter. In one embodiment, the avian is selected from the group consisting of a chicken, a turkey, a duck and a quail. In one embodiment, the avian is chicken.

In another aspect, the invention provides a vector comprising a nucleotide sequence encoding a human NaGlu operably linked to an ovalbumin promoter. In another aspect, the invention provides a host cell comprising the vector of the invention. In another aspect, the invention provides an isolated nucleic acid comprising the nucleic acid sequence of 5232-10248 of SEQ ID NO:4.

In one aspect, the invention provides a pharmaceutical formulation comprising a composition of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

In another aspect, the invention provides a composition comprising recombinant human NaGlu protein that crosses the blood brain barrier of a mammal having NaGlu deficiency when administered intravenously.

In yet another aspect, the invention provides a method of treating a subject suffering from NaGlu deficiency, the method comprising administering to the subject a therapeutically effective amount of the composition of the invention.

In yet another aspect, the invention provides a method of delivering recombinant human NaGlu protein to the brain of a subject suffering from NaGlu deficiency, the method comprising intravenously administering recombinant human NaGlu protein to the subject.

In another aspect, the invention provides a method of transporting a recombinant human NaGlu protein from the circulation across the blood brain barrier in a

therapeutically effective amount, the method comprising intravenously administering a recombinant human NaGlu protein to a subject having NaGlu deficiency.

In one embodiment, the NaGlu deficiency is Sanfilippo Syndrome B. In another embodiment, the subject is a human.

In another embodiment, the recombinant human NaGlu protein is administered intravenously to the subject at a dosage of about 0.5 to about 50 mg/kg body weight. In another embodiment, the recombinant human NaGlu protein is administered intravenously to the subject at a dosage of about 1 to about 30 mg/kg body weight. In another embodiment, the recombinant human NaGlu protein is administered intravenously to the subject at a dosage of about 6 to about 27 mg/kg body weight.

In yet another embodiment, the recombinant human NaGlu protein is intrathecally administered to the subject. In one embodiment, the recombinant human NaGlu protein is intrathecally administered at a dosage of at least about 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, or 0.9 mg/kg body weight. In another embodiment, the recombinant human NaGlu protein is intrathecally administered at a dosage of about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg/kg body weight. In another embodiment, the recombinant human NaGlu protein is administered intrathecally at a dosage of about 10 to about 30 mg/kg body weight.

In another embodiment, the therapeutically effective amount is an amount effective to reduce heparan sulfate levels in the brain, the kidney, or the liver of the subject. In another embodiment, the therapeutically effective amount is an amount effective to increase NaGlu activity in the brain or the liver of the subject.

In another embodiment, the method further comprises administering a second therapeutic agent. In one embodiment, the second therapeutic is an immunosuppressant.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts the amino acid sequence of human recombinant NaGlu (amino acid residues 1-23, signal peptide).

Fig. 2 depicts the nucleic acid sequence (cDNA) of human recombinant NaGlu, including the nucleic acid sequence encoding the signal peptide.

Fig. 3 depicts the nucleic acid sequence of 1.1kb ovalbumin promoter.

Figs. 4A-D depict the nucleic acid sequence of pSIN-OV-1.1-I-rhNaGlu vector used in transgenesis of an avian.

Fig. 5 is a schematic representation of pSIN-OV-1.1-I-rhNaGlu vector.

Fig. 6 depicts Western analysis of rhNaGlu isolated and purified from egg white of a transgenic *Gallus*.

Fig. 7 depicts the average concentration of rhNaGlu deposited in egg white of transgenic *Gallus*.

Fig. 8 depicts an oligosaccharide profile of rhNaGlu produced from a transgenic *Gallus* using HPAEC-PAD.

Fig. 9 depicts uptake analysis of rhNaGlu by human skin fibroblasts (MPS IIIB, NaGlu deficient; Normal, wild-type human skin fibroblast; 1U of enzymatic activity = nmol of protein/hr).

Fig. 10 depicts uptake inhibition analysis of rhNaGlu (*Gallus*) using various concentrations of M6P monosaccharide (1U of enzymatic activity = 1μmol of protein/min).

Fig. 11 depicts a schematic representation of pTT22 vector containing a recombinant human NaGlu fusion construct (AAA-NaGlu: acidic amino acid residues fused to N-terminus of the full length NaGlu).

Fig. 12 depicts a schematic representation of pTT22 vector containing a recombinant human NaGlu fusion construct (NaGlu-TfRL: transferrin receptor ligand fused to C-terminus of the full length NaGlu).

Fig. 13 depicts enzymatic activity of AAA-NaGlu produced from HEK293 as compared to rhNaGlu produced from *Gallus*.

Fig. 14 depicts enzymatic activity of NaGlu-TfRL produced from HEK293 as compared to AAA-NaGlu produced from HEK293.

Fig. 15 depicts uptake levels of rhNaGlu (*Gallus*) into a macrophage cell line (NR8383) over time (48 hours). Cellular NaGlu activity was measured in units/mg of protein.

Fig. 16 depicts heparan sulfate substrate levels (μg/mg tissue) in the kidney of *naglu* ($^{-/-}$) mice following intravenous administration of vehicle (KO); rhNaGlu *gallus* at

a dosage concentration of 6.25 mg/kg; or rhNaGlu *gallus* at a dosage concentration of 27 mg/kg. Wild type (WT) mice were untreated.

Fig. 17 depicts heparan sulfate substrate levels (µg/mg tissue) in the brain of *naglu* ($^{-/-}$) mice following intravenous administration of vehicle (KO); rhNaGlu *gallus* at a dosage concentration 6.25 mg/kg; or rhNaGlu *gallus* at a dosage concentration of 27 mg/kg. Wild type (WT) mice were untreated.

Fig. 18 depicts heparan sulfate substrate levels (µg/mg tissue) in the liver of *naglu* ($^{-/-}$) mice following intravenous administration of vehicle (KO); rhNaGlu *gallus* at a dosage concentration of 6.25 mg/kg; or rhNaGlu *gallus* at a dosage concentration of 27 mg/kg. Wild type (WT) mice were untreated.

Fig. 19 depicts heparan sulfate substrate levels (µg/mg tissue) in the brain of *naglu* ($^{-/-}$) mice following intrathecal administration of vehicle (KO) or rhNaGlu *gallus* at a dosage concentration of 0.31 mg/kg. Wild type (WT) mice were untreated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions comprising recombinant human NaGlu protein (rhNaGlu) useful for therapy, for example, in the treatment of NaGlu associated diseases, *e.g.,* Sanfilippo Syndrome B. The present invention is based on a discovery that the rhNaGlu protein described herein contains sufficient amount of oligosaccharides (*e.g.,* mannose and phosphorylated mannose (*i.e.,* M6P)) to allow efficient cellular uptake via mannose and/or M6P receptor-mediated endocytosis and be correctly targeted into human cells. Since the rhNaGlu of the invention is more efficiently taken up into a human cell, the rhNaGlu of the invention exhibits increased enzymatic activity as compared to NaGlu proteins produced and secreted from unmodified mammalian cells that are not designed to produce specific glycosylation. Additionally, the rhNaGlu described herein has one or more glycosylation patterns that allow the rhNaGlu to efficiently cross the blood brain barrier (BBB) when administered intravenously. The increased cellular uptake of the rhNaGlu protein of the invention minimizes the need for large and frequent dosing, thereby greatly reducing the potential risk of immunogenicity.

Some of the definitions and abbreviations used herein include the following: aa, amino acid(s); bp, base pair(s); CDS, coding sequence cDNA, DNA complementary to an RNA; GalNac, N-acetylgalactosamine; Gal, galactose; GlcNac, N-acetylglucosamine; nt, nucleotide(s); kb, 1,000 base pairs; µg, microgram; mL, milliliter; ng, nanogram; and nt, nucleotide.

Certain definitions are set forth herein to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

The term "avian" as used herein refers to any species, subspecies or strain of organism of the taxonomic class *ava*, such as, but not limited to, chicken, turkey, duck, goose, quail, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary. The term includes the various known strains of *Gallus gallus*, or chickens, (for example, White Leghorn, Brown Leghorn, Barred-Rock, Sussex, New Hampshire, Rhode Island, Ausstralorp, Minorca, Amrox, California Gray, Italian Partridge-colored), as well as strains of turkeys, pheasants, quails, duck, ostriches and other poultry commonly bred in commercial quantities.

The phrases "based on" and "derived from" typically mean obtained from, in whole or in part. For example, a retroviral vector being based on or derived from a particular retrovirus or based on a nucleotide sequence of a particular retrovirus mean that the genome of the retroviral vector contains a substantial portion of the nucleotide sequence of the genome of the particular retrovirus. The substantial portion can be a particular gene or nucleotide sequence such as the nucleotide sequence encoding the gag, pol and/or env proteins or other structural or functional nucleotide sequence of the virus genome such as sequences encoding the long terminal repeats (LTRs) or can be substantially the complete retrovirus genome, for example, most (*e.g.,* more than 60% or more than 70% or more than 80% or more than 90%) or all of the retrovirus genome, as will be apparent from the context in the specification as the knowledge of one skilled in the art. Examples of retroviral vectors that are based on or derived from a retrovirus are the NL retroviral vectors (*e.g.,* NLB) which are derived from the avian leukosis retrovirus ("ALV") as disclosed in Cosset *et al.*, Journal of Virology (1991) vol. 65, p 3388-3394.

The term "coding sequence" and "coding region" as used herein refer to nucleotide sequences and nucleic acid sequences, including both RNA and DNA, that encode genetic information for the synthesis of an RNA, a protein, or any portion of an RNA or protein.

Nucleotide sequences that are not naturally part of a particular organism's genome or are introduced at a non-native site in the organism's genome are referred to as "foreign" nucleotide sequences, "heterologous" nucleotide sequences, "recombinant" nucleotide sequences or "exogenous" nucleotide sequences. In addition, a nucleotide sequence that has been isolated and then reintroduced into the same type (*e.g.,* same species) of organism is not considered to be a naturally occurring part of a particular organism's genome and is therefore considered exogenous or heterologous. "Heterologous proteins" or "exogenous proteins" can be proteins encoded by foreign, heterologous or exogenous nucleotide sequences and therefore are often not naturally expressed in a cell of the host organism.

As used herein, the terms "exogenous," "heterologous" and "foreign" with reference to nucleic acids, such as DNA and RNA, are used interchangeably and refer to nucleic acid that does not occur naturally as part of a chromosome, a genome or cell in which it is present or which is found in a location(s) and/or in amounts that differ from the location(s) and/or amounts in which it occurs in nature. It can be nucleic acid that is not endogenous to the genome, chromosome or cell and has been exogenously introduced into the genome, chromosome or cell. Examples of heterologous DNA include, but are not limited to, DNA that encodes a gene product or gene product(s) of interest, for example, for production of an encoded protein. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, DNA that encodes therapeutic proteins. The terms "heterologous" and "exogenous" can refer to a biomolecule such as a nucleic acid or a protein which is not normally found in a certain cell, tissue or substance produced by an organism or is not normally found in a certain cell, tissue or substance produced by an organism in an amount or location the same as that found to occur naturally. For example, a protein that is heterologous or exogenous to an egg is a protein that is not normally found in the egg.

The term "construct" as used herein refers to a linear or circular nucleotide sequence such as DNA that has been assembled from more than one segments of nucleotide sequence which have been isolated from a natural source or have been chemically synthesized, or combinations thereof.

The term "complementary" as used herein refers to two nucleic acid molecules that can form specific interactions with one another. In the specific interactions, an adenine base within one strand of a nucleic acid can form two hydrogen bonds with thymine within a second nucleic acid strand when the two nucleic acid strands are in opposing polarities. Also in the specific interactions, a guanine base within one strand of a nucleic acid can form three hydrogen bonds with cytosine within a second nucleic acid strand when the two nucleic acid strands are in opposing polarities. Complementary nucleic acids as referred to herein, can further comprise modified bases wherein a modified adenine may form hydrogen bonds with a thymine or modified thymine, and a modified cytosine may form hydrogen bonds with a guanine or a modified guanine.

The term "expressed" or "expression" as used herein refers to the transcription of a coding sequence to yield an RNA molecule at least complementary in part to a region of one of the two nucleic acid strands of the coding sequence. The term "expressed" or "expression" as used herein can also refer to the translation of an mRNA to produce a protein or peptide.

The term "expression vector" as used herein refers to a nucleic acid vector that comprises a gene expression controlling region, such as a promoter or promoter component, operably linked to a nucleotide sequence encoding at least one polypeptide.

The term "fragment" as used herein can refer to, for example, an at least about 10, 20, 50, 75, 100, 150, 200, 250, 300, 500, 1000, 2000, 5000, 6,000, 8,000, 10,000, 20,000, 30,000, 40,000, 50,000 or 60,000 nucleotide long portion of a nucleic acid that has been constructed artificially (*e.g.,* by chemical synthesis) or by cleaving a natural product into multiple pieces, using restriction endonucleases or mechanical shearing, or enzymatically, for example, by PCR or any other polymerizing technique known in the art, or expressed in a host cell by recombinant nucleic acid technology known to one of skill in the art. The term "fragment" as used herein can also refer to, for example, an at

least about 5, 10, 15, 20, 25, 30, 40, or 50 amino acid residues less than a full length amino acid sequence for NaGlu (*i.e.,* amino acid sequence 24-743 of SEQ ID NO:1), which portion is cleaved from a naturally occurring amino acid sequence by proteolytic cleavage by at least one protease, or is a portion of the naturally occurring amino acid sequence synthesized by chemical methods or using recombinant DNA technology (*e.g.,* expressed from a portion of the nucleotide sequence encoding the naturally occurring amino acid sequence) known to one of skill in the art. "Fragment" may also refer to a portion, for example, of about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or about 99% of a particular nucleotide sequence or amino acid sequence.

"Functional portion" and "functional fragment" can be used interchangeably and as used herein mean a portion or fragment of a whole capable of performing, in whole or in part, a function of the whole. For example, a biologically functional portion of a molecule means a portion of the molecule that performs a biological function of the whole or intact molecule. Functional portions may be of any useful size. For example, a functional fragment may range in size from about 20 bases in length to a length equal to the entire length of the specified sequence minus one nucleotide. In another example, a functional fragment may range in size from about 50 bases in length to a length equal to the entire length of the specified sequence minus one nucleotide. In another example, a functional fragment may range in size from about 50 bases in length to about 20 kb in length. In another example, a functional fragment may range in size from about 500 bases in length to about 20 kb in length. In another example, a functional fragment may range in size from about 1 kb in length to about 20 kb in length. In another example, a functional fragment may range in size from about 0.1 kb in length to about 10 kb in length. In another example, a functional fragment may range in size from about 20 bases kb in length to about 10 kb in length.

The term "fully transgenic" or "germline transgenic" refers to an animal such as an avian that contains at least one copy of a transgene in essentially all of its cells.

The term "gene expression controlling region" as used herein refers to nucleotide sequences that are associated with a coding sequence and which regulate, in whole or in part, expression of the coding sequence, for example, regulate, in whole or in part, the transcription of the coding sequence. Gene expression controlling regions may be

isolated from a naturally occurring source or may be chemically synthesized and can be incorporated into a nucleic acid vector to enable regulated transcription in appropriate cells. The "gene expression controlling regions" may precede, but is not limited to preceding, the region of a nucleic acid sequence that is in the region 5' of the end of a coding sequence that may be transcribed into mRNA.

As used herein, "host cells" refers to cells that harbor vectors constructed using recombinant DNA techniques and encoding at least one heterologous gene.

The term "isolated nucleic acid" as used herein covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic molecule but is not flanked by at least one of the sequences that flank that part of the molecule in the genome of the species in which it naturally occurs; (b) a nucleic acid which has been incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting vector or genomic DNA is not identical to naturally occurring DNA from which the nucleic acid was obtained; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), ligase chain reaction (LCR) or chemical synthesis, or a restriction fragment; (d) a recombinant nucleotide sequence that is part of a hybrid gene, *i.e.,* a gene encoding a fusion protein, and (e) a recombinant nucleotide sequence that is part of a hybrid sequence that is not naturally occurring. Isolated nucleic acid molecules of the present invention can include, for example, natural allelic variants as well as nucleic acid molecules modified by nucleotide deletions, insertions, inversions, or substitutions.

The term "nucleic acid" as used herein refers to any linear or sequential array of nucleotides and nucleosides, for example cDNA, genomic DNA, mRNA, tRNA, oligonucleotides, oligonucleosides and derivatives thereof. For ease of discussion, non-naturally occurring nucleic acids may be referred to herein as constructs. Nucleic acids can include bacterial plasmid vectors including expression, cloning, cosmid and transformation vectors such as, animal viral vectors such as, but not limited to, modified adenovirus, influenza virus, polio virus, pox virus, retroviruses such as avian leukosis virus (ALV) retroviral vector, a murine leukemia virus (MLV) retroviral vector, and a lentivirus vector, and the like and fragments thereof. In addition, the nucleic acid can be an LTR of an avian leukosis virus (ALV) retroviral vector, a murine leukemia virus

(MLV) retroviral vector, or a lentivirus vector and fragments thereof. Nucleic acids can also include NL vectors such as NLB, NLD and NLA and fragments thereof and synthetic oligonucleotides such as chemically synthesized DNA or RNA. Nucleic acids can include modified or derivatized nucleotides and nucleosides such as, but not limited to, halogenated nucleotides such as, but not only, 5-bromouracil, and derivatized nucleotides such as biotin-labeled nucleotides.

As used herein, the terms "glycan," "glycan structure," "glycan moiety," "oligosaccharide," "oligosaccharide structure," "glycosylation pattern," "glycosylation profile," and "glycosylation structure" have essentially the same meaning and each refers to one or more structures which are formed from sugar residues and are attached to glycosylated protein such as human NaGlu. For example, "N-glycan" or "N-linked glycan" refers to a glycan structure attached to a nitrogen of asparagine or arginine side-chain of the glycosylated protein. "O-glycan" or "O-linked glycan" refers to a glycan structure attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side chain of the glycosylate protein.

The term "vector" and "nucleic acid vector" as used herein refers to a natural or synthetic single or double stranded plasmid or viral nucleic acid molecule that can be transfected or transformed into cells and replicate independently of, or within, the host cell genome. A circular double stranded vector can be linearized by treatment with an appropriate restriction enzyme based on the nucleotide sequence of the vector. A nucleic acid can be inserted into a vector by cutting the vector with restriction enzymes and ligating the desired pieces together, as is understood in the art. A typical vector can be comprised of the following elements operatively linked at appropriate distances for allowing functional gene expression: replication origin, promoter, enhancer, 5' mRNA leader sequence, ribosomal binding site, nucleic acid cassette, termination and polyadenylation sites, and selectable marker sequences. One or more of these elements can be omitted in specific applications. The nucleic acid cassette can include a restriction site for insertion of the nucleic acid sequence to be expressed. In a functional vector the nucleic acid cassette contains the nucleic acid sequence to be expressed including translation initiation and termination sites. An intron optionally can be included in the construct, for example, 5' to the coding sequence. A vector is

constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control or regulatory sequences. Modification of the sequences encoding the particular protein of interest can be desirable to achieve this end. For example, in some cases it can be necessary to modify the sequence so that it can be attached to the control sequences with the appropriate orientation, or to maintain the reading frame. The control sequences and other regulatory sequences can be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

The term "operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Gene expression controlling regions or promoter(s) (*e.g.,* promoter components) operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The controlling sequence(s) or promoter need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Overexpression", as used herein, refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

The term "oviduct" or "oviduct tissue" refers to a tissue of an avian oviduct, such as the magnum, *e.g.,* tubular gland cells, where proteins are produced with N-linked oligosaccharides that contain increased amounts of mannose and mammose-6-phosphate (M6P) and substantially reduced amounts of galactose and/or sialic acid relative to that of proteins produced in other tissue of the avian such as liver or kidney tissue.

The term "oviduct-specific promoter" as used herein refers to promoters and promoter components which are functional, *i.e.,* provide for transcription of a coding

sequence, to a large extent, for example, primarily (*i.e.,* more than 50% of the transcription product produced in the animal by a particular promoter type being produced in oviduct cells) or exclusively in oviduct cells of a bird. Examples of oviduct specific promoters include, but are not limited to, ovalbumin promoter, ovomucoid promoter, ovoinhibitor promoter, lysozyme promoter and ovotransferrin promoter and functional portions of these promoters, *e.g.,* promoter components. By limiting the expression of NaGlu protein to the magnum using oviduct specific promoters, deleterious physiological effects to the bird as result of expression of these enzymes in other tissues of the bird can be minimized.

The terms "percent sequence identity," "percent identity," "% identity," "percent sequence homology," "percent homology," "% homology" and "percent sequence similarity" can each refer to the degree of sequence matching between two nucleic acid sequences or two amino acid sequences. Such sequence matching can be determined using the algorithm of Karlin & Altschul (1990) Proc. Natl. Acad. Sci. 87: 2264-2268, modified as in Karlin & Altschul (1993) Proc. Natl. Acad. Sci. 90: 5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (1990) T. Mol. Biol. Q15: 403-410. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecule of the invention. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a reference amino acid sequence. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (1997) Nucl. Acids Res. 25: 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.,* XBLAST and NBLAST) are used. Other algorithms, programs and default settings may also be suitable such as, but not only, the GCG-Sequence Analysis Package of the U.K. Human Genome Mapping Project Resource Centre that includes programs for nucleotide or amino acid sequence comparisons. A sequence may be at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to another sequence, *e.g.,* the NaGlu protein sequence identified herein.

The term "avian derived" refers to a composition or substance produced by or obtained from a bird, poultry or avian. "Avian" refers to birds that can be kept as livestock, including but not limited to, chickens, duck, turkey, quail and ratites. For example, "avian derived" can refer to chicken derived, turkey derived and/or quail derived.

The terms "polynucleotide," "oligonucleotide", "nucleotide sequence" and "nucleic acid sequence" can be used interchangeably herein and include, but are not limited to, coding sequences, *i.e.,* polynucleotide(s) or nucleic acid sequence(s) which are transcribed and translated into polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory or control sequences; controlling sequences, *e.g.,* translational start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription factor binding sites, transcription termination sequences, upstream and downstream regulatory domains, enhancers, silencers, DNA sequences to which a transcription factor(s) binds and alters the activity of a gene's promoter either positively (induction) or negatively (repression) and the like. No limitations as to length or to synthetic origin are suggested by the terms described herein.

As used herein the terms "polypeptide" and "protein" refer to a polymer of amino acids, for example, three or more amino acids, in a serial array, linked through peptide bonds. The term "polypeptide" includes proteins, protein fragments, protein analogues, oligopeptides and the like. The term "polypeptides" includes polypeptides as defined above that are encoded by nucleic acids, produced through recombinant technology (*e.g.,* isolated from a transgenic bird), or synthesized. The term "polypeptides' further contemplates polypeptides as defined above that include chemically modified amino acids or amino acids covalently or noncovalently linked to labeling ligands.

The term "promoter" as used herein refers to a DNA sequence useful to initiate transcription by an RNA polymerase in an avian cell. A "promoter component" is a DNA sequence that can, by itself or in combination with other DNA sequences, effect or facilitate transcription. Promoter components can be functional fragments of promoters.

The terms "recombinant nucleic acid" and "recombinant DNA" as used herein refer to combinations of at least two nucleic acid sequences that are not naturally found in a eukaryotic or prokaryotic cell. The nucleic acid sequences may include, but are not limited to, nucleic acid vectors, gene expression regulatory elements, origins of replication, suitable gene sequences that when expressed confer antibiotic resistance, protein-encoding sequences and the like. The term "recombinant polypeptide" is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location, purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

As used herein, the term "regulatory" sequences or elements include promoters, enhancers, terminators, stop codons, and other elements that can control gene expression.

A "retrovirus", "retroviral particle," "transducing particle," or "transduction particle" refers to a replication-defective or replication-competent virus capable of transducing non-viral DNA or RNA into a cell.

A "SIN vector" refers to a self-inactivating vector. In particular, a SIN vector is a retroviral vector having an altered genome such that upon integration into genomic DNA of the target cell (*e.g.,* avian embryo cells), the 5' LTR of the integrated retroviral vector will not function as a promoter. For example, a portion or all of the nucleotide sequence of the retroviral vector that results in the U3 region of the 5' LTR of the retroviral vector once integrated can be deleted or altered in order to reduce or eliminate promoter activity of the 5' LTR. In certain examples, deletion of the CAAT box and/or the TAATA box from U3 of the 5' LTR can result in a SIN vector, as is understood in the art.

The term "sense strand" as used herein refers to a single stranded DNA molecule from a genomic DNA that can be transcribed into RNA and translated into the natural polypeptide product of the gene. The term "antisense strand" as used herein refers to the single strand DNA molecule of a genomic DNA that is complementary with the sense strand of the gene.

A "therapeutic protein" or "pharmaceutical protein" is a substance that, in whole or in part, makes up a drug. In particular, "therapeutic proteins" and "pharmaceutical proteins" include an amino acid sequence which in whole or in part makes up a drug.

The terms "promoter," "transcription regulatory sequence" and "promoter component" as used herein refer to nucleotide which regulates the transcriptional expression of a coding sequence. Exemplary transcription regulatory sequences include enhancer elements, hormone response elements, steroid response elements, negative regulatory elements, and the like. The "transcription regulatory sequence" can be isolated and incorporated into a vector to enable regulated transcription in appropriate cells of portions of the vector DNA. The "transcription regulatory sequence" can precede, but is not limited to, the region of a nucleic acid sequence that is in the region 5' of the end of a protein coding sequence that is transcribed into mRNA. Transcriptional regulatory sequence can also be located within a protein coding region, for example, in regions of a gene that are identified as "intron" regions.

The terms "transformation" and "transfection" as used herein refer to the process of inserting a nucleic acid into a host. Many techniques are well known to those skilled in the art to facilitate transformation or transfection of a nucleic acid into a prokaryotic or eukaryotic organism. These methods involve a variety of techniques, such as treating the cells with certain concentrations of salt, for example, but without limitation, a calcium or magnesium salt, or exposing the cells to an electric field, detergent, or liposome material, to render the host cell competent for the uptake of the nucleic acid molecules.

As used herein, a "transgenic animal" is any non-human animal, such as an avian species, including the chicken, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques known in the art (see, for example, U.S. patent publication No. 2007/0243165, published October 18, 2007, the disclosure of which is incorporated in its entirety herein by reference) including those disclosed herein. The nucleic acid is introduced into an animal, directly or indirectly by introduction into a cell (*e.g.*, egg or embryo cell) by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include

classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule can be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animal, the transgene can cause cells to express a recombinant form of the target protein or polypeptide. The terms "chimeric animal" or "mosaic animal" are used herein to refer to animals in which a transgene is found, or in which the recombinant nucleotide sequence is expressed, in some but not all cells of the animal. A germ-line chimeric animal contains a transgene in its germ cells and can give rise to an offspring transgenic animal in which most or all cells of the offspring will contain the transgene.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, for example, a human NaGlu protein) that is partly or entirely heterologous, *i.e.,* foreign, to the animal or cell into which it is introduced, or, is partly or entirely homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal or cell genome in such a way as to alter the genome of the organism into which it is inserted (*e.g.,* it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout).

As used herein, the term "enzyme replacement therapy (ERT)" refers to a therapeutic strategy for correcting an enzyme deficiency in a subject by administering the missing enzyme to a subject. For lysosomal enzyme replacement therapy to be effective, the therapeutic enzyme must be delivered to lysosomes in the appropriate cells in tissues where the storage defect is manifested. In one embodiment, the enzyme may be administered to the subject intravenously, intrathecally, intracerebrally, intraventricularly, or intraparenchymaly. In one embodiment, the enzyme is able to cross the blood brain barrier (BBB). Without intending to be limited by mechanism, it is believed that as the blood perfuses patient tissues, enzyme is taken up by cells and transported to the lysosome, where the enzyme acts to eliminate material that has accumulated in the lysosomes due to the enzyme deficiency.

I. Composition of NaGlu

The present invention provides novel compositions of recombinant human NaGlu (rhNaGlu or NaGlu) (amino acid sequence 24-743 set forth in SEQ ID NO:1) having patterns of glycosylation that confer an increased cellular uptake and an increased subcellular activity which are particularly useful for therapy, for example, in the treatment of Sanfilippo Syndrome B (mucopolysaccharidosis (MPS) IIIB).

In some aspects, the composition can be an isolated mixture of rhNaGlu comprising the amino acid sequence 24-743 of SEQ ID NO:1. In one embodiment, the mixture contains a sufficient amount of rhNaGlu having at least one glycan structure that contains phosphorylated mannose (*e.g.,* M6P) or mannose such that the rhNaGlu containing M6P or mannose is internalized into a human cell deficient in NaGlu and restores at least 50 % of NaGlu activity observed in a wild-type human cell of the same type that actively expresses endogenous NaGlu. In one aspect, at least 10 %, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93% 94%, 95%, 96%, 97%, 98% or 99% of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose. In one embodiment, at least 10 % of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose. In one embodiment, at least 20 % of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose. In one embodiment, at least 30 % of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose. In one embodiment, at least 30 % of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose. In one embodiment, at least 40 % of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose. In one embodiment, at least 50 % of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose. In one embodiment, at least 60 % of rhNaGlu in the mixture contains at least one glycan structure having phosphorylated mannose and/or mannose.

In some aspects, the NaGlu contains one or more N-linked glycan structure. The NaGlu contains at least one phosphorylated mannose (*e.g.,* M6P or bis-M6P) which allows the protein to be recognized by the Mannose 6-phosphate receptor (M6P

receptor), and subsequently taken up into a human cell, including but not limited to, a skin fibroblast, an endothelial, a neuronal cell, a hepatocyte, a macrophage or any cell that expresses M6P receptor on the cell surface via M6P receptor-mediated endocytosis. In one embodiment, the NaGlu contains at least one mannose (Man). In another embodiment, the NaGlu contains at least one N-acetylglucosamine (GlcNAc).

In some aspects, the NaGlu contains a glycan structure comprising a phosphorylated mannose (M6P). As used herein, M6P can encompass any phosphorylated mannose residue and includes mono- and bis-phosphorylated mannose. In one embodiment, the M6P is present at a concentration that is about 1, about 2, about 3, about 4, about 5 or about 6 mole(s) per mole of protein. In one embodiment, the NaGlu contains M6P at a concentration that is about 2, about 3, about 4, or about 5 moles per mole of protein. In one embodiment, the NaGlu contains M6P at a concentration that is about 2 moles per mole of protein. In one embodiment, the NaGlu contains M6P at a concentration that is about 3 moles per mole of protein. In one embodiment, the NaGlu contains M6P at a concentration that is about 4 moles per mole of protein. In one embodiment, the NaGlu contains M6P at a concentration that is about 5 moles per mole of protein. In one embodiment, the NaGlu contains M6P at a concentration that is about 6 moles per mole of protein.

In some aspects, the rhNaGlu contains a sufficient amount of M6P for cellular uptake into a human cell having a M6P receptor on the cell surface via M6P receptor-mediated endocytosis. In one embodiment, a sufficient amount of M6P for uptake into a human cell is about 1, 2, 3, 4, 5 or 6 moles per mole of protein. The rhNaGlu can be internalized into a human cell deficient in NaGlu such that the internalized protein fully (100% or more) restores a normal level of NaGlu activity in the human cell deficient in NaGlu. In one embodiment, the internalized rhNaGlu protein fully restores a normal level of NaGlu activity in the human cell at a concentration that is at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1.0 µg/mL. In one embodiment, the internalized protein fully restores a normal level of NaGlu activity in the human cell deficient in NaGlu at a concentration that is at least 2, 3, 4, 5, 6, 7, 8, 9 or 10 µg/mL. In one embodiment, the internalized protein fully restores a normal level of NaGlu activity in the human cell at a concentration that is at least 20, 30, 40, 50, 60, 70, 80, 90 or 100 µg/mL. As used

herein, the normal level of NaGlu activity is a level of NaGlu activity measured in a wild-type human cell of the same type that actively expresses a normal NaGlu enzyme.

In some aspects, the rhNaGlu can be internalized into a human cell deficient in NaGlu such that the protein restores at least about 50%, about 60%, about 70%, about 80%, about 90% or about 95% of NaGlu activity of a normal human cell of the same type. In some embodiments, the rhNaGlu can be internalized into a human cell deficient in NaGlu such that the internalized rhNaGlu provides a higher enzymatic activity than that observed in a normal human cell of the same type. In one embodiment, the rhNaGlu is internalized into a human cell deficient in NaGlu such that the internalized rhNaGlu provides about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9 and about 10-fold higher activity than that observed in a normal human cell of the same type. In one embodiment, the rhNaGlu is internalized into a human cell deficient in NaGlu such that the internalized rhNaGlu provides about 15, about 20, about 25, about 30, about 40, about 50, about 60, about 70, about 80, about 90 or about 100-fold higher activity than that observed in a normal human cell.

In one embodiment, the human cell deficient in NaGlu is any human cell deficient in NaGlu that expresses one or more M6P receptors on the cell surface. In one embodiment, the human cell deficient in NaGlu is a human mucopolysaccharidosis (MPS) IIIB fibroblast that accumulates heparan sulfate. In one embodiment, the human cell deficient in NaGlu is a hepatocyte. In one embodiment, the human cell deficient in NaGlu is a neuronal cell. In one embodiment, the human cell deficient in NaGlu is an endothelial cell. In one embodiment, the human cell deficient in NaGlu is a macrophage.

In some aspects, uptake of rhNaGlu into a human cell is inhibited by the presence of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9 or about 10 mM of competing M6P monosaccharide. In some aspects, the cellular uptake of rhNaGlu is inhibited by the presence of about 0.1, about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 0.8, about 0.9 or about 1.0 mM of M6P monosaccharide. In one embodiment, the cellular uptake of rhNaGlu is inhibited by the presence of about 0.01, about 0.02, about 0.03, about 0.04, about 0.05, about 0.06, about 0.07, about 0.08, or about 0.09 mM of M6P monosaccharide.

In some aspects, the rhNaGlu contains mannose in its glycan structures at a concentration that is about 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 moles per mole of protein. In one embodiment, the rhNaGlu has mannose at a concentration that is about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 moles per mole of protein. The rhNaGlu contains mannose at a concentration that is about 22, 23, 24, 25, 26, 27 or 28 moles per mole of protein. The rhNaGlu contains mannose at a concentration that is about 24 moles per mole of protein. The rhNaGlu protein contains mannose at a concentration that is about 25 moles per mole of protein. The rhNaGlu contains mannose at a concentration that is about 26 moles per mole of protein. The rhNaGlu contains mannose at a concentration that is about 27 moles per mole of protein. In one embodiment, the rhNaGlu has mannose at a concentration that is between about 20 and about 30 moles per mole of protein.

In some aspects, the rhNaGlu comprises N-acetylglucosamine (GlcNAc). In one embodiment, the rhNaGlu contains GlcNAc at a concentration that is between about 28 and about 42 moles per mole of protein. In one embodiment, the NaGlu protein has GlcNAc at a concentration that is between about 30 and about 40 moles per mole of protein. In one embodiment, the NaGlu protein comprises GlcNAc at a concentration that is between about 32 and about 38 moles per mole of protein. In one embodiment, the NaGlu protein comprises GlcNAc at a concentration that is between about 34 and about 36 moles per mole of protein. In one embodiment, the NaGlu protein has GlcNAc at a concentration that is about 35 moles per mole of protein. In one embodiment, the rhNaGlu protein contains GlcNAc at a concentration that is about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 moles per mole of protein.

In some aspects, the rhNaGlu contains N-acetylgalactosamine (GalNAc) and/or galactose (Gal). The presence of the GalNAc and Gal typically indicates that the NaGlu may contain one or more O-linked glycan structures which are added to the protein in the Golgi compartment. Accordingly, the present invention optionally includes a composition comprising a recombinant human NaGlu that contains one or more O-linked glycan structure.

In one embodiment, the rhNaGlu contains galactose at a concentration that is about 1, 2, 3, 4, 5, 6 or 7 moles per mole of protein. In one embodiment, the rhNaGlu

has galactose at a concentration that is about 2, 3, 4, 5 or 6 moles per mole of protein. In one embodiment, the rhNaGlu has galactose at a concentration that is about 3 moles per mole of protein. In one embodiment, the rhNaGlu has galactose at a concentration that is about 4 moles per mole of protein.

In one embodiment, the NaGlu comprises at least one GalNAc molecule per mole of protein. In one embodiment, the NaGlu comprises GalNAc at a concentration that is about 1 or 2 moles per mole of protein.

In one embodiment, the NaGlu contains no fucose. In yet another embodiment, the NaGlu contains no glucose. In yet another embodiment, rhNaGlu contains neither fucose nor glucose.

The present invention also contemplates compositions of modified rhNaGlu proteins produced from modified nucleic sequences of rhNaGlu. The modified nucleic acid sequences include deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that encodes a functionally equivalent polynucleotide or polypeptide. The encoded protein may also contain deletions, insertions, or substitutions of amino acid residues that produce a silent change and result in a functionally equivalent protein or polypeptide. Deliberate amino acid substitutions can be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological activity of the NaGlu is retained. For example, negatively charged amino acids can include aspartic acid and glutamic acid; positively charged amino acids can include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values can include leucine, isoleucine, and valine; glycine and alanine; asparagine and glutamine; serine and threonine; phenylalanine and tyrosine.

In other aspects, the rhNaGlu can be modified such that it contains an additional moiety or second peptide. Although unmodified NaGlu protein may cross the blood brain barrier at a high serum concentration, modifications of the protein can be performed to increase the efficiency of central nervous system (CNS) targeting. In one embodiment, transferrin receptor ligand (TfRL) can be attached to human NaGlu at N- or C-terminus of NaGlu protein. A non-limiting example of TrRL is THRPPMWSPVWP (SEQ ID NO:5). In one embodiment, the transferrin receptor

ligand can be attached to human NaGlu C-terminus of the NaGlu protein. In another embodiment, human NaGlu is fused to insulin-like growth factor receptor (IGF2R) ligand at N- or C-terminus of the NaGlu protein. In yet another embodiment, the NaGlu protein is fused to low density lipoprotein (LDL) receptor ligand at N- or C-terminus of the NaGlu protein. In one embodiment, the NaGlu protein is fused to a stretch of five to ten consecutive acidic amino acid residues. The acidic amino acid residues can include aspartic acid (D) or glutamic acid (E).

In one embodiment, the rhNaGlu is produced in a transgenic avian that contains a transgene encoding the NaGlu protein. In one embodiment, the rhNaGlu is produced in an oviduct cell (*e.g.,* a tubular gland cell) of a transgenic avian (*e.g.,* chicken (*Gallus*)). In one embodiment, the rhNaGlu is glycosylated in the oviduct cell (*e.g.,* tubular gland cell) of the transgenic avian. In one embodiment, the rhNaGlu has a glycosylation pattern resulting from the rhNaGlu being produced in an oviduct cell of a transgenic avian. In one embodiment, the rhNaGlu can be isolated and purified from the content of the hard shell eggs laid by the transgenic avian. In one embodiment, the rhNaGlu can be isolated and purified from egg white of the transgenic avian.

The present invention also includes compositions of an isolated mixture of NaGlu proteins, such as a mixture of one or more fragments and full-length rhNaGlu (*e.g.,* 24-743 set forth in SEQ ID NO:1). In one embodiment, a substantial portion of the mixture contains phosphorylated M6P. In one embodiment, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90% 95%, 97%, 98% or 99% of the rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 50% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 60% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 70% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 80% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 90% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 95% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 96% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 97% of the isolated rhNaGlu in the mixture contains M6P. In yet another embodiment, at least 98% of the isolated rhNaGlu in the mixture

contains M6P. In yet another embodiment, at least 99% of the isolated rhNaGlu in the mixture contains M6P.

Optionally, the rhNaGlu protein produced from an avian or mammalian expression system (*e.g.,* CHO, HEK293, or human skin fibroblast cell-line) can be further modified to achieve a favorable glycosylation pattern (*i.e.,* an increased amount of M6P) for cellular uptake while retaining the biological activity. Additional terminal M6P can be introduced to the rhNaGlu by the general methods applied to other hydrolases as described in U.S. Pat. No. 6,679,165, U.S. Pat. No. 7,138,262, or U.S. Publication No. 2009/0022702, the entire teachings of each of which are incorporated herein by reference. For example, a highly phosphorylated mannopyranosyl oligosaccharide compound can be derivatized with a chemical compound containing a carbonyl-reactive group, followed by oxidizing the rhNaGlu protein to generate carbonyl (aldehyde) group on one glycan structure of the protein, and reacting the oxidized NaGlu protein with the glycan with the derivatized highly phosphorylated mannopyranosyl oligosaccharide compound to form a new compound having hydrazine bond.

II. Vectors

Methods which are well-known to those skilled in the art can be used to construct expression vectors containing sequences encoding NaGlu and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook, J. *et al.* (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., and Ausubel, F. M. *et al.* (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., the entire teachings of which are incorporated herein by reference.

A variety of expression vector/host systems can be utilized to express nucleic acid sequences encoding rhNaGlu. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.,* baculovirus) or with bacterial expression

vectors (*e.g.,* Ti or pBR322 plasmids); or mammalian cell culture systems (*e.g.,* pTT22 vector). Non-limiting examples of the pTT22 vector containing human NaGlu cDNA fused to a nucleic acid sequence encoding acidic amino acid residue and TfRL are shown in Figs. 11 and 12.

Polynucleotide and nucleic acid coding regions of the present invention may be associated with additional coding regions which encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide of the present invention. According to the signal hypothesis, proteins secreted by vertebrate (*e.g.,* avian or mammalian) cells have a signal peptide or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum (ER) has been initiated. Those of ordinary skill in the art are aware that polypeptides produced in the ER by vertebrate cells generally have a signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the complete or "full length" polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, *e.g.,* the MEAVAVAAAVGVLLLAGAGGAAG (1-23 of SEQ ID NO:1) signal peptide of human NaGlu is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous signal peptide (*e.g.,* a heterologous mammalian or avian signal peptide), or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of, for example, human tissue plasminogen activator (tPA) or mouse ß-glucuronidase.

The control elements or regulatory sequences can includes those non-translated regions of the vector-enhancers, promoters, 5' and 3' untranslated regions that interact with host cellular proteins to carry out transcription and translation. Such elements can vary in their strength and specificity. Depending on the vector system and host cell utilized, any number of suitable transcription and translation elements can be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid *lac*-Z promoter of the Bluescript™ phagemid (Stratagene, LaJolla, California) or pSport1™ plasmid (Gibco BRL) and the like can be used. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferred. If it is necessary to

generate a cell line that contains multiple copies of the sequence encoding NaGlu, vectors based on SV40 or EBV can be also used with an appropriate selectable marker such as puromycin and ampicillin (see, *e.g.,* Figs. 11 and 12).

When the rhNaGlu is produced in a transgenic avian, the present invention contemplates that the rhNaGlu sequence be placed downstream of a promoter such that the sequence encoding the rhNaGlu can be expressed in a tissue-specific manner in a transgenic avian. For example, the promoter can be an oviduct-specific promoter that is largely, but not entirely, specific to the magnum, such as the oviduct-specific promoter, including but not limited to, ovalbumin, lysozyme, conalbumin, ovomucoid, ovomucoid, ovomucin and ovotransferrin promoters. In one embodiment, the promoter is an ovalbumin promoter, a lysozyme promoter, a conalbumin promoter, an ovomucoid promoter, an ovomucin promoter and/or an ovotransferrin promoter or any functional portion thereof.

Alternatively, a constitutive promoter can be used to express the coding sequence of human NaGlu in an avian. In this case, expression is not limited to the magnum; expression also occurs in other tissues within the avian (*e.g.,* blood). The use of such a transgene, which includes a constitutive promoter and the coding sequence of NaGlu, is also suitable for effecting or driving the expression of a protein in the oviduct and the subsequent secretion of the protein into the egg. In one embodiment, the constitutive promoter can be, for example, a cytomegalovirus (CMV) promoter, a rous-sarcoma virus (RSV) promoter, a murine leukemia virus (MLV) promoter, and β-actin promoter. In one embodiment, the promoter is a CMV promoter, a MDOT promoter, a RSV promoter, a MLV promoter, or a mouse mammary tumor virus (MMTV) promoter of any functional portion thereof.

The invention also contemplates any useful fragment or component of the promoters described herein. The promoter can be at least one segment, fragment or component of a promoter region, such as a segment of the ovalbumin, lysozyme, conalbumin, ovomucoid, ovomucin, ovotransferrin, CMV, RSV or MLV promoter region. In a preferred embodiment, the promoter is a segment of the oviduct-specific promoter region which contains essential elements to direct expression of the coding sequence in the tubular gland cells. For example, included in the scope of the present

invention is a segment, portion or fragment of an oviduct-specific promoter and/or condensing the critical regulatory elements of the oviduct-specific promoter so that it retains sequences required for expression in the tubular gland cells of the magnum of the oviduct. In one embodiment, a segment of the ovalbumin promoter region is used. This segment comprises the 5'-flanking region of the ovalbumin gene.

A vector that contains a coding sequence for human NaGlu can be used for transfecting blastodermal cells of an avian or mammalian cell to generate stable integrations into the avian or mammalian genome and to create a germline transgenic avian or mammalian cell line. A non-limiting example of such vector is shown in Figs. 4A-D and 5. In the avian expression system, the human NaGlu coding sequence is operably linked to a promoter in a positional relationship to express the coding sequence in a transgenic avian, particularly in the tubular gland cell of the magnum of the avian oviduct, such that the recombinant human NaGlu protein is expressed and deposited in egg white of a hard shell egg laid by the transgenic avian. Additional suitable vectors and methods to making vectors for expressing rhNaGlu in an avian system are also disclosed in U.S. Pat. No. 6,730,822; U.S. Pat. No. 6,825,396; U.S. Pat. No. 6,875,588; U.S. Pat. No. 7,294,507; U.S. Pat. No. 7,521,591; U.S. Pat. No. 7,534,929; U.S. Publication No. 2008/0064862A1; and U.S. Patent Publication No. 2006/0185024, the entire teachings of which are incorporated herein by reference. Non-limiting examples of other promoters which can be also useful in the present invention include Pol III promoters (for example, type 1, type 2 and type 3 Pol III promoters) such as H1 promoters, U6 promoters, tRNA promoters, RNase MPR promoters and functional portions of each of these promoters. Typically, functional terminator sequences are selected for use in the present invention in accordance with the promoter that is employed.

In one embodiment, the vector is a retroviral vector, in which the coding sequence and the promoter are both positioned between the 5' and 3' LTRs of the retroviral vector. In one useful embodiment, the LTRs or retroviral vector is derived from an avian leukosis virus (ALV), a murine leukemia virus (MLV) or a lentivirus. One useful retrovirus for randomly introducing a transgene into the avian genome is the

replication-deficient ALV, the replication-deficient MLV, or the replication-deficient lentivirus.

The present invention also contemplates the use of self-inactivating (SIN) vectors. SIN vectors can be useful for increasing the quantity of human NaGlu produced in the oviduct of a transgenic avian. This effect can be further enhanced when the SIN vector does not contain any selectable marker cassette with a functional promoter (SIN/SC negative vector). In one embodiment, a SIN vector is a retroviral vector having altered genome so that the 5' LTR of the integrated retroviral vector does not function as a promoter. In one particular embodiment, a portion or all of the nucleotide sequence of the retroviral vector that results in the U3 region of the 5' LTR of the retroviral vector once integrated can be deleted or altered in order to reduce or eliminate promoter activity of the 5' LTR. A non-limiting example of SIN vector which contains an ovalbumin promoter region fused to the coding sequence of human rhNaGlu is shown in Figs. 4A-D and 5. Functional components of the vector are also tabulated in Table 1.

Table 1. Functional components in pSIN-OV-1.1kb-I-rhNaGlu

Functional components	Nucleotide Sequence in SEQ ID NO:4
poly A site	634-639
Partial gag	692-945
LTR (RAV2)	1243-1588
Partial LTR (RAV2)	4691-4863
ALV CTE	4899-4986
1.1 kb Ovalbumin promoter	5232-6363
DHS II	5334-5714
DHS I	6064-6364
Exon L	6364-6410
Intron 1	6411-7999
NaGlu	8017-10248

Any of the vectors described herein can include a sequence encoding a signal peptide that directs secretion of the protein expressed by the vector's coding sequence from, for example, the tubular gland cells of the oviduct of an avian. Where a recombinant human NaGlu protein would not otherwise be secreted, the vector containing the coding sequence is modified to comprise a DNA sequence comprising

about 60 bp encoding a signal peptide from, for example, the lysozyme gene. The DNA sequence encoding the signal peptide is inserted in the vector such that it is located at the N-terminus of the rhNaGlu protein encoded by the DNA.

Further, the coding sequences of vectors used in any of the methods of the present invention can be provided with a 3' untranslated region (3' UTR) to confer stability to the RNA produced. When a 3' UTR is added to a retroviral vector, the orientation of the promoter, the coding sequence and the 3' UTR is preferably reversed with respect to the direction of the 3' UTR, so that the addition of the 3' UTR does not interfere with transcription of the full-length genomic RNA. In one embodiment, the 3' UTR may be that of the ovalbumin gene, lysozyme gene or any 3' UTR that is functional in a magnum cell, *i.e.,* the SV40 late region.

III. Transgenic Avians

Transgenes described herein can be introduced into avian embryonic blastodermal cells to produce a transgenic chicken, transgenic turkey, transgenic quail and other avian species that carry the transgene encoding recombinant human NaGlu in the genome of its germ-line tissue. In one aspect of the invention, a transgenic avian that produces rhNaGlu is created by transduction of embryonic blastodermal cells with replication-defective or replication-competent retroviral particles carrying the transgene between the 5' and 3' LTRs of the retroviral vector. For instance, an avian leukosis virus (ALV) retroviral vector or a murine leukemia virus (MLV) retroviral vector can be used. An RNA copy of the modified retroviral vector packaged into viral particles can be used to infect embryonic blastoderms which develop into transgenic avians.

By the methods of the present invention, transgenes can be introduced into embryonic blastodermal cells of various avian species. For example, the methods can be applied to produce a transgenic chicken, transgenic turkey, transgenic quail, transgenic duct, and other avian species, that carry the transgene in the genome of its germ-line tissue in order to produce proteins of the invention. The blastodermal cells are typically stage VII-XII cells as defined by Eyal-Giladi and Kochav (1976), or the equivalent thereof. In a preferred embodiment, the blastoderm cells are at or near stage X.

In one method of transfecting blastodermal cells, a packaged retroviral-based vector can be used to deliver the vector into embryonic blastodermal cells so that the vector is integrated into the avian genome. Such viral particles (*i.e.,* transduction particles) are produced for the vector and titered to determine the appropriate concentration that can be used to inject embryos. In one embodiment, avian eggs are windowed according to the procedure described in U.S. Pat. No. 5,897,998, the disclosure of which is incorporated herein by reference in its entirety, and the eggs are injected with transducing particles at or near stage X.

The transgenic avians of the invention which produce rhNaGlu are developed from the blastodermal cells into which the vector has been introduced. The resulting embryo is allowed to develop and the chick allowed to mature. At this stage, the transgenic avian produced from blastodermal cells is known as a founder and is chimeric with respect to the cells carrying the transgene and is referred to G0. G0 founder avians are typically chimeric for each inserted transgene. That is, only some of the cells of the G0 transgenic bird contain the transgene. Some founders carry the transgene in tubular gland cells in the magnum of their oviducts. These avians express the rhNaGlu protein encoded by the transgene in their oviducts. The NaGlu protein may also be expressed in other tissues (*e.g.,* blood) in addition to the oviduct. Some founders are germ-line founders that carry the transgene in the genome of the germ-line tissues, and may also carry the transgene in oviduct magnum tubular gland cells that express the exogenous protein.

The transgenic avian can carry the transgene in its germ-line providing transmission of the exogenous transgene to the avian's offspring stably in a Mendelian fashion. The G0 generation is typically hemizygous for the transgene encoding rhNaGlu. The G0 generation can be bred to non-transgenic animals to give rise to G1 transgenic offspring which are also hemizygous for the transgene and contain the transgene in essentially all of the bird's cells. The G1 hemizygous offspring can be bred to non-transgenic animals giving rise to G2 hemizygous offspring or may be bred together to give rise to G2 offspring homozygous for the transgene. Substantially all of the cells of avians which are positive for the transgene that are derived from G1 offspring contain the transgene. In one embodiment, hemizygotic G2 offspring from the

same line can be bred to produce G3 offspring homozygous for the transgene. In another embodiment, hemizygous G0 or G1 animals, for example, are bred together to give rise to homozygous G1 offspring containing two copies of the transgene(s) in each cell of the animal. These are merely examples of certain useful breeding methods and the present invention contemplates the employment of any useful breeding method such as those known to individuals of ordinary skill in the art.

IV. Production of rhNaGlu

The rhNaGlu can be produced using a transgenic avian that contains in the genome a transgene encoding rhNaGlu. In one embodiment, the transgenic avian is a germline transgenic chicken, quail, duck or turkey. In one particularly useful embodiment, the invention is drawn to the production of NaGlu which can be produced in the oviduct of a chicken.

Production of rhNaGlu with or without modification in the avian system (*e.g.,* in the avian oviduct) is within the scope of the invention. In one embodiment, the unmodified rhNaGlu comprises the wild-type amino acid sequence (24-743 of SEQ ID NO:1) with a glycosylation structure (*i.e.,* M6P) that enables efficient uptake by human cells. In another embodiment, the modified protein can be an rhNaGlu fusion protein having a glycosylation pattern (*i.e.,* M6P) that enables efficient uptake by human cells.

A suitable avian vector that contains a nucleic acid sequence encoding a NaGlu protein, operably linked to a tissue-specific or constitutive promoter that drives expression of the encoding sequence in the chicken oviduct are introduced into chicken embryonic cells at or near stage X as described herein. The transformed embryonic cells are incubated under conditions conducive to hatching live chicks. Live chicks are nurtured into a mature chimeric chicken which are mated with a non-transgenic chicken naturally or via artificial insemination. A transgenic chicken is identified by screening progeny for germline incorporation of the protein encoding sequence. The transgenic progeny can be mated with another transgenic or a non-transgenic chicken to produce a fully germline transgenic hen that lays eggs.

The rhNaGlu can be produced in a tissue-specific manner. For example, rhNaGlu can be expressed in the oviduct, blood and/or other cells or tissues of the

transgenic avian. In one embodiment, the NaGlu is expressed in the tubular gland cells of the magnum of the oviduct of the transgenic avian, secreted into the lumen of the oviduct, and deposited into egg white. In one embodiment, egg white containing rhNaGlu is harvested and stored in bulk at a temperature ranging from 4°C to -20°C. The NaGlu is then isolated and purified from the contents of the eggs using various methods known in the art.

One aspect of the present invention relates to avian hard shell eggs (*e.g.,* chicken hard shell eggs) which contain the rhNaGlu protein. The rhNaGlu produced and secreted by the transgenic avian is glycosylated in a manner favorable to cellular uptake by a human cell. The protein may be present in any useful amount. In one embodiment, the protein is present in an amount in a range between about 0.01 μg per hard-shell egg and about 1 gram per hard-shell egg. In another embodiment, the protein is present in an amount in a range of between about 1 μg per hard-shell egg and about 1 gram per hard-shell egg. For example, the protein may be present in an amount in a range of between about 10 μg per hard-shell egg and about 1 gram per hard-shell egg (*e.g.,* a range of between about 10 μg per hard-shell egg and about 400 milligrams per hard-shell egg).

In one embodiment, the rhNaGlu is present in the egg white of the egg. In one embodiment, the rhNaGlu is present in an amount in a range of between about 1 ng per milliliter of egg white and about 0.2 gram per milliliter of egg white. For example, the rhNaGlu may be present in an amount in a range of between about 0.1 μg per milliliter of egg white and about 0.2 gram per milliliter of egg white (*e.g.,* the rhNaGlu may be present in an amount in a range of between about 1 μg per milliliter of egg white and about 100 milligrams per milliliter of egg white. In one embodiment, the rhNaGlu is present in an amount in a range of between about 1 μg per milliliter of egg white and about 50 milligrams per milliliter of egg white. For example, the rhNaGlu may be present in an amount in a range of about 1 μg per milliliter of egg white and about 10 milligrams per milliliter of egg white (*e.g.,* the rhNaGlu may be present in an amount in a range of between about 1 μg per milliliter of egg white and about 1 milligrams per milliliter of egg white). In one embodiment, the rhNaGlu is present in an amount of more than 0.1 μg per milliliter of egg white. In one embodiment, the rhNaGlu is present in an amount of more than 0.5 μg per milliliter of egg white. In one embodiment, the

rhNaGlu is present in an amount of more than 1 μg per milliliter of egg white. In one embodiment, the protein is present in an amount of more than 1.5 μg per milliliter of egg white. In one embodiment, the rhNaGlu is present in an amount of more than 0.5 μg per milliliter of egg white. In one embodiment, the protein is present in an amount of more than 0.1 μg per milliliter of egg white.

In one embodiment, the rhNaGlu is present in an amount of 20 mg/L, 30mg/L, 40mg/L, 50 mg/L, 60 mg/L, 70 mg/L, 80 mg/L, 90 mg/L, 100 mg/L, 120 mg/L, 130 mg/L, 140 mg/L, 150 mg/L, 160 mg/L, 170 mg/L, 200 mg/L, 300mg/L, 400 mg/L, 500 mg/L, 600 mg/L, 700 mg/L, 800 mg/L, 900 mg/L, or 1,000 mg/L egg white. In one embodiment, the rhNaGlu is present in an amount of about 100 mg/L of egg white. In one embodiment, the rhNaGlu is present in an amount of about 200 mg/L of egg white.

V. Host Cells

The present invention also contemplates rhNaGlu produced in any useful protein expression system including, without limitation, cell culture (*e.g.,* avian cells, CHO cells, HEK293 cells and COS cells), yeast, bacteria, and plants.

A host cell strain can be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed NaGlu in the desired fashion. Such modifications of the polypeptide of NaGlu include, without limitation, glycosylation, phosphorylation, or lipidation. Different host cells such as CHO, COS, HeLa, MDCK, HEK293 and W138, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, can be chosen to ensure the correct modification and processing of the fusion protein of the present invention. An avian tumor cell line is also contemplated as a host cell for expressing the polypeptide of the present invention. Examples of a useful avian cell line (*e.g.,* an avian oviduct tumor cell line) are described in U.S. Pat. Publication No. 2009/0253176, the entire teachings of which are incorporated herein by reference.

VI. Pharmaceutical Compositions

The present invention also features pharmaceutical compositions comprising isolated and substantially purified rhNaGlu or a pharmaceutically acceptable salt thereof.

The recombinant human NaGlu proteins may be administered using one or more carriers, *e.g.,* as part of a pharmaceutical formulation, or without a carrier. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Compositions comprising such carriers, including composite molecules, are formulated by well-known conventional methods (*see*, for example, Remington's Pharmaceutical Sciences, 14th Ed., Mack Publishing Co., Easton, Pa.), the entire teachings of which are incorporated herein by reference. The carrier may comprise a diluent. In one embodiment, the pharmaceutical carrier can be a liquid and the protein may be in the form of a solution. The pharmaceutical carrier can be wax, fat, or alcohol. In another embodiment, the pharmaceutically acceptable carrier may be a solid in the form of a powder, a lyophilized powder, or a tablet. In one embodiment, the carrier may comprise a liposome or a microcapsule.

In some embodiments, a pharmaceutical composition comprising recombinant human NaGlu protein further comprises a buffer. Exemplary buffers include acetate, phosphate, citrate and glutamate buffers. Exemplary buffers also include lithium citrate, sodium citrate, potassium citrate, calcium citrate, lithium lactate, sodium lactate, potassium lactate, calcium lactate, lithium phosphate, sodium phosphate, potassium phosphate, calcium phosphate, lithium maleate, sodium maleate, potassium maleate, calcium maleate, lithium tartarate, sodium tartarate, potassium tartarate, calcium tartarate, lithium succinate, sodium succinate, potassium succinate, calcium succinate, lithium acetate, sodium acetate, potassium acetate, calcium acetate, and mixtures thereof. In some embodiments, the buffer is trisodium citrate dihydrate. In some embodiments, the buffer is citric acid monohydrate. In some embodiments, a pharmaceutical composition comprises trisodium citrate dehydrate and citric acid monohydrate.

In some embodiments, a pharmaceutical composition comprising recombinant human NaGlu protein further comprises a stabilizer. Exemplary stabilizers include albumin, trehalose, sugars, amino acids, polyols, cyclodextrins, salts such as sodium chloride, magnesium chloride, and calcium chloride, lyoprotectants, and mixtures

thereof. In some embodiments, a pharmaceutical composition comprises human serum albumin.

In some embodiments, it is desirable to add a surfactant to the pharmaceutical composition. Exemplary surfactants include nonionic surfactants such as Polysorbates (*e.g.,* Polysorbates 20 or 80); poloxamers (*e.g.,* poloxamer 188); Triton; sodium dodecyl sulfate (SDS); sodium laurel sulfate; sodium octyl glycoside; lauryl-, myristyl-, linoleyl-, or stearyl-sulfobetaine; lauryl-, myristyl-, linoleyl- or stearyl-sarcosine; linoleyl-, myristyl-, or cetyl-betaine; lauroamidopropyl-, cocamidopropyl-, linoleamidopropyl-, myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-betaine (*e.g.,* lauroamidopropyl); myristamidopropyl-, palmidopropyl-, or isostearamidopropyl-dimethylamine; sodium methyl cocoyl-, or disodium methyl ofeyl-taurate; and the MONAQUAT™ series (Mona Industries, Inc., Paterson, N.J.), polyethyl glycol, polypropyl glycol, and copolymers of ethylene and propylene glycol (*e.g.,* Pluronics, PF68, etc). Typically, the amount of surfactant added is such that it reduces aggregation of the protein and minimizes the formation of particulates or effervescences. For example, a surfactant may be present in a formulation at a concentration from about 0.001 - 0.5% (*e.g.,* about 0.005 - 0.05%, or 0.005 - 0.01%). In particular, a surfactant may be present in a formulation at a concentration of approximately 0.005%, 0.01%, 0.02%, 0.1%, 0.2%, 0.3%, 0.4%, or 0.5%, etc. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, suitable pharmaceutical compositions of the invention may further include one or more bulking agents, in particular, for lyophilized formulations. A "bulking agent" is a compound which adds mass to the lyophilized mixture and contributes to the physical structure of the lyophilized cake. For example, a bulking agent may improve the appearance of lyophilized cake (*e.g.,* essentially uniform lyophilized cake). Suitable bulking agents include, but are not limited to, sodium chloride, lactose, mannitol, glycine, sucrose, trehalose, hydroxyethyl starch. Exemplary concentrations of bulking agents are from about 1% to about 10% (*e.g.,* 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0%, 7.5%, 8.0%, 8.5%, 9.0%, 9.5%, and 10.0%). Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention. The pharmaceutical

compositions can be in the form of a sterile lyophilized powder for injection upon reconstitution with a diluent. The diluent can be water for injection, bacteriostatic water for injection, or sterile saline. The lyophilized powder may be produced by freeze drying a solution of the fusion protein to produce the protein in dry form. As is known in the art, the lyophilized protein generally has increased stability and a longer shelf-life than a liquid solution of the protein.

Pharmaceutical formulations include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral administration. Preferably, the pharmaceutical formulations of the invention include those suitable for administration by injection including intrathecal, intraparenchymal, intracerebral, intraventricular, intramuscular, sub-cutaneous and intravenous administration. In one embodiment, the formulations of the invention are suitable for intravenous administration. In another embodiment, the formulations of the invention are suitable for intrathecal administration. The pharmaceutical formulations of the invention also include those suitable for administration by inhalation or insufflation. The formulations can, where appropriate, be conveniently presented in discrete dosage units and can be prepared by any of the methods well known in the art of pharmacy. The methods of producing the pharmaceutical formulations typically include the step of bringing the therapeutic proteins into association with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation.

Recombinant human NaGlu proteins of the invention can also be formulated for parenteral administration (*e.g.,* by injection, for example bolus injection or continuous infusion) and can be presented in unit dose form in ampoules, pre-filled syringes, small volume infusion or in multi-dose containers with an added preservative. The therapeutic proteins can be injected by, for example, subcutaneous injections, intramuscular injections, intrathecal injections, intracerebral injections, intraparenchymal injections, intraventricular injections, and intravenous (IV) infusions or injections.

In one embodiment, the recombinant human NaGlu protein is administered intravenously by IV infusion by any useful method. In one example, the recombinant human NaGlu protein can be administered by intravenous infusion through a peripheral line. In another example, the recombinant human NaGlu protein can be administered by

intravenous infusion through a peripherally inserted central catheter. In another example, the recombinant human NaGlu protein can be administered by intravenous infusion facilitated by an ambulatory infusion machine attached to a venous vascular access port. In one embodiment of intravenous infusion, the medication is administered over a period of 1 to 8 hours depending on the amount of medication to be infused and the patient's previous infusion-related reaction history, as determined by a physician skilled in the art. In another embodiment, the recombinant human NaGlu protein is administered intravenously by IV injection. In another embodiment, the recombinant human NaGlu protein can be administered via intraperitoneal or intrathecal injection.

In some embodiments, the therapeutic proteins are administered by infusion, and the infusion can occur over an extended time period, for example, 30 minutes to 10 hours. Thus, the infusion can occur, for example, over a period of about 1 hour, about 2 hours, about 3 hours, about 4 hours, or about 5 hours. The infusion can also occur at various rates. Thus, for example, the infusion rate can be about 1 mL per hour to about 20 mL per hour. In some embodiments, the infusion rate is 5 mL to 10 mL per hour. In one embodiment, the infusion rate is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 mL per hour. In one embodiment, the infusion rate is 0.1 to 5 mg/kg/hr. In one embodiment, the infusion rate is about 0.1, about 0.2, about 0.3, about 0.5, about 1.0, about 1.5, about 2.0, or about 3 mg/kg/hr. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

The therapeutic proteins can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. The recombinant human NaGlu proteins can be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, *e.g.,* sterile, pyrogen-free water, before use.

Formulations in accordance with the present invention can be assessed based on product quality analysis, reconstitution time (if lyophilized), quality of reconstitution (if lyophilized), high molecular weight, moisture, and glass transition temperature. Typically, protein quality and product analysis include product degradation rate analysis using methods including, but not limited to, size exclusion HPLC (SE-HPLC), cation

exchange-HPLC (CEX-HPLC), X-ray diffraction (XRD), modulated differential scanning calorimetry (mDSC), reversed phase HPLC (RP-HPLC), multi-angle light scattering (MALS), fluorescence, ultraviolet absorption, nephelometry, capillary electrophoresis (CE), SDS-PAGE, and combinations thereof. In some embodiments, evaluation of product in accordance with the present invention may include a step of evaluating appearance (either liquid or cake appearance).

Generally, formulations (lyophilized or aqueous) can be stored for extended periods of time at room temperature. Storage temperature may typically range from 0°C to 45°C (*e.g.,* 4°C, 20°C, 25°C or 45°C). Formulations may be stored for a period of months to a period of years. Storage time generally will be 24 months, 12 months, 6 months, 4.5 months, 3 months, 2 months or 1 month. Formulations can be stored directly in the container used for administration, eliminating transfer steps. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

Formulations can be stored directly in the lyophilization container (if lyophilized), which may also function as the reconstitution vessel, eliminating transfer steps. Alternatively, lyophilized product formulations may be measured into smaller increments for storage. Storage should generally avoid circumstances that lead to degradation of the proteins, including but not limited to exposure to sunlight, UV radiation, other forms of electromagnetic radiation, excessive heat or cold, rapid thermal shock, and mechanical shock. The pharmaceutical compositions according to the invention can also contain other active ingredients such as immunosuppressive agents, antimicrobial agents, or preservatives, discussed in more detail below.

VII. Methods of Treatment

The present invention also provides methods of treating NaGlu-associated diseases, *e.g.,* Sanfilippo Syndrome B. Recombinant NaGlu employed in accordance with the invention includes recombinant NaGlu which can be produced in any useful protein expression system including, without limitation, cell culture (*e.g.,* CHO cells, COS cells), bacteria such as *E. coli*, transgenic animals such as mammals and avians (*e.g.,* chickens, duck, and turkey) and in plant systems (*e.g.,* duck weed and tobacco

plants). In one embodiment, the recombinant NaGlu is produced in a transgenic animal, such as an avian.

In one embodiment, the method comprises administering to the subject a recombinant human NaGlu protein (rhNaGlu), for instance a recombinant human NaGlu protein containing a sufficient amount of oligosaccharides (*e.g.,* mannose and phosphorylated mannose (*i.e.,* M6P)), in an amount sufficient to treat (*e.g.,* reduce, ameliorate) or prevent one or more symptoms of a NaGlu deficiency or NaGlu associated disease. The recombinant human NaGlu protein can be administered therapeutically or prophylactically, or both. The recombinant human NaGlu protein (rhNaGlu) can be administered to the subject, alone or in combination with other therapeutic modalities as described herein.

The terms "treat," "treating," and "treatment" refer to methods of alleviating, abating, or ameliorating a disease or symptom, preventing an additional symptom, ameliorating or preventing an underlying cause of a symptom, inhibiting a disease or condition, arresting the development of a disease or condition, relieving a disease or condition, causing regression of a disease or condition, relieving a condition caused by the disease or condition, or stopping a symptom of the disease or condition either prophylactically and/or after the symptom has occurred.

"Therapeutically effective dose" as used herein refers to the dose (*e.g.,* amount and/or interval) of drug required to produce an intended therapeutic response (*e.g.*, reduction of heparan sulfate levels and/or increase in NaGlu activity in a target tissue). A therapeutically effective dose refers to a dose that, as compared to a corresponding subject who has not received such a dose, results in improved treatment, healing, prevention, or amelioration of a disease, disorder, or side effect, or a decrease in the rate of the occurrence or advancement of a disease or disorder. The term also includes within its scope, doses effective to enhance physiological functions.

As used herein, the term "subject" or "patient" is intended to include human and non-human animals. Non-human animals include all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dogs, cats, cows, horses, chickens, amphibians, and reptiles. Preferred subjects include human subjects having a NaGlu deficiency or NaGlu associated disease.

As used herein a "NaGlu associated disease" is a disease or condition which is mediated by NaGlu activity or is associated with aberrant NaGlu expression or activity. An example of an NaGlu associated disease includes, but is not limited to, NaGlu deficiency such as Sanflippo Syndrome B (also known as mucopolysaccharidosis type IIIB).

The therapeutic methods of the present invention encompass any route of administration which facilitates the uptake or transport of the recombinant human NaGlu protein into the pertinent organs and tissues. In one embodiment, the methods of the invention include delivering the recombinant human NaGlu proteins of the invention to the CNS (central nervous system), the kidney, or the liver of a subject for the treatment of a NaGlu associated disease (*e.g.*, NaGlu deficiency). For example, the recombinant human NaGlu protein may be administered to the patient intravenously (*e.g.,* via intravenous injection or intravenous infusion) and surprisingly crosses the blood brain barrier (BBB) of the subject having NaGlu deficiency. In another embodiment of the invention, the recombinant human NaGlu protein is administered to the patient intrathecally.

A. Device for Intrathecal Delivery

Various devices may be used for intrathecal delivery according to the present invention. In some embodiments, a device for intrathecal administration contains a fluid access port (*e.g.,* injectable port); a hollow body (*e.g.,* catheter) having a first flow orifice in fluid communication with the fluid access port and a second flow orifice configured for insertion into spinal cord; and a securing mechanism for securing the insertion of the hollow body in the spinal cord. As a non-limiting example, a suitable securing mechanism contains one or more nobs mounted on the surface of the hollow body and a sutured ring adjustable over the one or more nobs to prevent the hollow body (*e.g.,* catheter) from slipping out of the spinal cord. In various embodiments, the fluid access port comprises a reservoir. In some embodiments, the fluid access port comprises a mechanical pump (*e.g.,* an infusion pump). In some embodiments, an implanted catheter is connected to either a reservoir (*e.g.,* for bolus delivery), or an infusion pump. The fluid access port may be implanted or external.

In some embodiments, intrathecal administration may be performed by either lumbar puncture (*i.e.,* slow bolus) or via a port-catheter delivery system (*i.e.,* infusion or bolus). In some embodiments, the catheter is inserted between the laminae of the lumbar vertebrae and the tip is threaded up the thecal space to the desired level (generally L3-L4).

Relative to intravenous administration, a single dose volume suitable for intrathecal administration is typically small. Typically, intrathecal delivery according to the present invention maintains the balance of the composition of the CSF as well as the intracranial pressure of the subject. In some embodiments, intrathecal delivery is performed absent the corresponding removal of CSF from a subject. In some embodiments, a suitable single dose volume may be *e.g.,* less than about 10 mL, 8 mL, 6 mL, 5 mL, 4 mL, 3 mL, 2 mL, 1.5 mL, 1 mL, or 0.5 mL. In some embodiments, a suitable single dose volume may be about 0.5-5 mL, 0.5-4 mL, 0.5-3 mL, 0.5-2 mL, 0.5-1 mL, 1-3 mL, 1-5 mL, 1.5-3 mL, 1-4 mL, or 0.5-1.5 mL. In some embodiments, intrathecal delivery according to the present invention involves a step of removing a desired amount of CSF first. In some embodiments, less than about 10 mL (*e.g.,* less than about 9 mL, 8 mL, 7 mL, 6 mL, 5 mL, 4 mL, 3 mL, 2 mL, 1 mL) of CSF is first removed before intrathecal administration. In those cases, a suitable single dose volume may be *e.g.,* more than about 3 mL, 4 mL, 5 mL, 6 mL, 7 mL, 8 mL, 9 mL, 10 mL, 15 mL, or 20 mL. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

Various other devices may be used to effect intrathecal administration of a therapeutic composition. For example, formulations containing desired enzymes may be given using an Ommaya reservoir which is in common use for intrathecally administering drugs for meningeal carcinomatosis (Lancet 2: 983-84, 1963). More specifically, in this method, a ventricular tube is inserted through a hole formed in the anterior horn and is connected to an Ommaya reservoir installed under the scalp, and the reservoir is subcutaneously punctured to intrathecally deliver the particular enzyme being replaced, which is injected into the reservoir. Other devices for intrathecal administration of therapeutic compositions or formulations to an individual are described in U.S. Pat. No. 6,217,552, the entire contents of which, as they relate to these

devices, are incorporated herein by reference. Alternatively, the drug may be intrathecally given, for example, by a single injection, or continuous infusion. It should be understood that the dosage treatment may be in the form of a single dose administration or multiple doses.

For injection, formulations of the invention can be formulated in liquid solutions. In addition, the NaGlu enzyme may be formulated in solid form and re-dissolved or suspended immediately prior to use. Lyophilized forms are also included. The injection can be, for example, in the form of a bolus injection or continuous infusion (*e.g.,* using infusion pumps) of the NaGlu enzyme.

In one embodiment of the invention, the NaGlu enzyme is administered by lateral cerebro ventricular injection into the brain of a subject. The injection can be made, for example, through a burr hole made in the subject's skull. In another embodiment, the enzyme and/or other pharmaceutical formulation is administered through a surgically inserted shunt into the cerebral ventricle of a subject. For example, the injection can be made into the lateral ventricles, which are larger. In some embodiments, injection into the third and fourth smaller ventricles can also be made.

In yet another embodiment, the pharmaceutical compositions used in the present invention are administered by injection into the cisterna magna, or lumbar area of a subject.

In another embodiment of the method of the invention, the pharmaceutically acceptable formulation provides sustained delivery, *e.g.,* "slow release" of the enzyme or other pharmaceutical composition used in the present invention, to a subject for at least one, two, three, four weeks or longer periods of time after the pharmaceutically acceptable formulation is administered to the subject.

As used herein, the term “sustained delivery” refers to continual delivery of a pharmaceutical formulation of the invention *in vivo* over a period of time following administration, preferably at least several days, a week or several weeks. Sustained delivery of the composition can be demonstrated by, for example, the continued therapeutic effect of the enzyme over time (*e.g.,* sustained delivery of the enzyme can be demonstrated by continued reduced amount of storage granules in the subject).

Alternatively, sustained delivery of the enzyme may be demonstrated by detecting the presence of the enzyme *in vivo* over time.

B. Intravenous Delivery

As discussed above, one of the surprising features of the present invention is that the recombinant human NaGlu proteins of the invention are able to effectively and extensively diffuse across the blood brain barrier (BBB) and brain surface and penetrate various layers or regions of the brain, including deep brain regions, when administered intravenously. The methods of the present invention effectively deliver the rhNaGlu proteins to various tissues, neurons or cells of the central nervous system (CNS), which are hard to target by existing CNS delivery methods. Furthermore, the methods of the present invention deliver sufficient amounts of the recombinant human NaGlu proteins to the blood stream and various peripheral organs and tissues.

"Intravenous injection," often medically referred to as IV push or bolus injection, refers to a route of administration in which a syringe is connected to the IV access device and the medication is injected directly, typically rapidly and occasionally up to a period of 15 minutes if it might cause irritation of the vein or a too-rapid effect. Once a medicine has been injected into the fluid stream of the IV tubing, there must be some means of ensuring that it gets from the tubing to the patient. Usually this is accomplished by allowing the fluid stream to flow normally and thereby carry the medicine into the bloodstream. However, in some cases a second fluid injection, sometimes called a "flush," is used following the first injection to facilitate the entering of the medicine into the bloodstream.

"Intravenous infusion" refers to a route of administration in which medication is delivered over an extended period of time. For example, the medication can be delivered to a patient over a period of time between 1 and 8 hours. The medication can also be delivered to a patient over a period of about 1, about 2, about 3, about 4, about 5, about 6, about 7, or about 8 hours. To accomplish an intravenous infusion, an IV gravity drip or an IV pump can be used. IV infusion is typically used when a patient requires medications only at certain times and does not require additional intravenous fluids (*e.g.*,

water solutions which can contain sodium, chloride, glucose, or any combination thereof) such as those that restore electrolytes, blood sugar, and water loss.

C. Target Tissues

In some embodiments, the rhNaGlu of the invention is delivered to the central nervous system (CNS) of a subject. In some embodiments, the rhNaGlu of the invention is delivered to one or more of target tissues of brain, spinal cord, and/or peripheral organs. As used herein, the term "target tissue" refers to any tissue that is affected by the NaGlu associated disease to be treated or any tissue in which the deficient NaGlu is normally expressed. In some embodiments, target tissues include those tissues in which there is a detectable or abnormally high amount of enzyme substrate, for example stored in the cellular lysosomes of the tissue, in patients suffering from or susceptible to the NaGlu associated disease. In some embodiments, target tissues include those tissues that display a disease-associated pathology, symptom, or feature. In some embodiments, target tissues include those tissues in which the deficient NaGlu is normally expressed at an elevated level. As used herein, a target tissue may be a brain target tissue, a spinal cord target tissue and/or a peripheral target tissue. Exemplary target tissues are described in detail below.

D. Brain Target Tissues

In general, the brain can be divided into different regions, layers and tissues. For example, meningeal tissue is a system of membranes which envelops the central nervous system, including the brain. The meninges contain three layers, including dura matter, arachnoid matter, and pia matter. In general, the primary function of the meninges and of the cerebrospinal fluid is to protect the central nervous system. In some embodiments, a therapeutic protein in accordance with the present invention is delivered to one or more layers of the meninges.

The brain has three primary subdivisions, including the cerebrum, cerebellum, and brain stem. The cerebral hemispheres, which are situated above most other brain structures and are covered with a cortical layer. Underneath the cerebrum lies the

brainstem, which resembles a stalk on which the cerebrum is attached. At the rear of the brain, beneath the cerebrum and behind the brainstem, is the cerebellum.

The diencephalon, which is located near the midline of the brain and above the mesencephalon, contains the thalamus, metathalamus, hypothalamus, epithalamus, prethalamus, and pretectum. The mesencephalon, also called the midbrain, contains the tectum, tegumentum, ventricular mesocoelia, and cerebral peduncels, the red nucleus, and the cranial nerve III nucleus. The mesencephalon is associated with vision, hearing, motor control, sleep/wake, alertness, and temperature regulation.

Regions of tissues of the central nervous system, including the brain, can be characterized based on the depth of the tissues. For example, CNS (*e.g.,* brain) tissues can be characterized as surface or shallow tissues, mid-depth tissues, and/or deep tissues.

According to the present invention, the rhNaGlu of the invention may be delivered to any appropriate brain target tissue(s) associated with a particular disease to be treated in a subject. In some embodiments, the rhNaGlu of the invention is delivered to surface or shallow brain target tissue. In some embodiments, the rhNaGlu of the invention is delivered to mid-depth brain target tissue. In some embodiments, the rhNaGlu of the invention is delivered to deep brain target tissue. In some embodiments, the rhNaGlu of the invention is delivered to a combination of surface or shallow brain target tissue, mid-depth brain target tissue, and/or deep brain target tissue. In some embodiments, the rhNaGlu of the invention is delivered to a deep brain tissue at least 4 mm, 5 mm, 6 mm, 7 mm, 8 mm, 9 mm, 10 mm or more below (or internal to) the external surface of the brain. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, the rhNaGlu of the invention is delivered to one or more surface or shallow tissues of cerebrum. In some embodiments, the targeted surface or shallow tissues of the cerebrum are located within 4 mm from the surface of the cerebrum. In some embodiments, the targeted surface or shallow tissues of the cerebrum are selected from pia mater tissues, cerebral cortical ribbon tissues, hippocampus, Virchow Robin space, blood vessels within the VR space, the hippocampus, portions of the hypothalamus on the inferior surface of the brain, the optic nerves and tracts, the olfactory bulb and projections, and combinations thereof.

In some embodiments, the rhNaGlu of the invention is delivered to one or more deep tissues of the cerebrum. In some embodiments, the targeted surface or shallow tissues of the cerebrum are located at least 4 mm (*e.g.,* 5 mm, 6 mm, 7 mm, 8 mm, 9 mm, or 10 mm) below (or internal to) the surface of the cerebrum. In some embodiments, targeted deep tissues of the cerebrum include the cerebral cortical ribbon. In some embodiments, targeted deep tissues of the cerebrum include one or more of the diencephalon (*e.g.,* the hypothalamus, thalamus, prethalamus or subthalamus), metencephalon, lentiform nuclei, the basal ganglia, caudate, putamen, amygdala, globus pallidus, and combinations thereof.

In some embodiments, the rhNaGlu of the invention is delivered to one or more tissues of the cerebellum. In certain embodiments, the targeted one or more tissues of the cerebellum are selected from the group consisting of tissues of the molecular layer, tissues of the Purkinje cell layer, tissues of the Granular cell layer, cerebellar peduncles, and combination thereof. In some embodiments, therapeutic agents (*e.g.,* enzymes) are delivered to one or more deep tissues of the cerebellum including, but not limited to, tissues of the Purkinje cell layer, tissues of the Granular cell layer, deep cerebellar white matter tissue (*e.g.,* deep relative to the Granular cell layer), and deep cerebellar nuclei tissue.

In some embodiments, the rhNaGlu of the invention is delivered to one or more tissues of the brainstem. In some embodiments, the targeted one or more tissues of the brainstem include brain stem white matter tissue and/or brain stem nuclei tissue.

In some embodiments, the rhNaGlu of the invention is delivered to various brain tissues including, but not limited to, gray matter, white matter, periventricular areas, pia-arachnoid, meninges, neocortex, cerebellum, deep tissues in cerebral cortex, molecular layer, caudate/putamen region, midbrain, deep regions of the pons or medulla, and combinations thereof.

In some embodiments, the rhNaGlu of the invention is delivered to various cells in the brain including, but not limited to, neurons, glial cells, perivascular cells and/or meningeal cells. In some embodiments, a therapeutic protein is delivered to oligodendrocytes of deep white matter.

E. Spinal Cord Target Tissue

In general, regions or tissues of the spinal cord can be characterized based on the depth of the tissues. For example, spinal cord tissues can be characterized as surface or shallow tissues, mid-depth tissues, and/or deep tissues.

In some embodiments, the rhNaGlu of the invention are delivered to one or more surface or shallow tissues of the spinal cord. In some embodiments, a targeted surface or shallow tissue of the spinal cord is located within 4 mm from the surface of the spinal cord. In some embodiments, a targeted surface or shallow tissue of the spinal cord contains pia matter and/or the tracts of white matter.

In some embodiments, the rhNaGlu of the invention are delivered to one or more deep tissues of the spinal cord. In some embodiments, a targeted deep tissue of the spinal cord is located internal to 4 mm from the surface of the spinal cord. In some embodiments, a targeted deep tissue of the spinal cord contains spinal cord grey matter and/or ependymal cells.

In some embodiments, replacement enzymes (*e.g.,* a NaGlu fusion protein) are delivered to neurons of the spinal cord.

F. Peripheral Target Tissues

As used herein, peripheral organs or tissues refer to any organs or tissues that are not part of the central nervous system (CNS). Peripheral target tissues may include, but are not limited to, blood system, liver, kidney, heart, endothelium, bone marrow and bone marrow derived cells, spleen, lung, lymph node, bone, cartilage, ovary and testis. In some embodiments, the rhNaGlu of the invention is delivered to one or more of the peripheral target tissues.

G. Biodistribution and Bioavailability

In various embodiments, once delivered to the target tissue, the rhNaGlu of the invention is localized intracellularly. For example, the rhNaGlu of the invention may be localized to exons, axons, lysosomes, mitochondria or vacuoles of a target cell (*e.g.,* neurons such as Purkinje cells). For example, in some embodiments the rhNaGlu of the invention demonstrates translocation dynamics such that the rhNaGlu moves within the

perivascular space (*e.g.,* by pulsation-assisted convective mechanisms). In addition, active axonal transport mechanisms relating to the association of the administered protein or enzyme with neurofilaments may also contribute to or otherwise facilitate the distribution of the rhNaGlu proteins of the invention into the deeper tissues of the central nervous system.

In some embodiments, the rhNaGlu of the invention delivered according to the present invention may achieve therapeutically or clinically effective levels or activities in various targets tissues described herein. As used herein, a therapeutically or clinically effective level or activity is a level or activity sufficient to confer a therapeutic effect in a target tissue. The therapeutic effect may be objective (*i.e.,* measurable by some test or marker) or subjective (*i.e.,* subject gives an indication of or feels an effect). For example, a therapeutically or clinically effective level or activity may be an enzymatic level or activity that is sufficient to ameliorate symptoms associated with the disease in the target tissue (*e.g.,* GAG storage).

In some embodiments, the rhNaGlu of the invention delivered according to the present invention may achieve an enzymatic level or activity that is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the normal level or activity of the corresponding NaGlu enzyme in the target tissue. In some embodiments, the rhNaGlu of the invention delivered according to the present invention may achieve an enzymatic level or activity that is increased by at least 1-fold, 2-fold, 3 -fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold or 10-fold as compared to a control (*e.g.,* endogenous levels or activities without the treatment). In some embodiments, the rhNaGlu delivered according to the present invention may achieve an increased enzymatic level or activity at least approximately 10 nmol/hr/mg, 20 nmol/hr/mg, 40 nmol/hr/mg, 50 nmol/hr/mg, 60 nmol/hr/mg, 70 nmol/hr/mg, 80 nmol/hr/mg, 90 nmol/hr/mg, 100 nmol/hr/mg, 150 nmol/hr/mg, 200 nmol/hr/mg, 250 nmol/hr/mg, 300 nmol/hr/mg, 350 nmol/hr/mg, 400 nmol/hr/mg, 450 nmol/hr/mg, 500 nmol/hr/mg, 550 nmol/hr/mg or 600 nmol/hr/mg in a target tissue. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, inventive methods according to the present invention are particularly useful for targeting the lumbar region. In some embodiments, the rhNaGlu delivered according to the present invention may achieve an increased enzymatic level or activity in the lumbar region of at least approximately 500 nmol/hr/mg, 600 nmol/hr/mg, 700 nmol/hr/mg, 800 nmol/hr/mg, 900 nmol/hr/mg, 1000 nmol/hr/mg, 1500 nmol/hr/mg, 2000 nmol/hr/mg, 3000 nmol/hr/mg, 4000 nmol/hr/mg, 5000 nmol/hr/mg, 6000 nmol/hr/mg, 7000 nmol/hr/mg, 8000 nmol/hr/mg, 9000 nmol/hr/mg, or 10,000 nmol/hr/mg. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In general, therapeutic agents (*e.g.,* the rhNaGlu) delivered according to the present invention have sufficiently long half time in CSF and target tissues of the brain, spinal cord, and peripheral organs. In some embodiments, the rhNaGlu delivered according to the present invention may have a half-life of at least approximately 30 minutes, 45 minutes, 60 minutes, 90 minutes, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 16 hours, 18 hours, 20 hours, 25 hours, 30 hours, 35 hours, 40 hours, up to 3 days, up to 7 days, up to 14 days, up to 21 days or up to a month. In some embodiments, In some embodiments, the rhNaGlu delivered according to the present invention may retain detectable level or activity in CSF or bloodstream after 12 hours, 24 hours, 30 hours, 36 hours, 42 hours, 48 hours, 54 hours, 60 hours, 66 hours, 72 hours, 78 hours, 84 hours, 90 hours, 96 hours, 102 hours, or a week following administration. Detectable level or activity may be determined using various methods known in the art. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In certain embodiments, the rhNaGlu delivered according to the present invention achieves a concentration of at least 30μg/mL in the CNS tissues and cells of the subject following administration (*e.g.,* one week, 3 days, 48 hours, 36 hours, 24 hours, 18 hours, 12 hours, 8 hours, 6 hours, 4 hours, 3 hours, 2 hours, 1 hour, 30 minutes, or less, following administration of the pharmaceutical composition to the subject). In certain embodiments, the rhNaGlu delivered according to the present invention achieves a concentration of at least 2 μg/mL, at least 15 μg/mL, at least 1 μg/mL, at least 7 μg/mL, at least 5 μg/mL, at least 2 μg/mL, at least 1 μg/mL or at least 0.5 μg/mL in the targeted

tissues or cells of the subject(*e.g.,* brain tissues or neurons) following administration to such subject (*e.g.,* one week, 3 days, 48 hours, 36 hours, 24 hours, 18 hours, 12 hours, 8 hours, 6 hours, 4 hours, 3 hours, 2 hours, 1 hour, 30 minutes, or less following administration of such pharmaceutical compositions to the subject). Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

H. Treatment of Sanfilippo Syndrome

Sanfilippo syndrome, or mucopolysaccharidosis III (MPS III), is a rare genetic disorder characterized by the deficiency of enzymes involved in the degradation of glycosaminoglycans (GAG). In the absence of enzyme, partially degraded GAG molecules cannot be cleared from the body and accumulate in lysosomes of various tissues, resulting in progressive widespread somatic dysfunction (Neufeld and Muenzer, 2001).

Four distinct forms of MPS III, designated MPS IIIA, B, C, and D, have been identified. Each represents a deficiency in one of four enzymes involved in the degradation of the GAG heparan sulfate. All forms include varying degrees of the same clinical symptoms, including coarse facial features, hepatosplenomegaly, corneal clouding and skeletal deformities. Most notably, however, is the severe and progressive loss of cognitive ability, which is tied not only to the accumulation of heparan sulfate in neurons, but also the subsequent elevation of the gangliosides GM2, GM3 and GD2 caused by primary GAG accumulation (Walkley 1998).

Mucopolysaccharidosis type IIIB (MPS IIIB; Sanfilippo syndrome B) is an autosomal recessive disorder that is characterized by a deficiency of the enzyme alpha-N-acetyl-glucosaminidase (NaGlu). In the absence of this enzyme, GAG heparan sulfate accumulates in lysosomes of neurons and glial cells, with lesser accumulation outside the brain.

A defining clinical feature of this disorder is central nervous system (CNS) degeneration, which results in loss of, or failure to attain, major developmental milestones. The progressive cognitive decline culminates in dementia and premature

mortality. The disease typically manifests itself in young children, and the lifespan of an affected individual generally does not extend beyond late teens to early twenties.

Compositions and methods of the present invention may be used to effectively treat individuals suffering from or susceptible to Sanfilippo syndrome B. The terms, "treat" or "treatment," as used herein, refers to amelioration of one or more symptoms associated with the disease, prevention or delay of the onset of one or more symptoms of the disease, and/or lessening of the severity or frequency of one or more symptoms of the disease.

In some embodiments, treatment refers to partial or complete alleviation, amelioration, relief, inhibition, delaying onset, reducing severity and/or incidence of neurological impairment in a Sanfilippo syndrome B patient. As used herein, the term "neurological impairment" includes various symptoms associated with impairment of the central nervous system (*e.g.,* the brain and spinal cord). Symptoms of neurological impairment may include, for example, developmental delay, progressive cognitive impairment, hearing loss, impaired speech development, deficits in motor skills, hyperactivity, aggressiveness and/or sleep disturbances, among others.

Thus, in some embodiments, treatment refers to decreased lysosomal storage (*e.g.,* of GAG) in various tissues. In some embodiments, treatment refers to decreased lysosomal storage in brain target tissues, spinal cord neurons, and/or peripheral target tissues. In certain embodiments, lysosomal storage is decreased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or more as compared to a control. In some embodiments, lysosomal storage is decreased by at least 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold or 10-fold as compared to a control. In some embodiments, lysosomal storage is determined by LAMP-1 staining. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, treatment refers to reduced vacuolization in neurons (*e.g.,* neurons containing Purkinje cells). In certain embodiments, vacuolization in neurons is decreased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or more as compared to a control. In some embodiments, vacuolization is decreased by at least 1-fold, 2-fold, 3-

fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold or 10-fold as compared to a control. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, treatment refers to increased NaGlu enzyme activity in various tissues. In some embodiments, treatment refers to increased NaGlu enzyme activity in brain target tissues, spinal cord neurons and/or peripheral target tissues. In some embodiments, NaGlu enzyme activity is increased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% 1000% or more as compared to a control. In some embodiments, NaGlu enzyme activity is increased by at least 1-fold, 2-fold, 3 -fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold or 10-fold as compared to a control. In some embodiments, increased NaGlu enzymatic activity is at least approximately 10 nmol/hr/mg, 20 nmol/hr/mg, 40 nmol/hr/mg, 50 nmol/hr/mg, 60 nmol/hr/mg, 70 nmol/hr/mg, 80 nmol/hr/mg, 90 nmol/hr/mg, 100 nmol/hr/mg, 150 nmol/hr/mg, 200 nmol/hr/mg, 250 nmol/hr/mg, 300 nmol/hr/mg, 350 nmol/hr/mg, 400 nmol/hr/mg, 450 nmol/hr/mg, 500 nmol/hr/mg, 550 nmol/hr/mg, 600 nmol/hr/mg or more. In some embodiments, NaGlu enzymatic activity is increased in the lumbar region. In some embodiments, increased NaGlu enzymatic activity in the lumbar region is at least approximately 2000 nmol/hr/mg, 3000 nmol/hr/mg, 4000 nmol/hr/mg, 5000 nmol/hr/mg, 6000 nmol/hr/mg, 7000 nmol/hr/mg, 8000 nmol/hr/mg, 9000 nmol/hr/mg, 10,000 nmol/hr/mg, or more. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In certain embodiments, treatment according to the present invention results in a reduction (*e.g.,* about a 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 90%, 95%, 97.5%, 99% or more reduction) or a complete elimination of the presence, or alternatively the accumulation, of one or more pathological or biological markers which are associated with the NaGlu associated disease. Such reduction or elimination may be particularly evident in the cells and tissues of the CNS (*e.g.,* neurons and oligodendrocytes). For example, in some embodiments, upon administration to a subject the pharmaceutical compositions of the present invention demonstrate or achieve a reduction in the accumulation of the biomarker lysosomal

associated membrane protein 1 (LAMPl) in the CNS cells and tissues of the subject (*e.g.,* in the cerebral cortex, cerebellum, caudate nucleus and putamen, white matter and/or thalamus). LAMPl is a glycoprotein highly expressed in lysosomal membranes and its presence is elevated many patients with a lysosomal storage disorder (Meikle et al., Clin. Chem. (1997) 43:1325-1335). The presence or absence of LAMP 1 in patients (*e.g.,* as determined by LAMP staining) with a lysosomal storage disease therefore may provide a useful indicator of lysosomal activity and a marker for both the diagnosis and monitoring of lysosomal storage diseases.

Accordingly, some embodiments of the present invention relate to methods of reducing or otherwise eliminating the presence or accumulation of one or more pathological or biological markers associated with the NaGlu associated disease. Similarly, some embodiments of the invention relate to methods of increasing the degradation (or the rate of degradation) of one or more pathological or biological markers (*e.g.,* LAMP1) associated with lysosomal storage diseases.

In some embodiments, treatment refers to decreased progression of loss of cognitive ability. In certain embodiments, progression of loss of cognitive ability is decreased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or more as compared to a control. In some embodiments, treatment refers to decreased developmental delay. In certain embodiments, developmental delay is decreased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or more as compared to a control. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, treatment refers to increased survival (*e.g.,* survival time). For example, treatment can result in an increased life expectancy of a patient. In some embodiments, treatment according to the present invention results in an increased life expectancy of a patient by more than about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 100%, about 105%, about 1 10%, about 115%, about 120%, about 125%, about 130%, about 135%, about 140%, about 145%, about 150%, about 155%,

about 160%, about 165%, about 170%, about 175%, about 180%, about 185%, about 190%, about 195%, about 200% or more, as compared to the average life expectancy of one or more control individuals with similar disease without treatment. In some embodiments, treatment according to the present invention results in an increased life expectancy of a patient by more than about 6 month, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 12 months, about 2 years, about 3 years, about 4 years, about 5 years, about 6 years, about 7 years, about 8 years, about 9 years, about 10 years or more, as compared to the average life expectancy of one or more control individuals with similar disease without treatment. In some embodiments, treatment according to the present invention results in long term survival of a patient. As used herein, the term "long term survival" refers to a survival time or life expectancy longer than about 40 years, 45 years, 50 years, 55 years, 60 years, or longer. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

The terms, “improve,” “increase” or “reduce,” as used herein, indicate values that are relative to a control. In some embodiments, a suitable control is a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of the treatment described herein. A "control individual" is an individual afflicted with Sanfilippo syndrome B, who is about the same age and/or gender as the individual being treated (to ensure that the stages of the disease in the treated individual and the control individual(s) are comparable).

The individual (also referred to as “patient” or “subject”) being treated is an individual (fetus, infant, child, adolescent, or adult human) having Sanfilippo syndrome B or having the potential to develop Sanfilippo syndrome B. The individual can have residual endogenous NaGlu expression and/or activity, or no measurable activity. For example, the individual having Sanfilippo Syndrome B may have NaGlu expression levels that are less than about 30-50%, less than about 25-30%, less than about 20-25%, less than about 15-20%, less than about 10-15%, less than about 5-10%, less than about 0.1-5% of normal NaGlu expression levels. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, the individual is an individual who has been recently diagnosed with the disease. Typically, early treatment (treatment commencing as soon as possible after diagnosis) is important to minimize the effects of the disease and to maximize the benefits of treatment.

I. Combination Therapies

Recombinant human NaGlu proteins, for instance a recombinant human NaGlu protein containing a sufficient amount of oligosaccharides (*e.g.,* mannose and phosphorylated mannose (*i.e.,* M6P)), can be used alone or in combination to treat NaGlu associated diseases (*e.g.,* Sanfilippo Syndrome B). It should be understood that the recombinant human NaGlu proteins of the invention can be used alone or in combination with an additional procedure, *e.g.,* surgical procedure, or agent, *e.g.,* therapeutic agent, the additional procedure or agent being selected by the skilled artisan for its intended purpose. For instance, the additional procedure or agent can be a therapeutic procedure or agent art-recognized as being useful to treat the disease or condition being treated by the recombinant human NaGlu protein of the present invention. The additional procedure or agent also can be an agent that imparts a beneficial attribute to the therapeutic composition, *e.g.,* an agent which affects the viscosity of the composition.

It should also be understood that the combinations which are included within this invention are those combinations useful for their intended purpose. The agents and procedures set forth below are for illustrative purposes and not intended to be limiting to the present invention. The combinations, which are part of this invention, can be the recombinant human NaGlu proteins of the present invention and at least one additional agent or procedure selected from the lists below. The combination can also include more than one additional agent or procedure, *e.g.,* two or three additional agents if the combination is such that the formed composition can perform its intended function.

The combination therapy can include surgical procedures, gene therapy, or enzyme-replacement therapy. Additionally, the recombinant human NaGlu protein can be coformulated with one or more additional therapeutic agents, *e.g.,* other recombinant

proteins or antibodies or drugs capable of preventing or reducing the accumulation of undegraded substrates (*e.g.,* substrate reduction therapy).

In one or more embodiments, the combination therapy can include co-administration with immunosuppresants, as discussed in further detail below. Immunosuppresants such as, but not limited to, antihistamines, corticosteroids, sirolimus, voclosporin, ciclosporin, methotrexate, IL-2 receptor directed antibodies, T-cell receptor directed antibodies, TNF-alpha directed antibodies or fusion proteins (*e.g.,* infliximab, etanercept, or adalimumab), CTLA-4-Ig (*e.g.,* abatacept), anti-OX-40 antibodies can also be administered before, during, or after administration of a recombinant human protein, such as a recombinant human NaGlu protein, for example, if an anaphylactic reaction or adverse immune response is expected or experienced by a patient.

J. Immunogenicity

The pharmaceutical compositions of the present invention are characterized by their tolerability. As used herein, the terms "tolerable" and "tolerability" refer to the ability of the pharmaceutical compositions of the present invention to not elicit an adverse reaction in the subject to whom such composition is administered, or alternatively not to elicit a serious adverse reaction in the subject to whom such composition is administered. In some embodiments, the pharmaceutical compositions of the present invention are well tolerated by the subject to whom such compositions is administered.

Generally, administration of a rhNaGlu protein according to the present invention does not result in severe adverse effects in the subject. As used herein, severe adverse effects induce, but are not limited to, substantial immune response, toxicity, or death. As used herein, the term "substantial immune response" refers to severe or serious immune responses, such as adaptive T-cell immune responses.

Thus, in many embodiments, inventive methods according to the present invention do not involve concurrent immunosuppressant therapy (*i.e.,* any immunosuppressant therapy used as pre-treatment/pre-conditioning or in parallel to the method). In some embodiments, inventive methods according to the present invention

do not involve an immune tolerance induction in the subject being treated. In some embodiments, inventive methods according to the present invention do not involve a pre-treatment or preconditioning of the subject using T-cell immunosuppressive agent.

However, in some embodiments, a subject mounts an immune response after being administered the rhNaGlu of the invention. Thus, in some embodiments, it may be useful to render the subject receiving the rhNaGlu of the invention tolerant to the enzyme replacement therapy. Immune tolerance may be induced using various methods known in the art. For example, an initial 30-60 day regimen of a T-cell immunosuppressive agent such as cyclosporin A (CsA) and an antiproliferative agent, such as, azathioprine (Aza), combined with weekly intrathecal infusions of low doses of a desired replacement enzyme may be used.

Any immunosuppressant agent known to the skilled artisan may be employed together with a combination therapy of the invention. Such immunosuppressant agents include but are not limited to cyclosporine, FK506, rapamycin, CTLA4-Ig, and anti-TNF agents such as etanercept (see *e.g.,* Moder, 2000, Ann. Allergy Asthma Immunol. 84, 280-284; Nevins, 2000, Curr. Opin. Pediatr. 12, 146-150; Kurlberg et al., 2000, Scand. J. Immunol. 51, 224-230; Ideguchi et al., 2000, Neuroscience 95, 217-226; Potteret al., 1999, Ann. N.Y. Acad. Sci. 875, 159-174; Slavik et al., 1999, Immunol. Res. 19, 1-24; Gaziev et al., 1999, Bone Marrow Transplant. 25, 689-696; Henry, 1999, Clin. Transplant. 13, 209-220; Gummert et al., 1999, J. Am. Soc. Nephrol. 10, 1366-1380; Qi et al., 2000, Transplantation 69, 1275-1283). The anti-IL2 receptor (α-subunit) antibody daclizumab (*e.g.,* Zenapax™), which has been demonstrated effective in transplant patients, can also be used as an immunosuppressant agent (see *e.g.,* Wiseman et al., 1999, Drugs 58, 1029-1042; Beniaminovitz et al., 2000, N. Engl J. Med. 342, 613-619; Ponticelli et al., 1999, Drugs R. D. 1, 55-60; Berard et al., 1999, Pharmacotherapy 19, 1 127-1 137; Eckhoff et al., 2000, Transplantation 69, 1867-1872; Ekberg et al., 2000, Transpl. Int. 13, 151-159). Additional immunosuppressant agents include but are not limited to anti-CD2 (Branco et al., 1999, Transplantation 68, 1588-1596; Przepiorka et al., 1998, Blood 92, 4066-4071), anti-CD4 (Marinova-Mutafchieva et al., 2000, Arthritis Rheum. 43, 638-644; Fishwild et al., 1999, Clin. Immunol. 92, 138-152), and anti-CD40

ligand (Hong et al., 2000, Semin. Nephrol. 20, 108-125; Chirmule et al., 2000, J. Virol. 74, 3345-3352; Ito et al., 2000, J. Immunol. 164, 1230-1235).

In other embodiments, the invention includes methods comprising co-administration of the NaGlu proteins of the present invention with agents which decrease or suppress an immune response to the NaGlu protein, *e.g.*, immunosuppresants. Immunosuppresants such as, but not limited to, antihistamines, corticosteroids, sirolimus, voclosporin, ciclosporin, methotrexate, IL-2 receptor directed antibodies, T-cell receptor directed antibodies, TNF-alpha directed antibodies or fusion proteins (*e.g.,* infliximab, etanercept, or adalimumab), CTLA-4-Ig (*e.g.,* abatacept), anti-OX-40 antibodies can also be administered before, during, or after administration of a recombinant human protein, such as a recombinant human NaGlu protein, for example, if an anaphylactic reaction or adverse immune response is expected or experienced by a patient.

In one embodiment, the invention provides for a pretreatment procedure to minimize or prevent any potential anaphylactic reactions that can be incurred by administration of the recombinant protein in accordance with the invention. In one embodiment, to prevent a potential anaphylactic reaction, an H-1 receptor antagonist, also known as an antihistamine (*e.g.,* diphenhydramine) is administered to the patient. In one embodiment, the H-1 receptor antagonist is administered in a dose of about 1 mg to about 10 mg per kilogram of body weight. For example, an antihistamine can be administered in a dose of about 5 mg per kilogram. In one embodiment, the antihistamine is administered in a dose of between about 0.1 mg and about 10 mg per kilogram of body weight. In one embodiment, the antihistamine is administered in a dose between about 1 mg and about 5 mg per kilogram of body weight. For example the dose can be 1 mg, 2 mg, 3 mg, 4 mg, or 5 mg per kilogram of body weight. The antihistamine can be administered by any useful method. In one embodiment, the antihistamine is administered intravenously. In another embodiment, the antihistamine is administered in pharmaceutically acceptable capsules.

Administration of the antihistamine can be prior to the administration of the recombinant NaGlu in accordance with the invention. In one embodiment, the H-1 receptor antagonist is administered about 10 to about 90 minutes, for example, about 30

to about 60 minutes prior to the administration of recombinant NaGlu. The H-1 receptor antagonist can be administered using an ambulatory system connected to a vascular access port. In one embodiment, the antihistamine is administered about 90 minutes prior to the administration of recombinant NaGlu. In one embodiment, the antihistamine is administered between about 10 and about 60 minutes prior to the administration of recombinant NaGlu. In another embodiment, the antihistamine is administered between about 20 and about 40 minutes prior to administering recombinant NaGlu. For example, the antihistamine can be administered 20, 25, 30, 35, or 40 minutes prior to the administration of recombinant NaGlu.

In one embodiment, the antihistamine administered is diphenhydramine. Any useful antihistamine can be used. Such antihistamines include, without limitation, clemastine, doxylamine, loratidine, desloratidine, fexofenadine, pheniramine, cetirizine, ebastine, promethazine, chlorpheniramine, levocetirizine, olopatadine, quetiapine, meclizine, dimenhydrinate, embramine, dimethidene, and dexchloropheniramine.

In another embodiment, with reference to intravenous infusion, the potential for anaphylactic reactions can be reduced by administering the infusions using a ramp-up protocol. In this context, a ramp-up protocol refers to slowly increasing the rate of the infusion over the course of the infusion in order to desensitize the patient to the infusion of the medication.

K. Administration

The methods of the present invention contemplate single as well as multiple administrations of a therapeutically effective amount of the rhNaGlu of the invention described herein. The rhNaGlu of the invention can be administered at regular intervals, depending on the nature, severity and extent of the subject's condition. In some embodiments, a therapeutically effective amount of the rhNaGlu protein of the present invention may be administered intravenously or intrathecally periodically at regular intervals (*e.g.,* once every year, once every six months, once every five months, once every three months, bimonthly (once every two months), monthly (once every month), biweekly (once every two weeks) or weekly).

In some embodiments, intrathecal administration may be used in conjunction with other routes of administration (*e.g.,* intravenous, subcutaneously, intramuscularly, parenterally, trans dermally, or transmucosally (*e.g.,* orally or nasally)). In some embodiments, those other routes of administration (*e.g.,* intravenous administration) may be performed no more frequent than biweekly, monthly, once every two months, once every three months, once every four months, once every five months, once every six months, annually administration.

As used herein, the term "therapeutically effective amount" is largely determined based on the total amount of the therapeutic agent contained in the pharmaceutical compositions of the present invention. Generally, a therapeutically effective amount is sufficient to achieve a meaningful benefit to the subject (*e.g.,* treating, modulating, curing, preventing and/or ameliorating the underlying disease or condition). For example, a therapeutically effective amount may be an amount sufficient to achieve a desired therapeutic and/or prophylactic effect, such as an amount sufficient to modulate lysosomal enzyme receptors or their activity to thereby treat such lysosomal storage disease or the symptoms thereof (*e.g.,* a reduction in or elimination of the presence or incidence of "zebra bodies" or cellular vacuolization following the administration of the compositions of the present invention to a subject). Generally, the amount of a therapeutic agent (*e.g.,* the rhNaGlu of the invention) administered to a subject in need thereof will depend upon the characteristics of the subject. Such characteristics include the condition, disease severity, general health, age, sex and body weight of the subject. One of ordinary skill in the art will be readily able to determine appropriate dosages depending on these and other related factors. In addition, both objective and subjective assays may optionally be employed to identify optimal dosage ranges.

A therapeutically effective amount is commonly administered in a dosing regimen that may comprise multiple unit doses. For any particular therapeutic protein, a therapeutically effective amount (and/or an appropriate unit dose within an effective dosing regimen) may vary, for example, depending on route of administration, on combination with other pharmaceutical agents. Also, the specific therapeutically effective amount (and/or unit dose) for any particular patient may depend upon a variety of factors including the disorder being treated and the severity of the disorder; the

activity of the specific pharmaceutical agent employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and/or rate of excretion or metabolism of the specific fusion protein employed; the duration of the treatment; and like factors as is well known in the medical arts.

In some embodiments, the therapeutically effective dose ranges from about 0.005 mg/kg body weight to 500 mg/kg body weight, *e.g.,* from about 0.005 mg/kg body weight to 400 mg/kg body weight, from about 0.005 mg/kg body weight to 300 mg/kg body weight, from about 0.005 mg/kg body weight to 200 mg/kg body weight, from about 0.005 mg/kg body weight to 100 mg/kg body weight, from about 0.005 mg/kg body weight to 90 mg/kg body weight, from about 0.005 mg/kg body weight to 80 mg/kg body weight, from about 0.005 mg/kg body weight to 70 mg/kg body weight, from about 0.005 mg/kg body weight to 60 mg/kg body weight, from about 0.005 mg/kg body weight to 50 mg/kg body weight, from about 0.005 mg/kg body weight to 40 mg/kg body weight, from about 0.005 mg/kg body weight to 30 mg/kg body weight, from about 0.005 mg/kg body weight to 25 mg/kg body weight, from about 0.005 mg/kg body weight to 20 mg/kg body weight, from about 0.005 mg/kg body weight to 15 mg/kg body weight, from about 0.005 mg/kg body weight to 10 mg/kg bra body in weight. Ranges and values intermediate to the above recited ranges and values (*e.g.,* 10-50 mg/kg, 1-5 mg/kg, 2-8 mg/kg, 5-10 mg/kg, 0.1-10 mg/kg, 0.3-30 mg/kg, 0.3-50 mg/kg, 0.5-10 mg/kg, 5-30 mg/kg, or 6-27 mg/kg) are also contemplated to be part of the invention.

In some embodiments, the therapeutically effective dose is greater than or at least about 0.1 mg/kg body weight, greater than or at least about 0.2 mg/kg body weight, greater than or at least about 0.3 mg/kg body weight, greater than or at least about 0.4 mg/kg body weight, greater than or at least about 0.5 mg/kg body weight, greater than or at least about 1.0 mg/kg body weight, greater than or at least about 3 mg/kg body weight, greater than or at least about 5 mg/kg body weight, greater than or at least about 6 mg/kg body weight, greater than or at least about 7 mg/kg body weight greater than or at least about 10 mg/kg body weight, greater than or at least about 15 mg/kg body weight, greater than or at least about 20 mg/kg body weight, greater than or at least

about 30 mg/kg body weight, greater than or at least about 40 mg/kg body weight, greater than or at least about 50 mg/kg body weight, greater than or at least about 60 mg/kg body weight, greater than or at least about 70 mg/kg body weight, greater than about or at least 80 mg/kg body weight, greater than or at least about 90 mg/kg body weight, greater than or at least about 100 mg/kg body weight. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

In some embodiments, the therapeutically effective dose may also be defined by mg/kg brain weight. As one skilled in the art would appreciate, the brain weights and body weights can be correlated (see, *e.g.,* Dekaban AS. "Changes in brain weights during the span of human life: relation of brain weights to body heights and body weights," Ann Neurol 1978; 4:345-56).

In some embodiments, the therapeutically effective dose may also be defined by mg/15 cc of CSF. As one skilled in the art would appreciate, therapeutically effective doses based on brain weights and body weights can be converted to mg/15 cc of CSF. For example, the volume of CSF in adult humans is approximately 150 mL (Johanson CE, *et al.* "Multiplicity of cerebrospinal fluid functions: New challenges in health and disease," Cerebrospinal Fluid Res. 2008 May 14;5: 10). Therefore, single dose injections of 0.1 mg to 50 mg protein to adults would be approximately 0.01 mg/15 cc of CSF (0.1 mg) to 5.0 mg/15 cc of CSF (50 mg) doses in adults.

It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the enzyme replacement therapy and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed invention.

VIII. Kits

The present invention further provides kits or other articles of manufacture which contain the recombinant human NaGlu of the present invention and provide instructions for its reconstitution (if lyophilized) and/or use. Kits or other articles of manufacture may include a container, a catheter and any other articles, devices or equipment useful in

intrathecal administration and associated surgery. Suitable containers include, for example, bottles, vials, syringes (*e.g.,* pre-filled syringes), ampules, cartridges, reservoirs, or lyo-jects. The container may be formed from a variety of materials such as glass or plastic. In some embodiments, a container is a pre-filled syringe. Suitable pre-filled syringes include, but are not limited to, borosilicate glass syringes with baked silicone coating, borosilicate glass syringes with sprayed silicone, or plastic resin syringes without silicone.

Typically, a label on, or associated with, the container may indicate directions for use and/or reconstitution. For example, the label may indicate that the formulation is reconstituted to protein concentrations as described above. The label may further indicate that the formulation is useful or intended for, for example, intravenous or intrathecal administration. In some embodiments, a container may contain a single dose of a stable formulation containing a replacement enzyme (*e.g.,* a recombinant NaGlu protein). In various embodiments, a single dose of the stable formulation is present in a volume of less than about 15 mL, 10 mL, 5.0 mL, 4.0 mL, 3.5 mL, 3.0 mL, 2.5 mL, 2.0 mL, 1.5 mL, 1.0 mL, or 0.5 mL. Alternatively, a container holding the formulation may be a multi-use vial, which allows for repeat administrations (*e.g.,* from 2-6 administrations) of the formulation. Kits or other articles of manufacture may further include a second container comprising a suitable diluent (*e.g.,* BWFI, saline, buffered saline). Upon mixing of the diluent and the formulation, the final protein concentration in the reconstituted formulation will generally be at least 1 mg/mL (*e.g.,* at least 5 mg/mL, at least 10 mg/mL, at least 25 mg/mL, at least 50 mg/ mL, at least 75 mg/mL, at least 100 mg/mL).

Kits or other articles of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, catheters, syringes, and package inserts with instructions for use. Ranges and values intermediate to the above recited ranges and values are also contemplated to be part of the invention.

EXAMPLES

The following specific examples are intended to illustrate the invention and should not be construed as limiting the scope of the claims. The contents of all figures and all references, patents and published patent applications cited throughout this application, as well as the Figures, are expressly incorporated herein by reference in their entirety.

Example 1

Purification of rhNaGlu

rhNaGlu protein was purified by using methods known in the art. Egg white (EW) containing rhNaGlu was solubilized at pH 6 overnight and clarified through centrifugation and/or depth filtration. The EW was adjusted with 1 M NaOAc buffer (pH 4) to pH 6. For the depth filtration process, T2600 filter (Pall™, 40 um) was used as a 1st filtration and then PDF1 (Pall™, K200P,15 um + EKS, 0.22 um) as a 2nd filtration step. The filters are single-use membrane with an optimized capacity 60 L EW/m^2 for each filter. The hold volume of membrane is 2 L/m^2 for T2600 and 4-5 L/m^2 for PDF1. In the process, the hold volume was discarded before the filtered EW collected. The buffer (20 mM Phosphate/137 mM NaCl, pH 6) equivalent to the membrane hold volume was used to chase EW left on the filters.

A phenyl-HIC (hydrophobic interaction chromatography) column was applied as a capture step. Since most of egg white proteins are hydrophilic, 99% of egg white proteins passed through the HIC column into flow through. rhNaGlu has a higher hydrophobicity binding to phenyl-HIC.

Egg white containing rhNaGlu was loaded onto the column with a ratio of 30:1. After completion of loading, the column was washed with the equilibration buffer, 5 mM phosphate buffer, pH 6, and 5 mM Tris buffer, pH 7.2. rhNaGlu was eluted with 30% propylene glycol, pH 7.2. After the completion of loading, the column was washed with equilibration buffer and 5 mM phosphate buffer (pH 6). rhNaGlu was eluted with 30% propylene glycol with 5 mM Tris buffer (pH 7.2). The column binding capacity is approximately 4.5 mg/mL. The purity of rhNaGlu through the phenyl-HIC column can

be reached to >95% (950 time increase). The recovery is approximately 80% with 30% of propylene glycol elution.

The eluted rhNaGlu fraction was adjusted to pH 5 with 1 M acetic acid and then loaded onto a GigaCap S column (EW: column size=10:1). The column was equilibrated with 50 mM NaOAc buffer (pH 5). After completion of loading, the column was washed with the equilibration buffer. The rhNaGlu was eluted with 50 mM NaOAc/60 mM NaCl (pH 5).

The protein characterization was performed using purified rhNaGlu. The molecular weight of rhNaGlu (~90 kDa) purified from egg white was analyzed on SDS-PAGE (Fig. 6). The average expression level of rhNaGlu in egg white is shown in Fig. 7. The characteristics of rhNaGlu produced from the transgenic avian are summarized in Table 2.

Table 2.

	rhNaGlu (*Gallus*)
Apparent Molecular Weight	~90 kDa
pI	6.1-6.9
pH Stability	pH 5-8
Stability in Egg White	> 50 days

Example 2

Stability of rhNaGlu in egg white

A single egg was cracked 7 days post-lay and analyzed for activity. Contents were divided in half and each half was subject to standard egg white clarification. Both untreated and clarified egg whites were aliquoted and stored at 4°C and -20°C for enzyme activity stability. rhNaGlu in egg white showed stable enzyme activity at least up to 50 days.

Freeze/thaw cycle stability was assessed. The purified rhNaGlu was frozen in liquid nitrogen for 10 seconds and thawed at 37 °C for 2 min. The enzyme activity showed no change for 10 cycles.

The purified rhNaGlu was dialyzed into different pH buffers to measure the stability of pure enzyme. The results showed that pure rhNaGlu was stable between pH 5-8 for 12 days.

Example 3

Oligosaccharide Profiling

Mannose-6-phosphate (M6P) is a terminal monosaccharide of N-linked oligosaccharides that is an important part of the tertiary structure of glycoprotein and, when incorporated in the glycoprotein's final oligosaccharide, is recognized by and bound to the M6P receptors present on the cell surface, subsequently allowing internalization into the lysosomes. Thus, M6P is an effective epitope for the targeting of glycoproteins to the lysosomes.

Analysis of protein glycosylation is an important part of glycoprotein characterization. Oligosaccharides can be linked to a protein through a serine or a threonine as O-lined glycans or through an asparagine as N-linked glycans.

To analyze the structure of oligosaccharides, various chromatographic and spectroscopic techniques were performed. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was employed. Using this technique, oligosaccharides were quickly separated into general groups based on charge (*i.e.,* neutral, singly charged, or multiply charged) and their structures were determined by comparison to pure standards.

All methods were based on protocols described by Hardy and Townsend (Hardy, M. R., and Townsend, R. R., "High-pH anion-exchange chromatography of glycoprotein-derived carbohydrates", 1994, *Methods Enzymol.* 230: 208-225). Purified samples of transgenic avian derived rhNaGlu were dialyzed using a Tube-O-Dialyzer against nanopure water at 4°C for about 24 hours to remove salts and other contaminants. Nanopure water was replaced four times during the entire dialysis period. After dialysis, each of the samples was divided into three aliquots. The aliquot intended

for neutral and amino sugars analysis was hydrolyzed with 2 N trifluoroacetic acid (TFA) at 100°C for 4 hours and the aliquot for mannose-6-phosphate analysis was hydrolyzed with 6.75 N TFA at 100°C for 1.5 hours. The hydrolysates were then dried under N_2, re-dissolved with 50 µL H_2O, sonicated for 7 min in ice and transferred to an injection vial.

A mix of standards for neutral and amino sugars, and for mannose-6-phosphate with a known number of moles was hydrolyzed in the same manner and at the same time as the sample. Four different concentrations of the neutral and amino sugar standard mix and mannose-6-phosphate were prepared to establish a calibration equation. The number of moles of each sugar in the sample was quantified by linear interpolation from the calibration equation.

The oligossacharide profile and mannose-6-phosphate profile were analyzed separately by HPAEC-PAD. Instrument control and data acquisition were accomplished using Dionex chromeleon software. HPAEC-PAD analysis of hydrolyzed rhNaGlu detected M6P. The mean measured amount of M6P was 3.8 µg (CV 3.7%) per 210 µg of hydrolyzed protein. Converting to moles resulted in 13.4 nmol of M6P per 2.8 nmol of protein which was equivalent to a ratio of 3.2 moles of M6P per mole of protein.

The oligosaccharide profile was also obtained for rhNaGlu (*Gallus*) using HPAEC-PAD (see Fig. 8). The profiles demonstrated good repeatability of the PNGase F reaction on the single sample. Peak clusters were observed in regions corresponding to neutral oligosaccharides (~10 min to ~20 min). A group of significantly smaller peaks eluting between ~25 and ~35 min were also observed, which were possibly attributed to singly charged species.

The monosaccharide composition analysis results obtained from samples of rhNaGlu produced from a transgenic avian (*Gallus*) are summarized in Table 3, which tabulates the average molar ratio of each monosaccharide analyzed for rhNaGlu.

Table 3. Monosaccharide Molar Ratios in rhNaGlu (*Gallus*)

N-acetylgalactosamine (GalNAc)	1.1*
N-acetylglucosamine (GlcNAc)	35.6*
Galactose (Gal)	4*
Mannose (Man)	25.5*
Mannose-6-phosphate (M6P)	3.2*
Fucose	Not detected
Glucose	Not detected

* mole of monosaccharide per mole of protein

Example 4

Cellular Uptake into Fibroblasts

Wild-type human fibroblasts and mucopolysaccharidosis III B (NaGlu deficient) human fibroblasts were placed in a 24-well plate (2.5 x 10^4 cells per well) and incubated for overnight at 37°C in 5% CO_2. Conditioned media containing fibroblast basal medium and fibroblast growth kit having low serum were used. Various amounts of rhNaGlu (30, 10, 3.0, 1.0, 0.3 and 0 µg/mL) were co-incubated for 24 hours at 37°C with 5% CO_2 to determine levels of cellular uptake by the human fibroblasts (see, Fig. 9). The wells were washed three times with PBS. 100µL lysis buffer was added per well and the plate was incubated for 10 min at 37°C. Cell lysate was transferred into 1.5 mL centrifuge tube. One cycle of freezing and thawing was performed. The cell lysate was centrifuged at 10,000 rpm for 10 min. 25µL of supernatants were used for the assay. The assay time was 2 hours. The enzyme activity was measured using the methods known in the art and according to the methods described in Marsh *et al.*, *Clinical Genetics* (1985) 27: 258-262, Chow *et al.*, *Carbohydrate Research* (1981) 96:87-93; Weber *et al.*, *Protein Expression and Purification*, (2001)21:251-259).

As shown in Fig. 9, negative control (*i.e.*, MPS IIIB) did not exhibit any NaGlu activity while positive control (*i.e.*, wild-type human fibroblast) showed NaGlu activity. MPS IIIB cells treated with 0.3 µg/mL of rhNaGlu exhibited approximately 50% of the normal activity level observed in wild-type fibroblast cells. MPS IIIB cells treated with 1 µg/mL of rhNaGlu demonstrated NaGlu activity that was approximately 4-fold higher than that observed in wild-type cells. Surprisingly, MPS IIIB cells treated with 30 µg/mL of rhNaGlu showed NaGlu activity that was at least 40-fold higher than that

observed in wild-type cells. This result indicated that rhNaGlu produced from a transgenic avian (*Gallus*) was efficiently internalized into human fibroblasts at a high level.

To determine whether internalization of rhNaGlu is via M6P receptor mediate endocytosis, M6P inhibition assays were performed. For the M6P inhibition assays, various concentrations of free M6P were added to human MPS IIIB fibroblasts treated with 30μg/mL of rhNaGlu and enzymatic activity was measured as described above. As shown in Fig. 10, human MPS IIIB fibroblasts did not exhibit any NaGlu activity, suggesting effective inhibition of NaGlu uptake by free M6P. In contrast, MPSIII fibroblasts treated with 30 μg/mL of rhNaGlu in the absence of free M6P exhibited a high level of enzymatic activity, suggesting that the protein was efficiently internalized into the NaGlu deficient fibroblasts and retained activity. This enzymatic activity was inhibited by the presence of M6P monosaccharide in the medium at the concentration 0.03 mM and higher. The presence of 1mM of M6P monosaccharide in conditioned medium inhibited more than 90% of cellular uptake of the protein.

These results indicated that the rhNaGlu produced from a transgenic avian was efficiently internalized into the MPS IIIB fibroblasts via M6P receptor-mediated endocytosis and the rhNaGlu competed with M6P monosaccharides for the receptor recognition. The results were consistent with the glycan analysis that revealed the presence of the M6P structures on the rhNaGlu produced from the transgenic avian.

Example 5

Generation of Active NaGlu Fusion Proteins

Two different rhNaGlu fusion constructs were designed to validate the feasibility of expressing rhNaGlu fusion proteins in the avian expression system.

In one construct, a nucleic acid sequence encoding 8 consecutive aspartic acid residues (DDDDDDDD) was fused to the nucleic sequence encoding NaGlu protein at the 5' end of the full-length NaGlu cDNA sequence (SEQ ID NO:2) using conventional PCR and DNA recombinant technology. In another construct, a nucleic acid sequence encoding TfRL (*i.e.,* THRPPMWSPVWP; SEQ ID NO:5) was fused to the nucleic sequence encoding NaGlu at the 3' end of the full-length NaGlu cDNA sequence. The

each construct was inserted into the pTT22 expression vector using EcoRI and HindIII restriction sites. The resulting vectors were each transfected into human embryonic kidney (HEK) 293 cells and stable clones expressing high levels of the fusion NaGlu proteins were obtained. An rhNaGlu protein fused to a stretch of 8 consecutive aspartic acid residues at N-terminus (AAA-NaGlu) and an rhNaGlu protein fused to transferrin receptor ligand (TfRL) at C-terminus (NaGlu-TfRL) were isolated from conditioned media.

The enzymatic activity of AAA-NaGlu and NaGlu-TfRL was measured using the methods known in the art (see, *e.g.,* Marsh *et al.*, *Clinical Genetics* (1985) 27:258-262; Chow *et al.*, *Carbohydrate Research*, (1981) 96:87-93; Weber *et al.*, *Protein Expression and Purification* (2001) 21:251-259; Neufeld *et al., Protein Expression and Purification* (2000) 19:202-211; and Weber *et al.*, *Human Molecular Genetics* (1996) 5:771-777.

As shown in Figs. 13 and 14, AAA-NaGlu and NaGlu-TfRL fusion proteins produced from HEK293 cells showed high levels of enzymatic activity. These results confirmed the possibility that these constructs can be used to produce NaGlu fusion proteins that have increased levels of phosphorylated mannose while retaining enzymatic activity from a transgenic avian expression system.

Example 6

Cellular Uptake into Macrophages

Internalization of rhNaGlu produced from *Gallus* into human macrophage cells was also measured. NR8383 macrophage cells were incubated with 10µg/mL of rhNaGlu in F12 growth media for 0, 4, 8, 24, 32 and 48 hours at 37 °C with 5% CO_2. Samples were recovered and washed with PBS prior to lysis. 2.5 x 10^5 cells were lysed in 1 mL of lysis buffer (10mM of Na Phosphate pH6.0, 0.05% NP40), and lysates transferred into 1.5 mL centrifuge tubes and centrifuged at 10,000 rpm for 10 min. Protein concentration was determined by the Bradford assay and aliquots were frozen for NaGlu enzyme assays.

Enzyme activity was measured using standard methods. 25mM of substrate (4-methylumbelliferyl 2-Acetamido-2-deoxy-a-D-glucopyranoside) was diluted to 2mM in nanopure water to form a working substrate stock. Dilutions of samples were prepared

in assay buffer (1% bovine serum albumin). 25µL of 200mM sodium acetate was distributed to wells of a multi-well plate. 25µL of standard and 25µL of samples were added to designated wells. 50µL of the working substrate stock was added to each well and the plate was gently tapped to mix. The plate was sealed with adhesive film and incubated at 37°C for 30 minutes. The reaction was then terminated by addition of 50µL of stop solution (1M Glycine pH 12.5). The plate was placed on a microplate reader using a fluorescence bottom and the intensity was measured at an excitation 360nm and an emission 460nm. The level of liberated 4-methylumbelliferone (4-MU) was measured by comparison with standards of 4-MU at 0.25mM, 0.125mM, 0.0625mM, 0.0312mM, 0.0156mM, and 0.0078mM.

As depicted in Fig. 15, levels of the NaGlu activity in macrophages incubated with 10µg/mL of rhNaGlu increased almost linearly over a 48 hour period: The rhNaGlu uptake by macrophages was rather slow, but steady throughout the entire time period measured. The relatively slow, extended uptake of NaGlu activity (as compared to other lysosomal enzymes containing M6P and/or mannose in their glycosylation structures) was unexpected and surprising. Equally surprising and unexpected was that a large amount of rhNaGlu proteins was taken up into the macrophages over the extended time period, resulting in intracellular enzymatic activity levels at least 10, 50, 100, 200, 300, 500, or even 1,000-fold higher than the basal levels observed in wild-type macrophages not exposed to rhNaGlu. The results demonstrate that rhNaGlu is extremely stable in extracellular as well as intracellular environments. Further, these results suggest that rhNaGlu may possess physicochemical characteristics that allow for longer serum half-life (*e.g.,* longer circulation) and high serum concentrations *in vivo*, properties which are ideal for enhanced uptake into the central nervous system (CNS).

Table 4. Summary of NaGlu Characteristics

	Avian (*gallus*) produced rhNaGlu	Natural human NaGlu	CHO produced human NaGlu
Apparent Molecular Mass (kDa)	~85 - ~90	~86	~79 - ~89
Enzymatic Activity (nmol/min/mg)	>1,000	~500	~1,057
Mannose-6-phosphate	High	High	None or very Low

Example 7

Administration of rhNaGlu into NaGlu deficient mice

Homozygous null mice were generated from breeding pairs of the strain B6.129S6-*NaGlu*^{tm1Efn}/J. Control wild-type mice were generated in the same manner. Genotyping was performed according to a standard PCR protocol. It is described in the art that at birth, homozygous *naglu*($^{-/-}$) null mice are viable, normal in size, and do not display any gross physical or behavioral abnormalities, though they exhibited no NaGlu in all tissues (*see*, Li et al., (1999) *Proc., Natl. Acad. Sci. USA* 96:14505-14510). At one month of age, vacuolated macrophages are found in most tissues. Epithelial cells in kidney and neurons in some parts of the brain are also affected. The vacuolation becomes more prominent with age. At 4-5 months, the mice show abnormal behavior in an open field test. Older animals may have urinary retention and difficulty walking. Typical life span of the homozygous null *naglu* $^{-/-}$ mice is 8-12 months (*see*, Li et al., (1999) *Proc., Natl. Acad. Sci. USA* 96:14505-14510).

Intravenous (IV) administration

The intravenous administration of test article and vehicle by tail vein injection was accomplished as follows. Before injection vasodilation was achieved by gently warming the animal with an incandescent lamp or by soaking the tail in warm water, approximately 43°C. The animal was then placed in restraint device. The surface of the tail was disinfected with 70% isopropanol prior to injection. The lateral veins of the tail are located just under the skin and are identified in the distal part of the tail with the application of tension. A 27G needle, bevel up, was inserted into the vein for 3 – 4 mm.

The test article or vehicle was then administered as a slow bolus injection over a period of ten seconds as evidenced by the observed clearing of the vein as the administered liquid momentarily occupies the vascular space. After removal of needle, gentle pressure was applied to the puncture site to provide hemostasis. The animal was monitored immediately following procedure to assure normal activity.

Intrathecal (IT) administration

The intrathecal administration of test article and vehicle by lumbar puncture injection was accomplished as follows. Before injection, animals were anesthetized using isoflurane that was maintained via nose cone throughout the procedure. The site of injection was prepared by shaving the fur, as necessary, prior to each injection. The animal was placed in a prone position on a platform, ensuring the hind limbs were straddling the platform forming a convex curve of the animals back. The surface of the back was swabbed with 70% isopropanol and allowed to dry prior to injection. Spinal column and hip bones were palpated to locate the L4-L5 or L5-L6 margin. A 30G needle, bevel facing cranially, was inserted into the intervertebral space. Placement was confirmed by the observation of a tail flick. The test article or vehicle was then administered as bolus injection. The animal was allowed to recover from anesthesia and monitored immediately following procedure to assure normal activity and use of limbs.

Results

Twelve-week old *naglu* $^{-/-}$ mice (B6.129S6-*Naglu*^{tm1Efn}/J) were administered rhNaGlu (*Gallus*) at dose levels of 6.75 or 27 mg/kg via tail vein injection (IV administration), once every other day, for a total of 5 doses (at rhNaGlu concentrations of 1.125, or 4.5 mg/mL, respectively). Similarly, twelve-week old *naglu* $^{-/-}$ mice were administered with rhNaGlu (*Gallus*) at a dose level of 0.31 mg/kg via lumbar puncture injection (IT administration), once every other day, for a total of 5 doses at NaGlu concentrations of 1.54 mg/mL. Vehicle (10 mM phosphate buffer, 150 mM NaCl and 0.02% Tween80, pH 5.5-5.8) was administered to *naglu* $^{-/-}$ knock-out mice at the same dose concentration for 5 doses every other day. Untreated wild-type and *naglu* $^{-/-}$ knock-out mice were also maintained for the duration of the study.

Animals were sacrificed 4 hours after the fifth and final injection. All animals were necropsied and the liver, brain, spleen, heart, lung and kidneys were excised. Each organ was divided sagittally, providing samples for both frozen (-80°C) and formalin-fixed storage.

Tissue samples were analyzed for: (1) heparan sulfate concentration using an analytical method based on SAX-HPLC analysis of heparan sulfate disaccharides; and (2) α-*N*-acetylglucosaminidase enzyme activity using a cell-based enzyme activity assay.

Histopathologic evaluation of brain, liver, kidney, spleen, heart and lung tissue was conducted using formalin-fixed tissue samples, embedded in paraffin, sectioned at 4 µm, mounted on glass slides and stained with hematoxylin and eosin (H&E).

Following the repeated intravenous administration (5 doses over a 10 day period) of rhNaglu (*Gallus*) to *naglu*$^{-/-}$ mice at dose levels of 6.25 and 27 mg/kg body weight, there was an apparent dose-dependent decrease in the concentration of Heparan Sulfate in the brain, liver and kidney of *naglu*$^{-/-}$ mice (Table 5; Figs. 16-18). The relative α-N-acetylglucosaminidase activity was increased in the brain and liver following intravenous administration (Table 6). These results were unexpected and surprising because the NaGlu enzymatic activities and resulting substrate clearance were observed in the brain of the treated *naglu*$^{-/-}$ mice with the IV administration, suggesting that rhNaGlu (*Gallus*) administered systemically was distributed to the brain of the *naglu*$^{-/-}$ mice and effective to elicit efficacy even in the present of the blood brain barrier (BBB).

Following the intrathecal administration (5 doses over a 10 day period) of rhNaGlu (*Gallus*) to *naglu*$^{-/-}$ mice at a dose level of 0.31 mg/kg, there was a decrease in the concentration of Heparan Sulfate in the brain of *naglu*$^{-/-}$ mice (Table 5; Fig. 19), suggesting that rhNaGlu (Gallus) was targeted to the brain and effective in reducing the accumulated substrate in the brain of *naglu*$^{-/-}$ mice.

Table 5: Tissue Substrate Level (rhNaGlu *Gallus*)

Tissue	Animal Number	Genotype	Age at sacrifice (wks)	Treatment	Dose (mg/kg)	Route	Heparan Sulfate ug/mg tissue	mean	sd
KIDNEY	253	WT	4	na	-	-	0.1		
	155	WT	12	na	-	-	0.045	0.0725	0.038891
	178	KO	12	na	-	-	1.882		

	242	KO	4	na	-	-	1.687		
	145	KO	13	na	-	-	1.904		
	474	KO	13	vehicle	0	IV	1.501		
	479	KO	13	vehicle	0	IV	1.983		
	484	KO	13	vehicle	0	IV	1.839	1.799333	0.175908
	487	KO	13	rhNaGlu	6.25	IV	0.928		
	492	KO	13	rhNaGlu	6.25	IV	0.737	0.8325	0.135057
	481	KO	13	rhNaGlu	27	IV	0.591		
	485	KO	13	rhNaGlu	27	IV	0.311		
	490	KO	13	rhNaGlu	27	IV	0.585	0.495667	0.159954
	86	KO	15	vehicle	0	IT	2.105		
	91	KO	14	vehicle	0	IT	1.704	1.9045	0.28355
	94	KO	14	rhNaGlu	0.31	IT	1.324		
	101	KO	14	rhNaGlu	0.31	IT	2.233	1.7785	0.64276
LIVER	253	WT	4	na	-	-	0.045		
	155	WT	12	na	-	-	0.092	0.0685	0.033234
	243	WT	4	na	-	-	0.045		
	178	KO	12	na	-	-	1.85		
	242	KO	4	na	-	-	2.263	2.0565	0.292035
	255	KO	4	na	-	-	1.85		
	474	KO	13	vehicle	0	IV	1.822		
	479	KO	13	vehicle	0	IV	1.981		
	484	KO	13	vehicle	0	IV	2.004	1.961667	0.165779
	487	KO	13	rhNaGlu	6.25	IV	0.748		
	492	KO	13	rhNaGlu	6.25	IV	0.444		
	504	KO	13	rhNaGlu	6.25	IV	0.494	0.562	0.163009
	481	KO	13	rhNaGlu	27	IV	0.491		
	485	KO	13	rhNaGlu	27	IV	0.172	0.3315	0.225567
BRAIN	253	WT	4	na	-	-	0.021		
	155	WT	12	na	-	-	0.013		
	243	WT	4	na	-	-	0.014308		
	10	WT	36	na	-	-	0.012649	0.015239	0.003906
	239	KO	4	na	-	-	0.095		
	178	KO	12	na	-	-	0.084		
	242	KO	4	na	-	-	0.099		
	255	KO	4	na	-	-	0.094538		
	165	KO	24	na	-	-	0.084015		
	474	KO	13	vehicle	0	IV	0.085447		
	479	KO	13	vehicle	0	IV	0.072		

	484	KO	13	vehicle	0	IV	0.073	0.085875	0.009972
	487	KO	13	rhNaGlu	6.25	IV	0.045		
	492	KO	13	rhNaGlu	6.25	IV	0.044119		
	504	KO	13	rhNaGlu	6.25	IV	0.044	0.044373	0.000546
	481	KO	13	rhNaGlu	27	IV	0.017796		
	485	KO	13	rhNaGlu	27	IV	0.016668		
	490	KO	13	rhNaGlu	27	IV	0.028	0.020821	0.006242
	86	KO	15	vehicle	0	IT	0.094521		
	91	KO	14	vehicle	0	IT	0.072623	0.083572	0.015484
	94	KO	14	rhNaGlu	0.31	IT	0.038866		
	101	KO	14	rhNaGlu	0.31	IT	0.028229	0.033548	0.007521

na: Not applicable (mice were untreated).

Table 6: Tissue enzymatic activity (rhNaGlu *Gallus*; U/ng protein)

Tissue	Animal Number	Genotype	Age at sacrifice (wks)	Treatment	Dose (mg/kg)	Route	Enzymatic Activity (U/ug protein)
BRAIN	253	WT	4	na	-	-	7.7
	178	KO	12	na	-	-	0
	474	KO	13	vehicle	0	IV	0
	479	KO	13	vehicle	0	IV	0
	484	KO	13	vehicle	0	IV	0.575
	487	KO	13	rhNaGlu	6.25	IV	10.58
	492	KO	13	rhNaGlu	6.25	IV	5.066666667
	504	KO	13	rhNaGlu	6.25	IV	4.033333333
	481	KO	13	rhNaglu	27	IV	87.91666667
	485	KO	13	rhNaGlu	27	IV	90.15
	490	KO	13	rhNaGlu	27	IV	17.35
LIVER	253	WT	4	na	-	-	36.69
	178	KO	12	na	-	-	0
	474	KO	13	vehicle	0	IV	0
	479	KO	13	vehicle	0	IV	0
	484	KO	13	vehicle	0	IV	0
	487	KO	13	rhNaGlu	6.25	IV	512.92
	492	KO	13	rhNaGlu	6.25	IV	378.805
	504	KO	13	rhNaGlu	6.25	IV	607.9225

	481	KO	13	rhNaGlu	27	IV	659.6825
	485	KO	13	rhNaGlu	27	IV	654.2475
	490	KO	13	rhNaGlu	27	IV	677.8725

na: not applicable (mice were untreated).

* * *

Each example in the above specification is provided by way of explanation of the invention, not limitation of the invention. In fact, it will be apparent to those skilled in the art that various modifications, combinations, additions, deletions and variations can be made in the present invention without departing from the scope or spirit of the invention. For instance, features illustrated or described as part of one embodiment can be used in another embodiment to yield a still further embodiment. It is intended that the present invention cover such modifications, combinations, additions, deletions, and variations.

All publications, patents, patent applications, internet sites, and accession numbers/database sequences (including both polynucleotide and polypeptide sequences) cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, internet site, or accession number/database sequence were specifically and individually indicated to be so incorporated by reference.

CLAIMS

What is claimed is:

1. A composition comprising an isolated mixture of recombinant human N-acetyl-alpha-D-glucosaminidase (rhNaGlu) comprising the amino acid sequence 24-743 of SEQ ID NO:1, wherein at least 10 % of said rhNaGlu in said mixture comprises at least one glycan structure having mannose-6-phosphate (M6P).

2. The composition of Claim 1, wherein said rhNaGlu having M6P is capable of being taken up into a mammalian cell deficient in NaGlu such that internalized rhNaGlu restores at least 50% of normal NaGlu activity observed in a wild-type mammalian cell of the same type.

3. The composition of Claim 2, wherein said glycan structure is an N-linked glycan.

4. The composition of Claim 3, wherein said rhNaGlu contains at least 1 mole of M6P per mole of protein.

5. The composition of Claim 3, wherein said rhNaGlu contains between about 1 and about 6 moles of M6P per mole of protein.

6. The composition of Claim 5, wherein said rhNaGlu contains about 2 moles of M6P per mole of protein.

7. The composition of Claim 5, wherein said rhNaGlu contains about 3 moles of M6P per mole of protein.

8. The composition of Claim 5, wherein said rhNaGlu contains about 4 moles of M6P per mole of protein.

9. The composition of Claim 5, wherein said rhNaGlu contains about 5 moles of M6P per mole of protein.

10. The composition of Claim 5, wherein said rhNaGlu contains about 6 moles of M6P per mole of protein.

11. The composition of Claim 2, wherein said mammalian cell deficient in NaGlu is a human cell.

12. The composition of Claim 11, wherein said human cell deficient in NaGlu is a skin fibroblast, a hepatocyte or a macrophage.

13. The composition of Claim 11, wherein said human cell deficient in NaGlu is a neuronal cell.

14. The composition of Claim 13, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when systemically administered.

15. The composition of Claim 14, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when intravenously administered.

16. The composition of Claim 13, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intrathecally.

17. The composition of Claim 2, wherein said rhNaGlu having M6P is internalized by a NaGlu deficient cell and restores at least 100% of normal NaGlu activity *in vivo*.

18. The composition of Claim 2, wherein said rhNaGlu having M6P contains at least 25 moles of mannose per mole of protein.

19. The composition of Claim 1, wherein at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of said rhNaGlu in said mixture contains M6P.

20. The composition of Claim 19, wherein at least 20% of said rhNaGlu in said mixture contains at least one M6P.

21. The composition of Claim 20, wherein at least 30% of said rhNaGlu in said mixture contains at least one M6P.

22. The composition of Claim 21, wherein at least 40% of said rhNaGlu in said mixture contains at least one M6P.

23. The composition of Claim 22, wherein at least 50% of said rhNaGlu in said mixture contains at least one M6P.

24. The composition of Claim 23, wherein at least 60% of said rhNaGlu in said mixture contains at least one M6P.

25. A composition comprising an isolated mixture of recombinant human N-acetyl-alpha-D-glucosaminidase (rhNaGlu) comprising the amino acid sequence 24-743 of SEQ ID NO:1, wherein said mixture comprises a sufficient amount of rhNaGlu containing one or more glycan structures comprising mannose-6-phosphate (M6P) such that said rhNaGlu containing M6P is internalized into a mammalian cell having NaGlu deficiency via M6P receptor-mediated endocytosis and restores at least 50 % of NaGlu activity observed in a wild-type cell of the same type expressing endogenous NaGlu.

26. The composition of Claim 25, wherein said rhNaGlu is N-linked glycosylated.

27. The composition of Claim 25, wherein said rhNaGlu is O-linked glycosylated.

28. The composition of Claim 25, wherein said rhNaGlu comprises at least 1 moles of M6P per mole of rhNaGlu.

29. The composition of Claim 26, wherein said rhNaGlu comprises about 1, 2, 3, 4, 5 or 6 moles of M6P per mole of rhNaGlu.

30. The composition of Claim 29, wherein said rhNaGlu comprises about 3 moles of M6P per mole of rhNaGlu.

31. The composition of Claim 29, wherein said rhNaGlu comprises about 4 moles of M6P per mole of rhNaGlu.

32. The composition of Claim 25, wherein said rhNaGlu comprises mannose.

33. The composition of Claim 25, wherein said rhNaGlu comprises N-acetylglucosamine (GlcNAc).

34. The composition of Claim 25, wherein said rhNaGlu comprises galactose.

35. The composition of Claim 25, wherein said rhNaGlu comprises N-acetylgalactosamine (GalNAc).

36. The composition of Claim 25, wherein said rhNaGlu contains no fucose.

37. The composition of Claim 25, wherein said rhNaGlu contains no glucose.

38. The composition of Claim 25, wherein said rhNaGlu restores at least 60, 70, 80, 90, 95 or 100% of normal NaGlu enzymatic activity.

39. The composition of Claim 25, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered systemically.

40. The composition of Claim 39, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intravenously.

41. The composition of Claim 25, wherein said mammalian cell deficient in NaGlu is a human cell.

42. The composition of Claims 41, wherein said human cell is a skin fibroblast, a hepatocyte or a macrophage.

43. The composition of Claim 41, wherein said human cell deficient in NaGlu is a neuronal cell.

44. The composition of Claim 25, wherein said rhNaGlu is a fusion protein comprising a second moiety.

45. The composition of Claim 44, wherein said second moiety is a polypeptide.

46. The composition of Claim 45, wherein said polypeptide is selected from the group consisting of transferrin receptor ligand (TfRL), insulin-like growth factor receptor (IGF2R) ligand, low density lipoprotein (LDL) receptor ligand and acidic amino acid (AAA) residues.

47. The composition of Claim 25, wherein said rhNaGlu is produced from a transgenic avian.

48. The composition of Claim 47, wherein said transgenic avian is a chicken, a turkey, a duck or a quail.

49. The composition of Claim 48, wherein said transgenic avian is a chicken.

50. The composition of Claim 49, wherein said rhNaGlu is produced from an oviduct cell.

51. A composition comprising an isolated recombinant human N-acetyl-alpha-D-glucosaminidase (rhNaGlu) comprising one or more glycan structures having sufficient amount of mannose-6-phosphate (M6P) that allows for internalization of said rhNaGlu into a mammalian cell having NaGlu deficiency via M6P receptor-mediated endocytosis, such that when internalized *in vivo*, said rhNaGlu restores at least 50% of NaGlu activity observed in a cell of the same type in a normal subject.

52. The composition of Claim 51, wherein said rhNaGlu protein is N-linked glycosylated.

53. The composition of Claim 51, wherein said rhNaGlu protein is O-linked glycosylated.

54. The composition of Claim 51, wherein said rhNaGlu comprises about 2, 3, 4, 5 or 6 moles of M6P per mole of rhNaGlu.

55. The composition of Claim 51, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered systemically.

56. The composition of Claim 55, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intravenously.

57. The composition of Claim 51, wherein said rhNaGlu is effectively delivered to the brain of a mammal having NaGlu deficiency when administered intrathecally.

58. A transgenic avian comprising a transgene containing a promoter operably linked to a nucleic acid sequence encoding a recombinant human NaGlu (rhNaGlu), wherein said transgene is contained in the genome of the transgenic avian and expressed in an oviduct cell such that said rhNaGlu is glycosylated in the oviduct cell of the transgenic avian, secreted into lumen of oviduct and deposited in egg white of an egg of the transgenic avian.

59. The transgenic avian of Claim 58, wherein said rhNaGlu comprises about 2, 3, 4 or 6 moles of M6P per mole of rhNaGlu.

60. The transgenic avian Claim 58, wherein said promoter component is an oviduct-specific promoter.

61. The transgenic avian Claim 60, wherein said oviduct-specific promoter is an ovalbumin promoter.

62. The transgenic avian of Claim 58, wherein said transgenic avian is selected from the group consisting of a chicken, a turkey, a duck and a quail.

63. An egg produced by the transgenic avian of Claim 58.

64. A method of producing a recombinant human NaGlu (rhNaGlu) comprising:

a) producing a transgenic avian comprising a transgene having a promoter component operably linked to a nucleic acid sequence encoding the rhNaGlu set forth in 24-743 of SEQ ID NO:1, wherein said transgene is contained in the genome of the transgenic avian and expressed in an oviduct cell, such that the

rhNaGlu is glycosylated in the oviduct cell of the transgenic avian, secreted into lumen of oviduct and deposited in egg white of an egg laid by the transgenic avian; and

b) isolating said rhNaGlu from said egg white.

65. The method of Claim 64, wherein said promoter component is an oviduct-specific promoter.

66. The method of Claim 65, wherein said oviduct-specific promoter is an ovalbumin promoter.

67. The method of Claim 64, wherein the avian is selected from the group consisting of a chicken, a turkey, a duck and a quail.

68. The method of Claim 67, wherein the avian is chicken.

69. A vector comprising a nucleotide sequence encoding a human NaGlu operably linked to an ovalbumin promoter.

70. A host cell comprising the vector of Claim 69.

71. An isolated nucleic acid comprising the nucleic acid sequence of 5232-10248 of SEQ ID NO:4.

72. A pharmaceutical formulation comprising a composition according to Claim 1 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

73. A composition comprising recombinant human NaGlu protein that crosses the blood brain barrier of a mammal having NaGlu deficiency when administered intravenously.

74. A method of treating a subject suffering from NaGlu deficiency, the method comprising administering to the subject a therapeutically effective amount of the composition of any one of Claims 1, 25 or 51.

75. A method of delivering recombinant human NaGlu protein to the brain of a subject suffering from NaGlu deficiency, the method comprising intravenously administering recombinant human NaGlu protein to the subject.

76. A method of transporting a recombinant human NaGlu protein from the circulation across the blood brain barrier in a therapeutically effective amount, the method comprising intravenously administering a recombinant human NaGlu protein to a subject having NaGlu deficiency.

77. The method of any one of Claims 74-76, wherein said NaGlu deficiency is Sanfilippo syndrome B.

78. The method of any one of Claims 74-76, wherein said subject is a human.

79. The method of any one of Claims 74-76, wherein said recombinant human NaGlu protein is administered intravenously to the subject at a dosage of about 1 to about 30 mg/kg body weight.

80. The method of Claim 79, wherein said recombinant human NaGlu protein is administered intravenously to the subject at a dosage of about 6 to about 27 mg/kg body weight.

81. The method of Claim 74, wherein said recombinant human NaGlu protein is intrathecally administered to the subject.

82. The method of Claim 81, wherein said recombinant human NaGlu protein is intrathecally administered at a dosage of at least about 0.3 mg/kg body weight.

83. The method of Claim 82, wherein said recombinant human NaGlu protein is intrathecally administered at a dosage of about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg/kg body weight.

84. The method of Claim 82, wherein said recombinant human NaGlu protein is administered intrathecally at a dosage of about 10 to about 30 mg/kg body weight.

85. The method of Claim 74, wherein said therapeutically effective amount is an amount effective to reduce heparan sulfate levels in the brain, the kidney, or the liver of the subject.

86. The method of Claim 74, wherein said therapeutically effective amount is an amount effective to increase NaGlu activity in the brain or the liver of the subject.

87. The method of Claim 74, further comprising administering a second therapeutic agent.

88. The method of Claim 87, wherein the second therapeutic is an immunosuppressant.

Human NaGlu Amino Acid Sequence (signal peptide: 1-23, underlined)

MEAVAVAAAV GVLLLAGAGG AAGDEAREAA AVRALVARLL GPGPAADFSV SVERALAAKP	60
GLDTYSLGGG GAARVRVRGS TGVAAAAGLH RYLRDFCGCH VAWSGSQLRL PRPLPAVPGE	120
LTEATPNRYR YYQNVCTQSY SFVWWDWARW EREIDWMALN GINLALAWSG QEAIWQRVYL	180
ALGLTQAEIN EFFTGPAFLA WGRMGNLHTW DGPLPPSWHI KQLYLQHRVL DQMRSFGMTP	240
VLPAFAGHVP EAVTRVFPQV NVTKMGSWGH FNCSYSCSFL LAPEDPIFPI IGSLFLRELI	300
KEFGTDHIYG ADTFNEMQPP SSEPSYLAAA TTAVYEAMTA VDTEAVWLLQ GWLFQHQPQF	360
WGPAQIRAVL GAVPRGRLLV LDLFAESQPV YTRTASFQGQ PFIWCMLHNF GGNHGLFGAL	420
EAVNGGPEAA RLFPNSTMVG TGMAPEGISQ NEVVYSLMAE LGWRKDPVPD LAAWVTSFAA	480
RRYGVSHPDA GAAWRLLLRS VYNCSGEACR GHNRSPLVRR PSLQMNTSIW YNRSDVFEAW	540
RLLLTSAPSL ATSPAFRYDL LDLTRQAVQE LVSLYYEEAR SAYLSKELAS LLRAGGVLAY	600
ELLPALDEVL ASDSRFLLGS WLEQARAAAV SEAEADFYEQ NSRYQLTLWG PEGNILDYAN	660
KQLAGLVANY YTPRWRLFLE ALVDSVAQGI PFQQHQFDKN VFQLEQAFVL SKQRYPSQPR	720
GDTVDLAKKI FLKYYPRWVA GSW	743

(SEQ ID NO:1)

Fig. 1

Human NaGlu Coding Sequence (cDNA)

```
atggaggcgg tggcggtggc cgcggcggtg ggggtccttc tcctggccgg ggccgggggc      60
gcggcaggcg acgaggcccg ggaggcggcg gccgtgcggg cgctcgtggc ccggctgctg     120
gggccaggcc ccgcggccga cttctccgtg tcggtggagc gcgctctggc tgccaagccg     180
ggcttggaca cctacagcct gggcggcggc ggcgcggcgc gcgtgcgggt gcgcggctcc     240
acgggcgtgg cagccgccgc ggggctgcac cgctacctgc gcgacttctg tggctgccac     300
gtggcctggt ccggctctca gctgcgcctg ccgcggccac tgccagccgt gccgggggag     360
ctgaccgagg ccacgcccaa caggtaccgc tattaccaga atgtgtgcac gcaaagctac     420
tctttcgtgt ggtgggactg ggcccggtgg gagcgagaga tagactggat ggcgctgaat     480
ggcatcaacc tggcactggc atggagcggc caggaggcca tctggcagcg ggtgtacctg     540
gccttgggcc tgacccaggc agagatcaat gagttcttta ctggtcctgc cttcttggca     600
tgggggcgaa tgggcaacct gcacacctgg gatggccccc tgccccctc ctggcacatc     660
aagcagcttt atctgcagca ccgggtcctg gaccagatgc gctccttcgg catgacccca     720
gtgctgcctg cattcgcggg gcatgttccc gaggctgtca ccagggtgtt ccctcaggtc     780
aatgtcacga agatgggcag ttggggccac tttaactgtt cctactcctg ctccttcctt     840
ctggctccgg aagaccccat attccccatc atcgggagcc tcttcttgcg agagctgatc     900
aaagagtttg gcacagacca catctatggg gccgacactt tcaatgagat gcagccacct     960
tcctcagagc cctcctatct tgccgcagcc accactgccg tctatgaggc catgactgca    1020
gtggatactg aggctgtgtg gctgctccaa ggctggctct tccagcacca gccgcagttc    1080
tgggggcccg cccagatcag ggctgtgctg ggagctgtgc cccgtggccg cctcctggtt    1140
ctggacctgt ttgctgagag ccagcctgtg tatacccgca ctgcctcctt ccaaggccag    1200
cccttcatct ggtgcatgct gcacaacttt gggggaaatc atggtctttt tggagccttg    1260
gaggccgtga acggaggccc agaagctgcc cgcctcttcc ccaactccac aatggtaggc    1320
acgggcatgg ccccgaggg catcagccag aacgaagtgg tctattccct catggctgag    1380
ctgggctggc gaaaggaccc agtgccagat ttggcagcct gggtgaccag ctttgccgcc    1440
cggcggtatg gggtctccca cccggacgca ggggcagcgt ggaggctact gctccggagt    1500
gtgtacaact gctccgggga ggcatgcagg ggccacaatc gtagcccgct ggtcaggcgg    1560
ccgtccctac agatgaatac cagcatctgg tacaaccgat ctgatgtgtt tgaggcctgg    1620
cggctgctgc tcacatctgc tccctccctg gccaccagcc ccgccttccg ctacgacctg    1680
ctggacctca ctcggcaggc agtgcaggag ctggtcagct tgtattatga ggaggcaaga    1740
agcgcctatc tgagcaagga gctggcctcc ttgttgaggg ctggaggcgt cctggcctat    1800
gagctgctgc cggcactgga cgaggtgctg gctagtgaca gccgcttctt gctgggcagc    1860
tggctagagc aggcccgagc agcggcagtc agtgaggccg aggccgattt ctacgagcag    1920
aacagccgct accagctgac cttgtggggg ccagaaggca acatcctgga ctatgccaac    1980
aagcagctgg cggggttggt ggccaactac tacacccctc gctggcggct tttcctggag    2040
gcgctggttg acagtgtggc ccagggcatc cctttccaac agcaccagtt tgacaaaaat    2100
gtcttccaac tggagcaggc cttcgttctc agcaagcaga ggtaccccag ccagccgcga    2160
ggagacactg tggacctggc caagaagatc ttcctcaaat attacccccg ctgggtggcc    2220
ggctcttggt gatt                                                      2234
```

(SEQ ID NO:2)

Fig. 2

1.1kb OV promoter

```
gttaagtcct cagacttggc aaggagaatg tagatttcca cagtatatat gttttcacaa      60
aaggaaggag agaaacaaaa gaaaatggca ctgactaaac ttcagctagt ggtataggaa     120
agtaattctg cttaacagag attgcagtga tctctatgta tgtcctgaag aattatgttg     180
tacttttttc ccccattttt aaatcaaaca gtgctttaca gaggtcagaa tggtttcttt     240
actgtttgtc aattctatta tttcaataca gaacaatagc ttctataact gaaatatatt     300
tgctattgta tattatgatt gtccctcgaa ccatgaacac tcctccagct gaatttcaca     360
attcctctgt catctgccag gccattaagt tattcatgga agatctttga ggaacactgc     420
aagttcatat cataaacaca tttgaaattg agtattgttt tgcattgtat ggagctatgt     480
tttgctgtat cctcagaata aaagtttgtt ataaagcatt cacacccata aaaagataga     540
tttaaatatt ccaactatag gaaagaaagt gtgtctgctc ttcactctag tctcagttgg     600
ctccttcaca tgcacgcttc tttatttctc ctattttgtc aagaaaataa taggtcaagt     660
cttgttctca tttatgtcct gtctagcgtg gctcagatgc acattgtaca tacaagaagg     720
atcaaatgaa acagacttct ggtctgttac tacaaccata gtaataagca cactaactaa     780
taattgctaa ttatgttttc catctccaag gttcccacat ttttctgttt tcttaaagat     840
cccattatct ggttgtaact gaagctcaat ggaacatgag caatatttcc cagtcttctc     900
tcccatccaa cagtcctgat ggattagcag aacaggcaga aaacacattg ttacccagaa     960
ttaaaaacta atatttgctc tccattcaat ccaaaatgga cctattgaaa ctaaaatcta    1020
acccaatccc attaaatgat ttctatggtg tcaaaggtca aacttctgaa gggaacctgt    1080
gggtgggtca caattcagac tatatattcc ccagggctca gccagtgtct gt             1132
```

(SEQ ID NO:3)

Fig. 3

pSIN-OV-1.1-I-rhNaGlu

```
ggccgcaaga agaaagctga aaaactctgt cccttccaac aagacccaga gcactgtagt      60
atcaggggta aaatgaaaag tatgttatct gctgcatcca gacttcataa aagctggagc     120
ttaattcaga aaaaaaatca gaaagaaatt acactgtgag aacaggtgca attcactttt     180
cctttacaca gagtaatact ggtaactcat ggatgaaggc ttaagggaat gaaattggac     240
tcacagtact gagtcatcac actgaaaaat gcaacctgat acatcagcag aaggtttatg     300
ggggaaaaat gcagccttcc aattaagcca gatatctgta tgaccaagct gctccagaat     360
tagtcactca aaatctctca gattaaatta tcaactgtca ccaaccattc ctatgctgac     420
aaggcaattg cttgttctct gtgttcctga tactacaagg ctcttcctga cttcctaaag     480
atgcattata aaaatcttat aattcacatt tctccctaaa ctttgactca atcatggtat     540
gttggcaaat atggtatatt actattcaaa ttgttttcct tgtacccata tgtaatgggt     600
cttgtgaatg tgctcttttg ttcctttaat cataataaaa acatgtttaa gcaaacactt     660
ttcacttgta gtatttgaag gtaccggatc tcgagccgcc ttcaatgccc ccaaaaccaa     720
tccccaggtt tttaactctc ccgattttcc aagtaccata gcccgctgag agagcgccgc     780
ggtaatggga tcccaggacc ccggggaata taagtctgag ggggacgtaa gcaacccttc     840
cttttgtaac agggacaaca tagccctat ttccttctta gaaggagagg ttttcccgca     900
ataggtctta cacgcggacg aaatcacctt tatgacggct tccatgcttg atccaccggg     960
cgaccggaat cacgcagagc aaccggaatc acgcctgggg tggaccgctc agtcgtcggg    1020
cttccttccc gtcttccaac gactctctga gttctcggta gggtatgttg gccccctgca    1080
gtagggctcc ctccgacgcc actcagcttc tgccctccta agccgcagcc ccctctacta    1140
gggtcatcgt ccgctccccg aataagcgag acggatgagg acaggatcgc cacgccgcct    1200
gtggccgacc actattccct aacgatcacg tcggggtcac caaatgaagc cttctgcttc    1260
atgcatgtgc tcgtagtcgt cagggaatca acggtccggc catcaaccca ggtgcacacc    1320
aatgtggtga atggtcaaat ggcgtttatt gtatcgagct aggcacttaa atacaatatc    1380
tctgcaatgc ggaattcagt ggttcgtcca atccgtgtta gacccgtctg ttgccttcct    1440
aacaaggcac gatcatacca cgatcatacc accttactcc caccaatcgg catgcacggt    1500
gcttttttctc tccttataag gcatgttgct aactcatcgt tacataagca tgttgcaaga    1560
ctacaagagt attgcataag actacatttc cccctcccta tgcaaaagcg aaactactat    1620
atcctgaggg gactcctaac cgcgtacaac cgaagccccg cttttcgcct aaacatgcta    1680
ttgtcccctc agtcaagcct tgcccgttac aacccgattc gcaagccttg ccctccccac    1740
attatccgta gcattatttc ctagcagtca tcagagctac agaagatact ctatgctgta    1800
gccaagtcta caagtttact attcagcgac ctcctatatt ccgcgtgcca gccgatcaat    1860
taccaatgcg cgcttggcgt aatcatggtc atagctgttt cctgtgtgaa attgttatcc    1920
gctcacaatt ccacacaaca tacgagccgg aagcataaag tgtaaagcct ggggtgccta    1980
atgagtgagc taactcacat taattgcgtt gcgctcactg cccgctttcc agtcgggaaa    2040
cctgtcgtgc cagctgcatt aatgaatcgg ccaacgcgcg gggagaggcg gtttgcgtat    2100
tgggcgctct tccgcttcct cgctcactga ctcgctgcgc tcggtcgttc ggctgcggcg    2160
```

Fig. 4A

```
agcggtatca gctcactcaa aggcggtaat acggttatcc acagaatcag gggataacgc      2220
aggaaagaac atgtgagcaa aaggccagca aaaggccagg aaccgtaaaa aggccgcgtt      2280
gctggcgttt ttccataggc tccgcccccc tgacgagcat cacaaaaatc gacgctcaag      2340
tcagaggtgg cgaaacccga caggactata aagataccag gcgtttcccc ctggaagctc      2400
cctcgtgcgc tctcctgttc cgaccctgcc gcttaccgga tacctgtccg cctttctccc      2460
ttcgggaagc gtggcgcttt ctcatagctc acgctgtagg tatctcagtt cggtgtaggt      2520
cgttcgctcc aagctgggct gtgtgcacga accccccgtt cagcccgacc gctgcgcctt      2580
atccggtaac tatcgtcttg agtccaaccc ggtaagacac gacttatcgc cactggcagc      2640
agccactggt aacaggatta gcagagcgag gtatgtaggc ggtgctacag agttcttgaa      2700
gtggtggcct aactacggct acactagaag gacagtattt ggtatctgcg ctctgctgaa      2760
gccagttacc ttcggaaaaa gagttggtag ctcttgatcc ggcaaacaaa ccaccgctgg      2820
tagcggtggt ttttttgttt gcaagcagca gattacgcgc agaaaaaaag gatctcaaga      2880
agatcctttg atcttttcta cggggtctga cgctcagtgg aacgaaaact cacgttaagg      2940
gattttggtc atgagattat caaaaaggat cttcacctag atccttttaa attaaaaatg      3000
aagttttaaa tcaatctaaa gtatatatga gtaaacttgg tctgacagtt accaatgctt      3060
aatcagtgag gcacctatct cagcgatctg tctatttcgt tcatccatag ttgcctgact      3120
ccccgtcgtg tagataacta cgatacggga gggcttacca tctggcccca gtgctgcaat      3180
gataccgcga gacccacgct caccggctcc agatttatca gcaataaacc agccagccgg      3240
aagggccgag cgcagaagtg gtcctgcaac tttatccgcc tccatccagt ctattaattg      3300
ttgccgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttgccat      3360
tgctacaggc atcgtggtgt cacgctcgtc gtttggtatg gcttcattca gctccggttc      3420
ccaacgatca aggcgagtta catgatcccc catgttgtgc aaaaaagcgg ttagctcctt      3480
cggtcctccg atcgttgtca gaagtaagtt ggccgcagtg ttatcactca tggttatggc      3540
agcactgcat aattctctta ctgtcatgcc atccgtaaga tgcttttctg tgactggtga      3600
gtactcaacc aagtcattct gagaatagtg tatgcggcga ccgagttgct cttgcccggc      3660
gtcaatacgg gataataccg cgccacatag cagaacttta aaagtgctca tcattggaaa      3720
acgttcttcg gggcgaaaac tctcaaggat cttaccgctg ttgagatcca gttcgatgta      3780
acccactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg      3840
agcaaaaaca ggaaggcaaa atgccgcaaa aaagggaata agggcgacac ggaaatgttg      3900
aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcagggtt attgtctcat      3960
gagcggatac atatttgaat gtatttagaa aaataaacaa atagggggttc cgcgcacatt      4020
tccccgaaaa gtgccacctg acgcgccctg tagcggcgca ttaagcgcgg cgggtgtggt      4080
ggttacgcgc agcgtgaccg ctacacttgc cagcgcccta gcgcccgctc ctttcgcttt      4140
cttcccttcc tttctcgcca cgttcgccgg ctttccccgt caagctctaa atcgggggct      4200
ccctttaggg ttccgattta gtgctttacg gcacctcgac cccaaaaaac ttgattaggg      4260
tgatggttca cgtagtgggc catcgccctg atagacggtt tttcgccctt tgacgttgga      4320
gtccacgttc tttaatagtg gactcttgtt ccaaactgga acaacactca accctatctc      4380
ggtctattct tttgatttat aagggatttt gccgatttcg gcctattggt taaaaaatga      4440
gctgatttaa caaaaattta acgcgaattt taacaaaata ttaacgctta caatttccat      4500
tcgccattca ggctgcgcaa ctgttgggaa gggcgatcgg tgcgggcctc ttcgctatta      4560
cgccagctgg cgaaaggggg atgtgctgca aggcgattaa gttgggtaac gccagggttt      4620
tcccagtcac gacgttgtaa aacgacggcc agtgagcgcg tattccctaa cgatcacgtc      4680
ggggtcacca aatgaagcct tctgcttcat gcatgtgctc gtagtcgtca gggaatcaac      4740
ggtccggcca tcaacccagg tgcacaccaa tgtggtgaat ggtcaaatgg cgtttattgt      4800
atcgagctag gcacttaaat acaatatctc tgcaatgcgg aattcagtgg ttcgtccaat      4860
ccgtcccctt ccctatgcaa aagcgaaact actatatcct gaggggactc ctaaccgcgt      4920
acaaccgaag ccccgctttt cgcctaaaca tgctattgtc ccctcagtca agccttgccc      4980
gttacaaccc gattcgcaag ccttgccctc cccacattat ccgtagcatt atttcctagc      5040
agtcatcaga gctacagaag atactctatg ctgtagccaa gtctacaagt ttactattca      5100
```

Fig. 4B

```
gcgacctcct atattccgcg tgccagccga tcaattacca atccaaccag ctatcacacg      5160
gaatacaaga actcgcctac gctcttcttt cgggctgctt ataagcctcc tgtaattttt      5220
ttatattcct cgttaagtcc tcagacttgg caaggagaat gtagatttcc acagtatata      5280
tgttttcaca aaaggaagga gagaaacaaa agaaaatggc actgactaaa cttcagctag      5340
tggtatagga aagtaattct gcttaacaga gattgcagtg atctctatgt atgtcctgaa      5400
gaattatgtt gtactttttt cccccatttt taaatcaaac agtgctttac agaggtcaga      5460
atggtttctt tactgtttgt caattctatt atttcaatac agaacaatag cttctataac      5520
tgaaatatat ttgctattgt atattatgat tgtccctcga accatgaaca ctcctccagc      5580
tgaatttcac aattcctctg tcatctgcca ggccattaag ttattcatgg aagatctttg      5640
aggaacactg caagttcata tcataaacac atttgaaatt gagtattgtt ttgcattgta      5700
tggagctatg ttttgctgta tcctcagaat aaaagtttgt tataaagcat tcacacccat      5760
aaaaagatag atttaaatat tccaactata ggaaagaaag tgtgtctgct cttcactcta      5820
gtctcagttg gctccttcac atgcacgctt ctttatttct cctattttgt caagaaaata      5880
ataggtcaag tcttgttctc atttatgtcc tgtctagcgt ggctcagatg cacattgtac      5940
atacaagaag gatcaaatga aacagacttc tggtctgtta ctacaaccat agtaataagc      6000
acactaacta ataattgcta attatgtttt ccatctccaa ggttcccaca tttttctgtt      6060
ttcttaaaga tcccattatc tggttgtaac tgaagctcaa tggaacatga gcaatatttc      6120
ccagtctttct ctcccatcca acagtcctga tggattagca gaacaggcag aaaacacatt      6180
gttacccaga attaaaaact aatatttgct ctccattcaa tccaaaatgg acctattgaa      6240
actaaaatct aacccaatcc cattaaatga tttctatggt gtcaaaggtc aaacttctga      6300
agggaacctg tgggtgggtc acaattcaga ctatatattc cccagggctc agccagtgtc      6360
tgtacataca gctagaaagc tgtattgcct ttagcagtca agctcgaaag gtaagcaact      6420
ctctggaatt accttctctc tatattagct cttacttgca cctaaacttt aaaaaattaa      6480
caattattgt gctatgtgtt gtatctttaa gggtgaagta cctgcgtgat acccccctata      6540
aaaacttctc acctgtgtat gcattctgca ctattttatt atgtgtaaaa gctttgtgtt      6600
tgttttcagg aggcttattc tttgtgctta aaatatgttt ttaatttcag aacatcttat      6660
cctgtcgttc actatctgat atgctttgca gtttgcttga ttaacttcta gccctacaga      6720
gtgcacagag agcaaaatca tggtgttcag tgaattctgg ggagttattt taatgtgaaa      6780
attctctaga agtttaattc ctgcaaagtg cagctgctga tcactacaca agataaaaat      6840
gtgggggtg cataaacgta tattcttaca ataatagata catgtgaact tatatacaga      6900
aaagaaaatg agaaaaatgt gtgtgtgtat actcacacac gtggtcagta aaaacttttg      6960
aggggtttaa tacagaaaat ccaatcctga ggccccagca ctcagtacgc atataaaggg      7020
ctgggctctg aaggacttct gactttcaca gattatataa atctcaggaa agcaactaga      7080
ttcatgctgg ctccaaaagc tgtgctttat ataagcacac tggctataca atagttgtac      7140
agttcagctc tttataatag aaacagacag aacaagtata aatcttctat tggtctatgt      7200
catgaacaag aattcattca gtggctctgt tttatagtaa acattgctat tttatcatgt      7260
ctgcatttct cttctgtctg aatgtcacca ctaaaattta actccacaga aagtttatac      7320
tacagtacac atgcatatct ttgagcaaag caaaccatac ctgaaagtgc aatagagcag      7380
aatatgaatt acatgcgtgt ctttctccta gactacatga ccccatataa attacattcc      7440
ttatctattc tgccatcacc aaaacaaagg taaaaatact tttgaagatc tactcatagc      7500
aagtagtgtg caacaaacag atatttctct acatttattt ttagggaata aaaataagaa      7560
ataaaatagt cagcaagcct ctgctttctc atatatctgt ccaaacctaa agtttactga      7620
aatttgctct ttgaatttcc agttttgcaa gcctatcaga ttgtgtttta atcagaggta      7680
ctgaaaagta tcaatgaatt ctagctttca ctgaacaaaa atatgtagag gcaactggct      7740
tctgggacag tttgctaccc aaaagacaac tgaatgcaaa tacataaata gatttatgaa      7800
tatggttttg aacatgcaca tgagaggtgg atatagcaac agacacatta ccacagaatt      7860
actttaaaac tacttgttaa catttaattg cctaaaaact gctcgtaatt tactgttgta      7920
gcctaccata gagtaccctg catggtacta tgtacagcat tccatcctta cattttcact      7980
```

Fig. 4C

```
gttctgctgt ttgctctaga caactcagag ttcaccatgg aggcggtggc ggtggccgcg      8040
gcggtggggg tccttctcct ggccggggcc gggggcgcgg caggcgacga ggcccgggag      8100
gcggcggccg tgcgggcgct cgtggcccgg ctgctggggc caggccccgc ggccgacttc      8160
tccgtgtcgg tggagcgcgc tctggctgcc aagccgggct tggacaccta cagcctgggc      8220
ggcggcggcg cggcgcgcgt gcgggtgcgc ggctccacgg gcgtggcagc cgccgcgggg      8280
ctgcaccgct acctgcgcga cttctgtggc tgccacgtgg cctggtccgg ctctcagctg      8340
cgcctgccgc ggccactgcc agccgtgccg ggggagctga ccgaggccac gcccaacagg      8400
taccgctatt accagaatgt gtgcacgcaa agctactctt tcgtgtggtg ggactgggcc      8460
cggtgggagc gagagataga ctggatggcg ctgaatggca tcaacctggc actggcatgg      8520
agcggccagg aggccatctg gcagcgggtg tacctggcct tgggcctgac ccaggcagag      8580
atcaatgagt tctttactgg tcctgccttc ttggcatggg ggcgaatggg caacctgcac      8640
acctgggatg gcccccctgcc cccctcctgg cacatcaagc agctttatct gcagcaccgg      8700
gtcctggacc agatgcgctc cttcggcatg accccagtgc tgcctgcatt cgcggggcat      8760
gttcccgagg ctgtcaccag ggtgttccct caggtcaatg tcacgaagat gggcagttgg      8820
ggccacttta actgttccta ctcctgctcc ttccttctgg ctccggaaga ccccatattc      8880
cccatcatcg ggagcctctt cttgcgagag ctgatcaaag agtttggcac agaccacatc      8940
tatggggccg acactttcaa tgagatgcag ccaccttcct cagagccctc ctatcttgcc      9000
gcagccacca ctgccgtcta tgaggccatg actgcagtgg atactgaggc tgtgtggctg      9060
ctccaaggct ggctcttcca gcaccagccg cagttctggg ggcccgccca gatcagggct      9120
gtgctgggag ctgtgccccg tggccgcctc ctggttctgg acctgtttgc tgagagccag      9180
cctgtgtata cccgcactgc ctccttccaa ggccagccct tcatctggtg catgctgcac      9240
aactttgggg gaaatcatgg tctttttgga gccttggagg ccgtgaacgg aggcccagaa      9300
gctgcccgcc tcttccccaa ctccacaatg gtaggcacgg gcatggcccc cgagggcatc      9360
agccagaacg aagtggtcta ttccctcatg gctgagctgg gctggcgaaa ggacccagtg      9420
ccagatttgg cagcctgggt gaccagcttt gccgcccggc ggtatggggt ctcccacccg      9480
gacgcagggg cagcgtggag gctactgctc cggagtgtgt acaactgctc cggggaggca      9540
tgcaggggcc acaatcgtag cccgctggtc aggcggccgt ccctacagat gaataccagc      9600
atctggtaca accgatctga tgtgtttgag gcctggcggc tgctgctcac atctgctccc      9660
tccctggcca ccagccccgc cttccgctac gacctgctgg acctcactcg gcaggcagtg      9720
caggagctgg tcagcttgta ttatgaggag gcaagaagcg cctatctgag caaggagctg      9780
gcctccttgt tgagggctgg aggcgtcctg gcctatgagc tgctgccggc actggacgag      9840
gtgctggcta gtgacagccg cttcttgctg ggcagctggc tagagcaggc ccgagcagcg      9900
gcagtcagtg aggccgaggc cgatttctac gagcagaaca gccgctacca gctgaccttg      9960
tgggggccag aaggcaacat cctggactat gccaacaagc agctggcggg gttggtggcc     10020
aactactaca cccctcgctg gcggcttttc ctggaggcgc tggttgacag tgtggcccag     10080
ggcatccctt tccaacagca ccagtttgac aaaaatgtct tccaactgga gcaggccttc     10140
gttctcagca agcagaggta ccccagccag ccgcgaggag acactgtgga cctggccaag     10200
aagatcttcc tcaaatatta ccccccgctgg gtggccggct cttggtgatt cgaagc         10256
```

(SEQ ID NO:4)

Fig. 4D

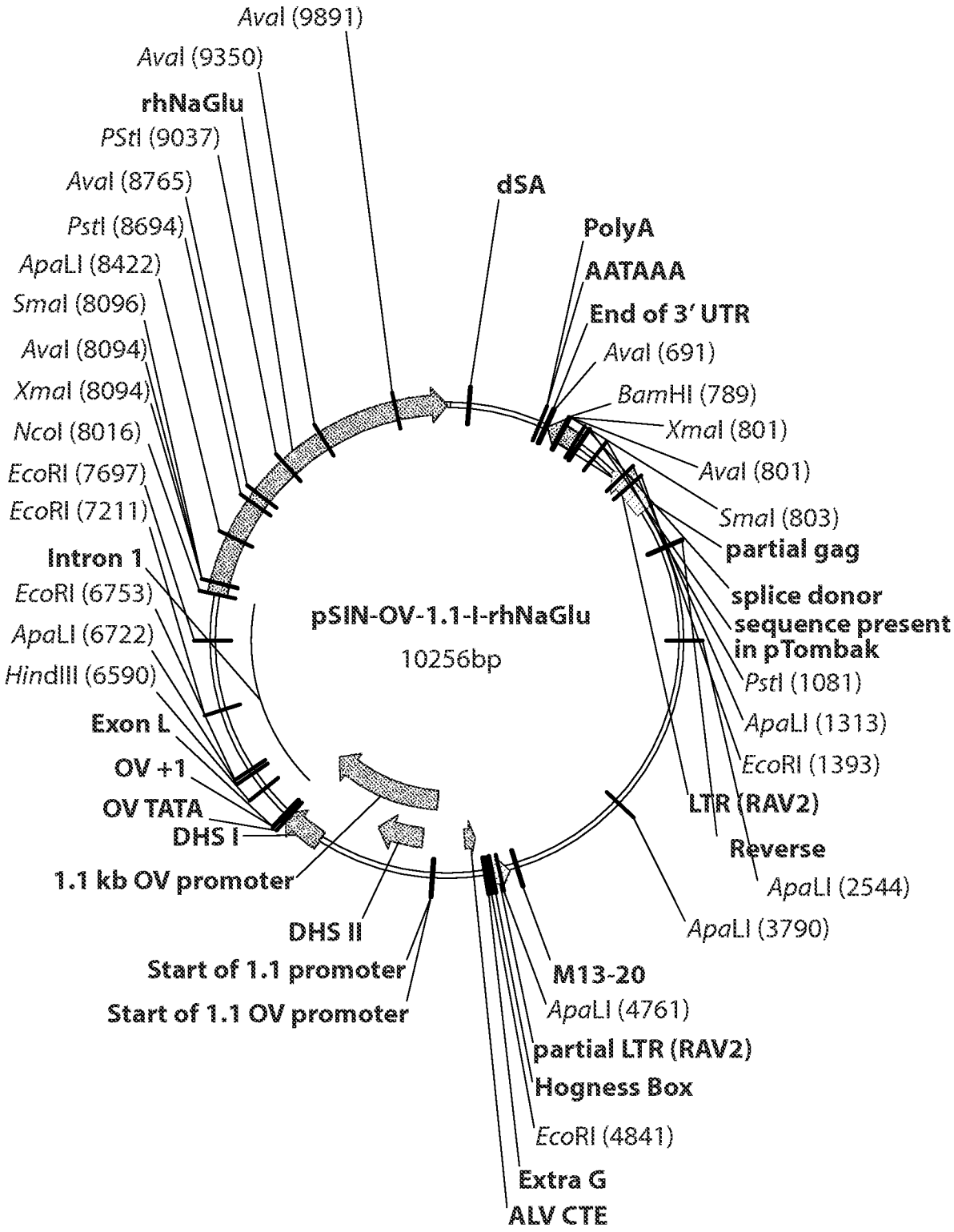
AvaI (9891)
AvaI (9350)
rhNaGlu
PStI (9037)
AvaI (8765)
PstI (8694)
ApaLI (8422)
SmaI (8096)
AvaI (8094)
XmaI (8094)
NcoI (8016)
EcoRI (7697)
EcoRI (7211)
Intron 1
EcoRI (6753)
ApaLI (6722)
HindIII (6590)
Exon L
OV +1
OV TATA
DHS I
1.1 kb OV promoter
DHS II
Start of 1.1 promoter
Start of 1.1 OV promoter
dSA
PolyA
AATAAA
End of 3' UTR
AvaI (691)
BamHI (789)
XmaI (801)
AvaI (801)
SmaI (803)
partial gag
splice donor
sequence present
in pTombak
PstI (1081)
ApaLI (1313)
EcoRI (1393)
LTR (RAV2)
Reverse
ApaLI (2544)
ApaLI (3790)
M13-20
ApaLI (4761)
partial LTR (RAV2)
Hogness Box
EcoRI (4841)
Extra G
ALV CTE
pSIN-OV-1.1-I-rhNaGlu
10256bp

Fig. 5

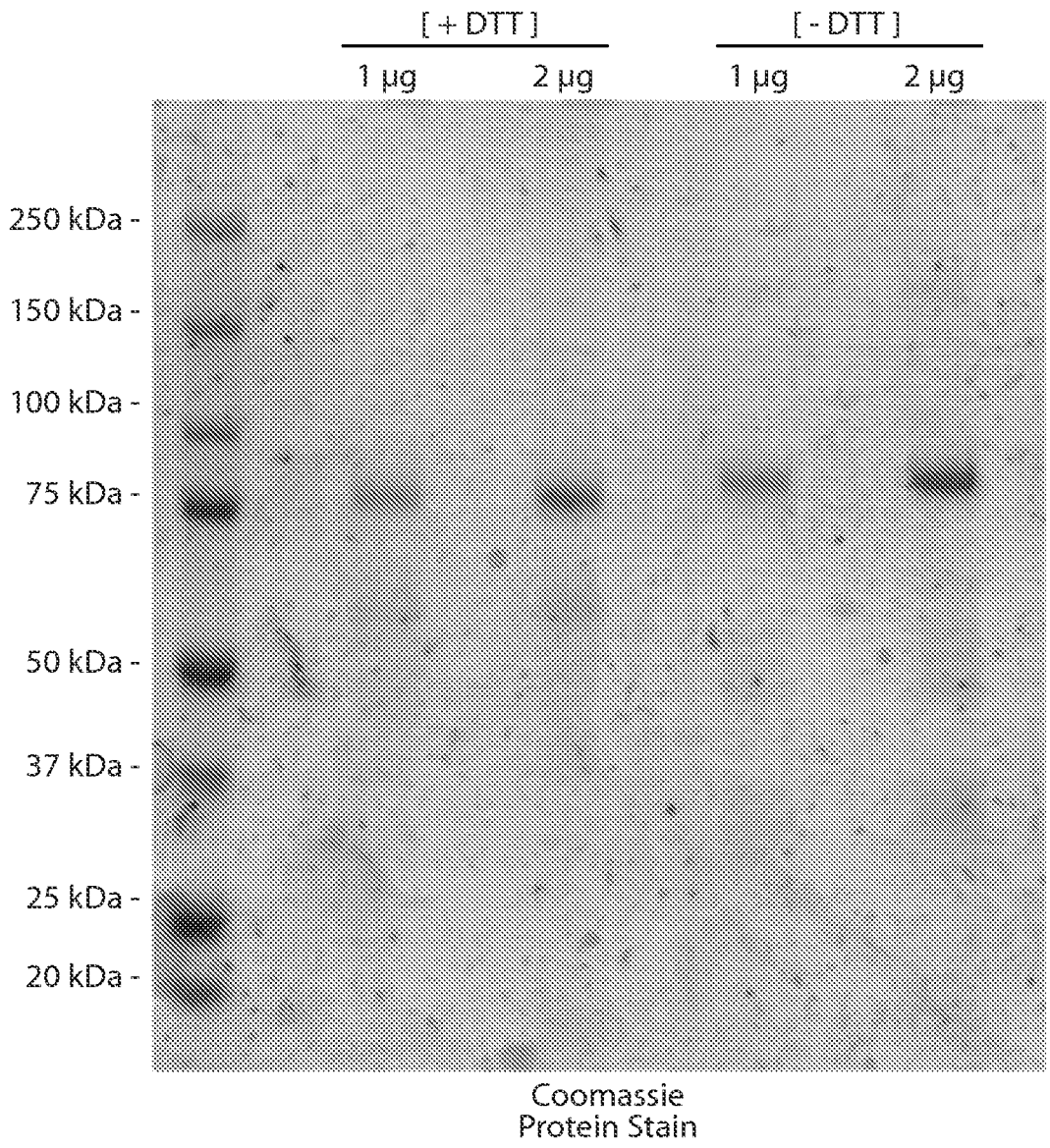
[+ DTT]
[- DTT]
1 µg
2 µg
1 µg
2 µg
250 kDa -
150 kDa -
100 kDa -
75 kDa -
50 kDa -
37 kDa -
25 kDa -
20 kDa -
Coomassie
Protein Stain

Fig. 6

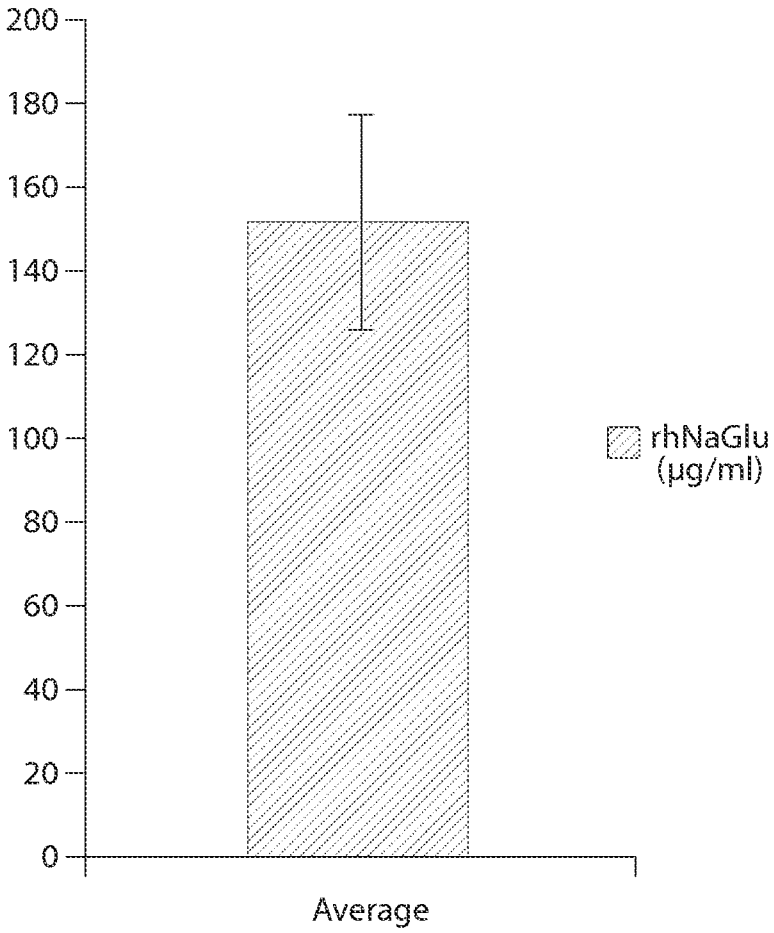

Fig. 7

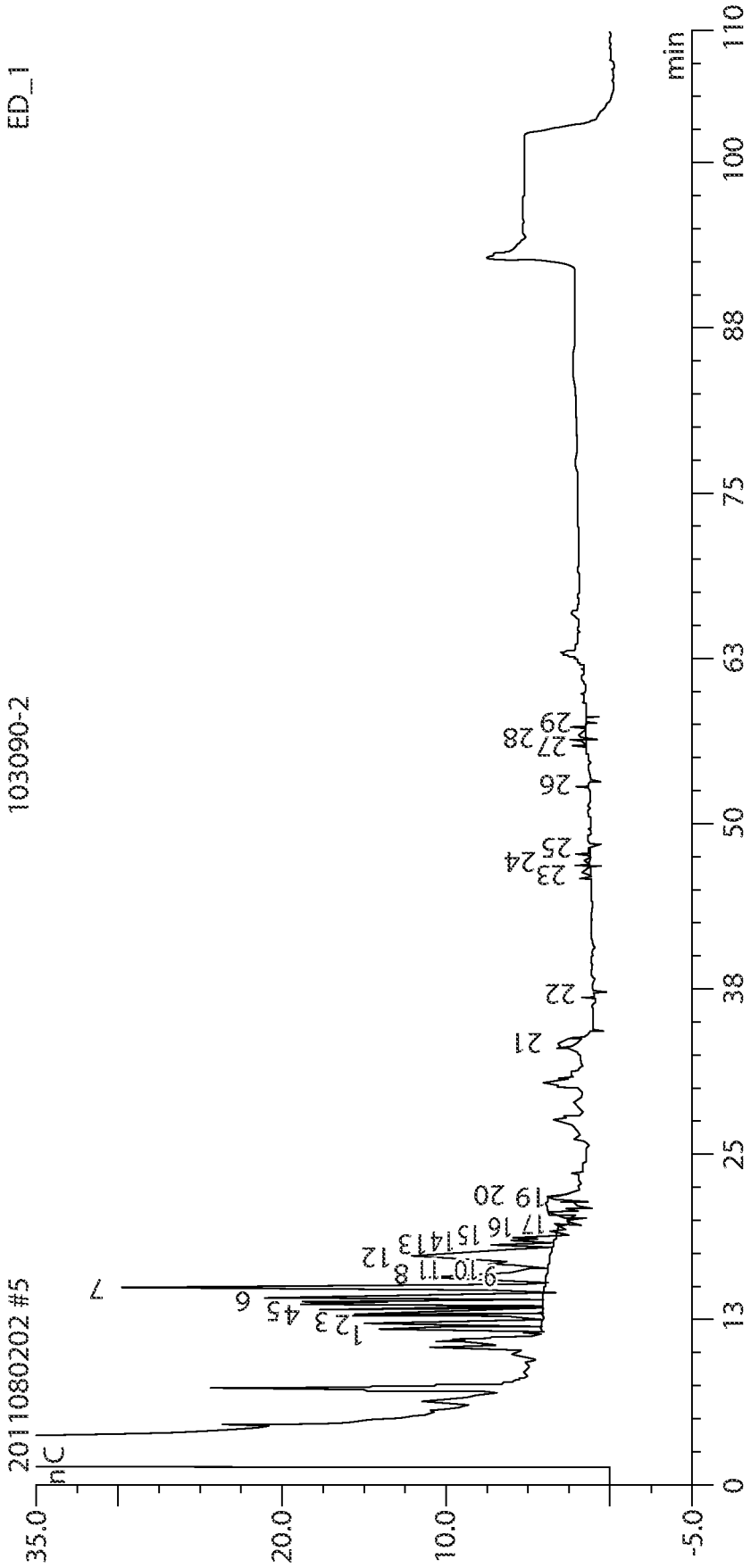
ED_1
103090-2
20110802O2 #5
nC
35.0
20.0
10.0
-5.0
min
0
13
25
38
50
63
75
88
100
110
1 2 3 4 5
6
7
8 9 10 11
12
13 14 15 16 17
19 20
21
22
23 24 25
26
27 28 29

Fig. 8

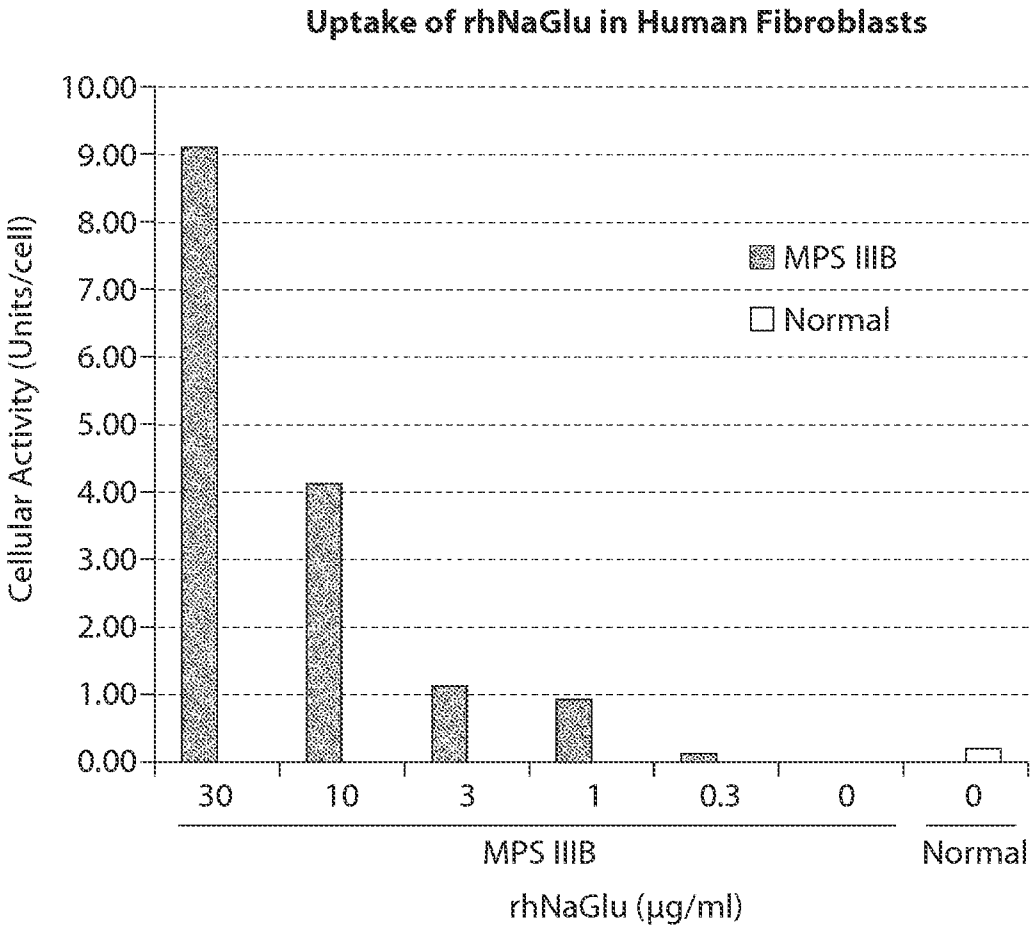
Uptake of rhNaGlu in Human Fibroblasts
Cellular Activity (Units/cell)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
MPS IIIB
Normal
30
10
3
1
0.3
0
0
MPS IIIB
Normal
rhNaGlu (µg/ml)

Fig. 9

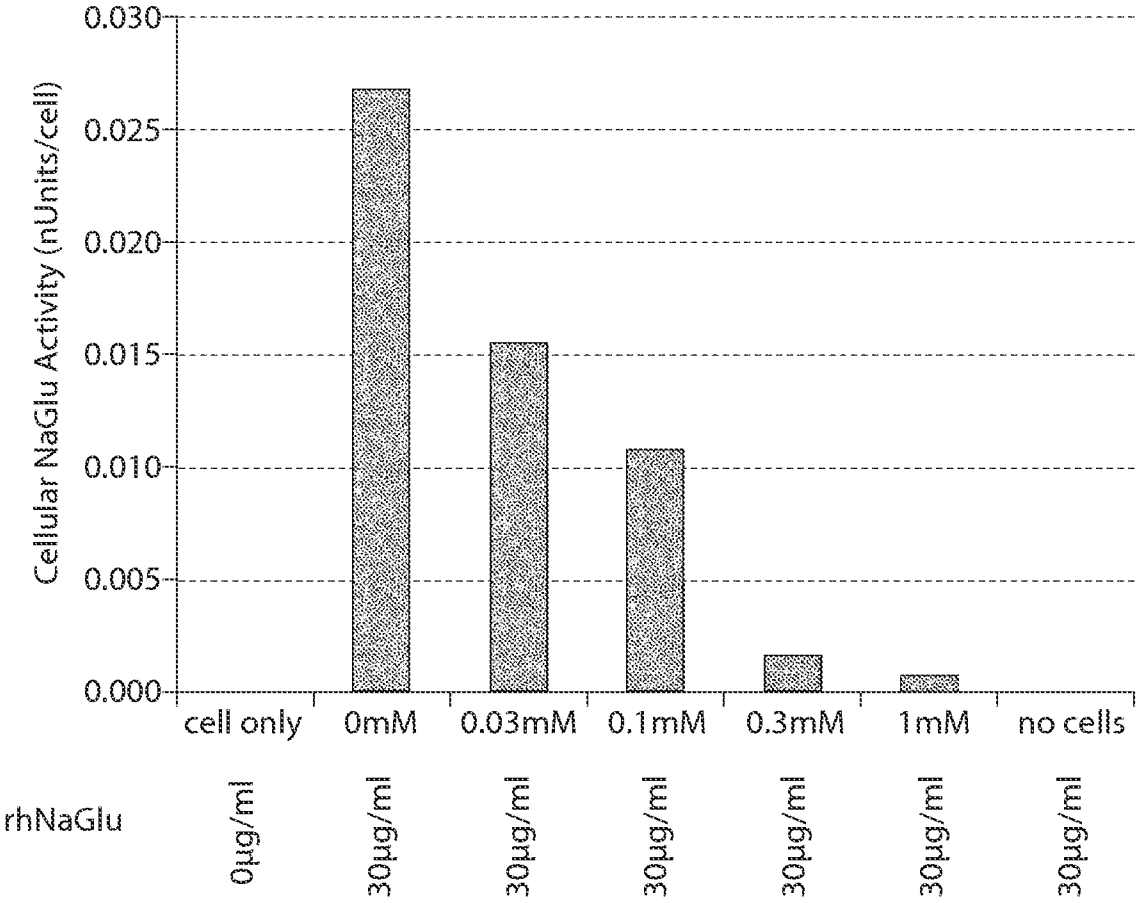
Cellular NaGlu Activity (nUnits/cell)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
cell only
0mM
0.03mM
0.1mM
0.3mM
1mM
no cells
rhNaGlu
0µg/ml
30µg/ml
30µg/ml
30µg/ml
30µg/ml
30µg/ml
30µg/ml

Fig. 10

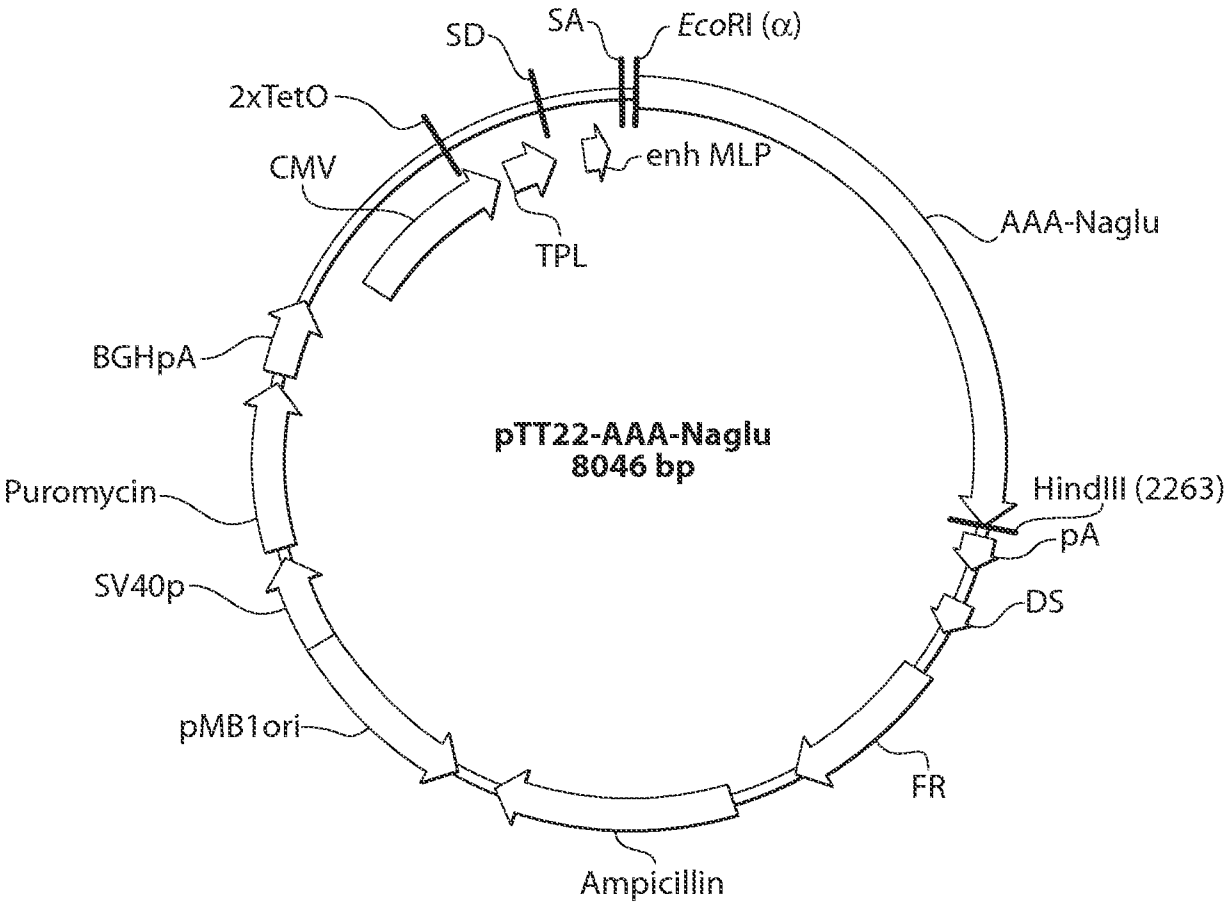
SD
SA
EcoRI (α)
2xTetO
CMV
enh MLP
TPL
AAA-Naglu
BGHpA
pTT22-AAA-Naglu
8046 bp
Puromycin
HindIII (2263)
pA
SV40p
DS
pMB1ori
FR
Ampicillin

Fig. 11

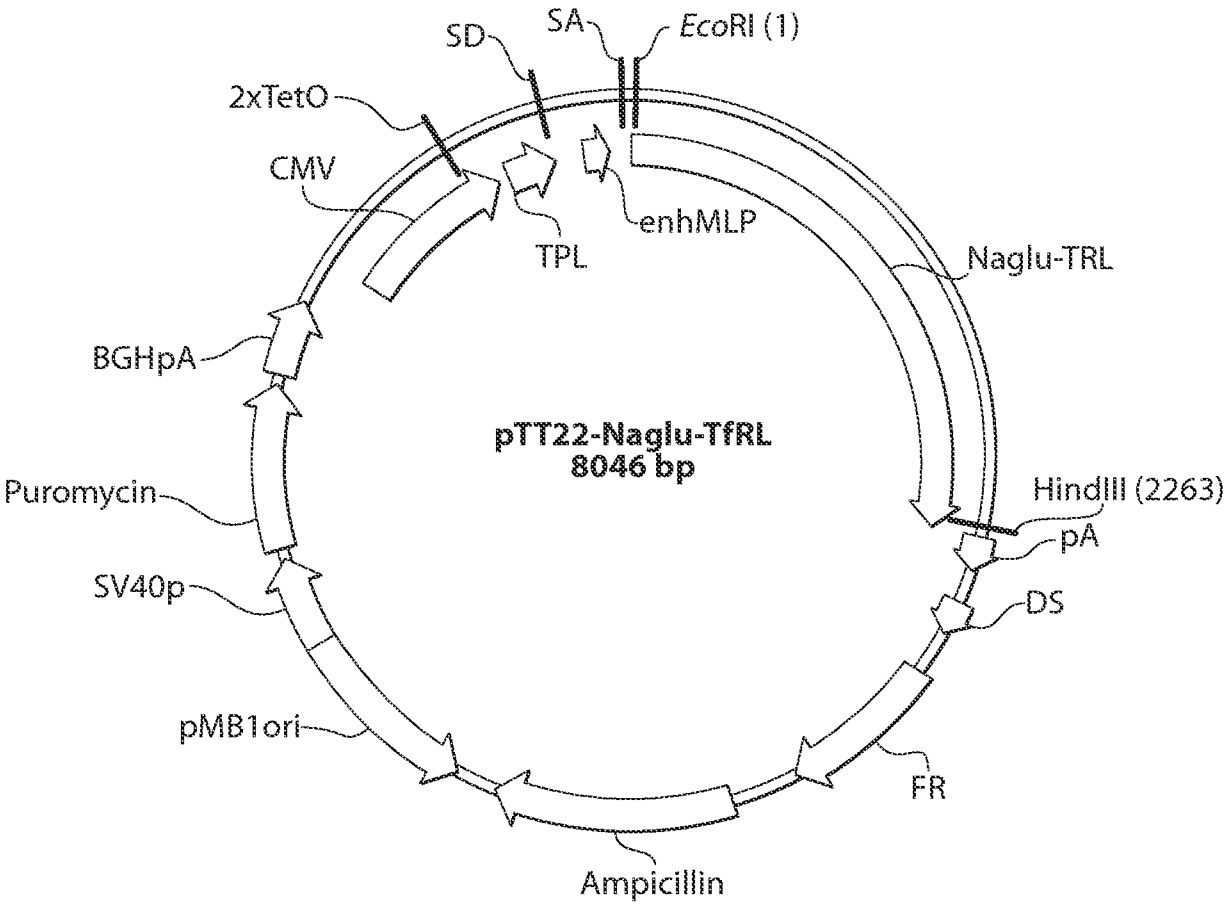
SD
SA
EcoRI (1)
2xTetO
CMV
TPL
enhMLP
Naglu-TRL
BGHpA
pTT22-Naglu-TfRL
8046 bp
HindIII (2263)
pA
Puromycin
SV40p
DS
pMB1ori
FR
Ampicillin

Fig. 12

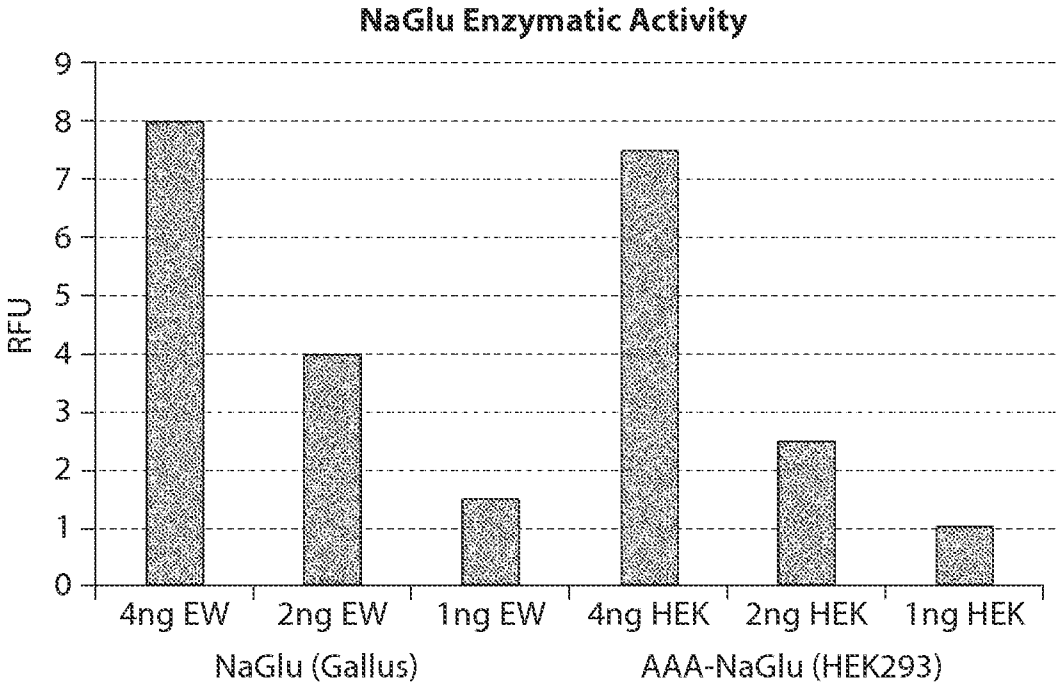
NaGlu Enzymatic Activity
RFU
9
8
7
6
5
4
3
2
1
0
4ng EW
2ng EW
1ng EW
4ng HEK
2ng HEK
1ng HEK
NaGlu (Gallus)
AAA-NaGlu (HEK293)

Fig. 13

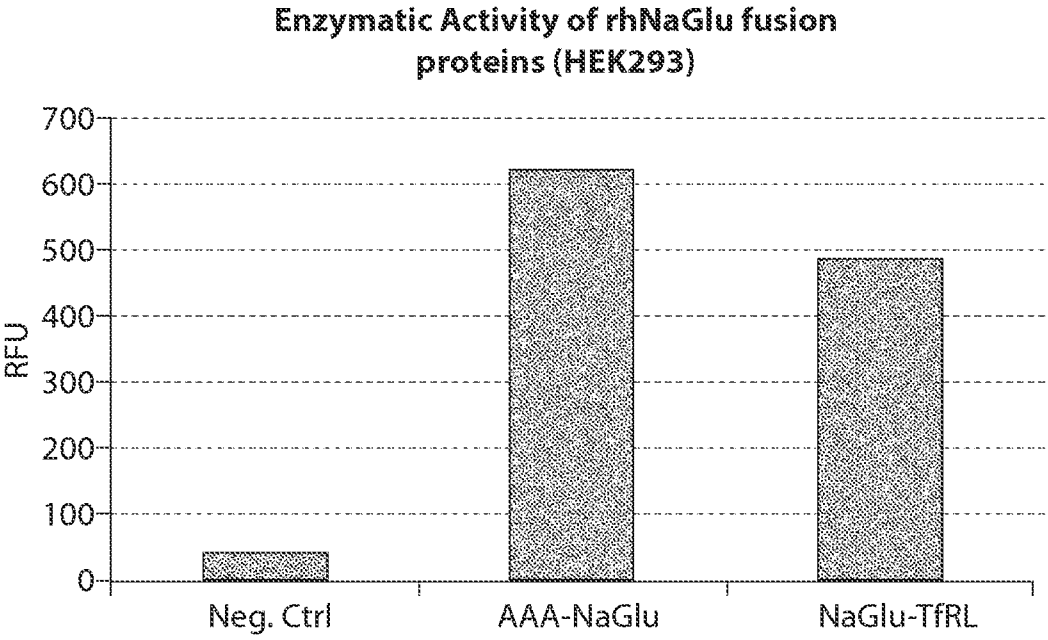
Enzymatic Activity of rhNaGlu fusion proteins (HEK293)
RFU
700
600
500
400
300
200
100
0
Neg. Ctrl
AAA-NaGlu
NaGlu-TfRL

Fig. 14

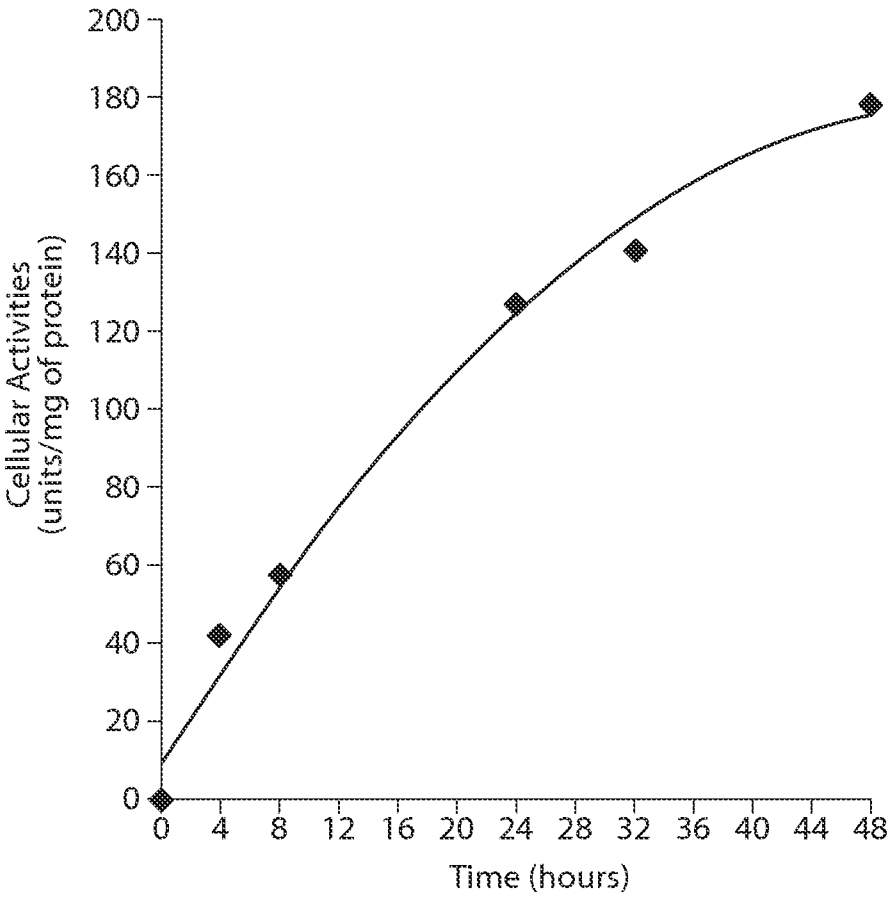
Cellular Activities
(units/mg of protein)
200
180
160
140
120
100
80
60
40
20
0
0 4 8 12 16 20 24 28 32 36 40 44 48
Time (hours)

Fig. 15

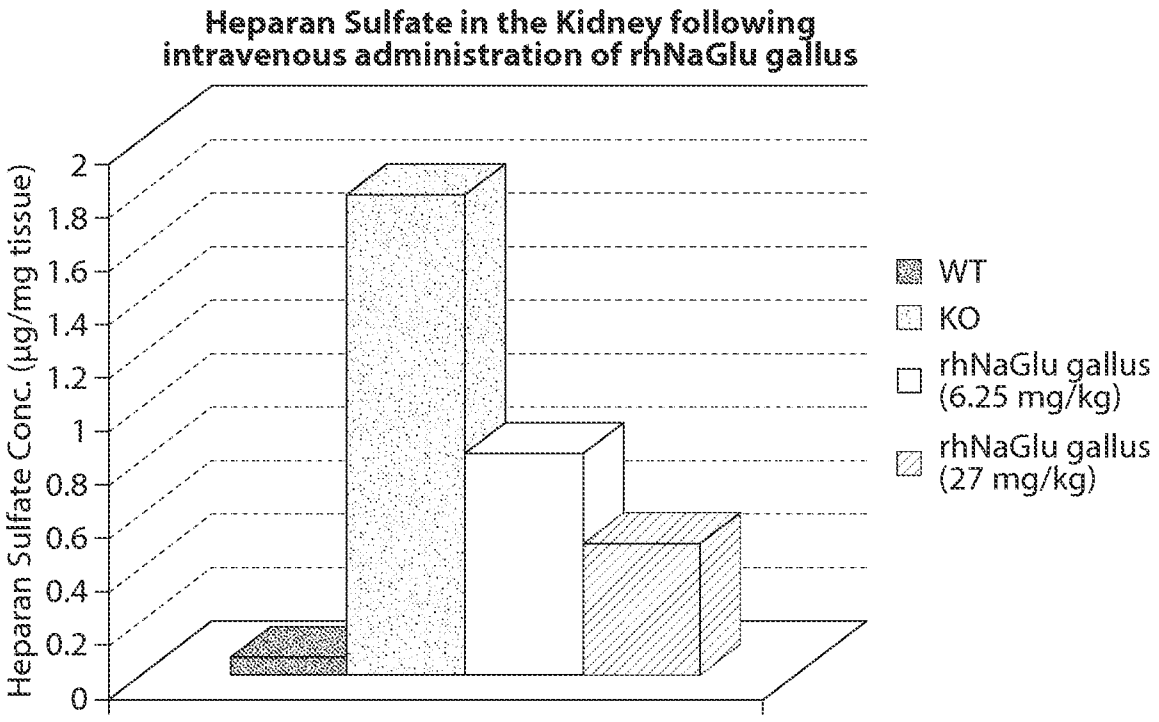
Heparan Sulfate in the Kidney following intravenous administration of rhNaGlu gallus
Heparan Sulfate Conc. (µg/mg tissue)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
WT
KO
rhNaGlu gallus (6.25 mg/kg)
rhNaGlu gallus (27 mg/kg)

Fig. 16

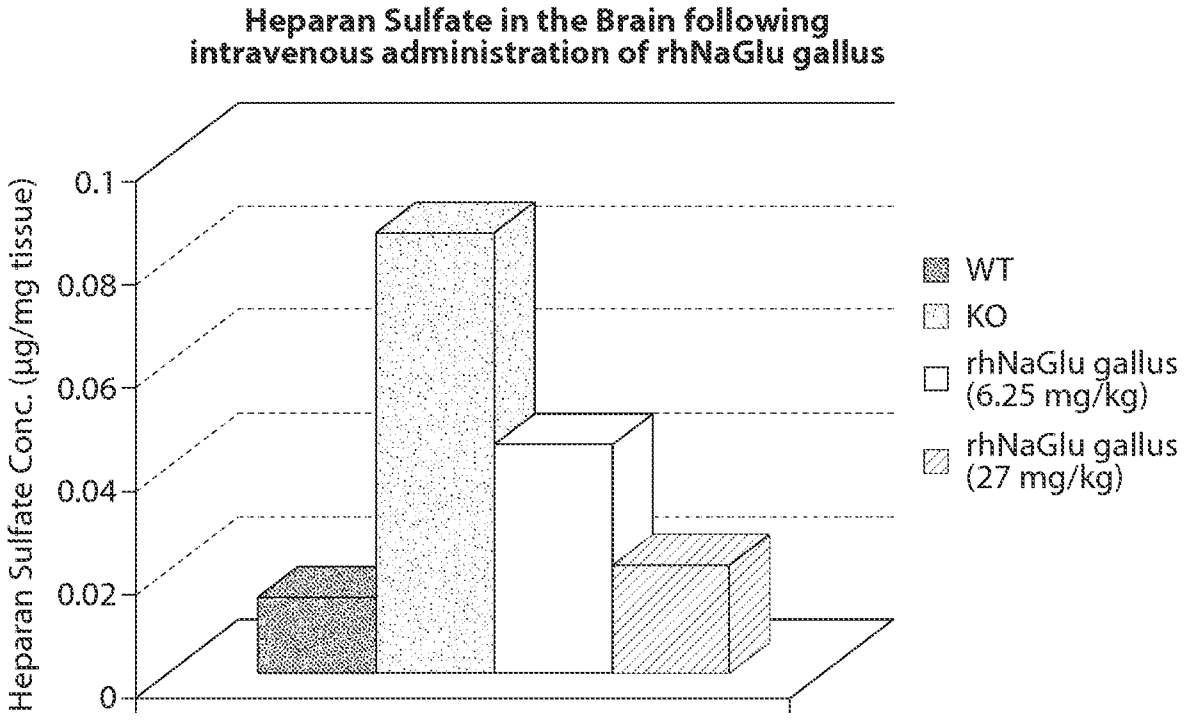
Heparan Sulfate in the Brain following intravenous administration of rhNaGlu gallus
Heparan Sulfate Conc. (µg/mg tissue)
0
0.02
0.04
0.06
0.08
0.1
WT
KO
rhNaGlu gallus (6.25 mg/kg)
rhNaGlu gallus (27 mg/kg)

Fig. 17

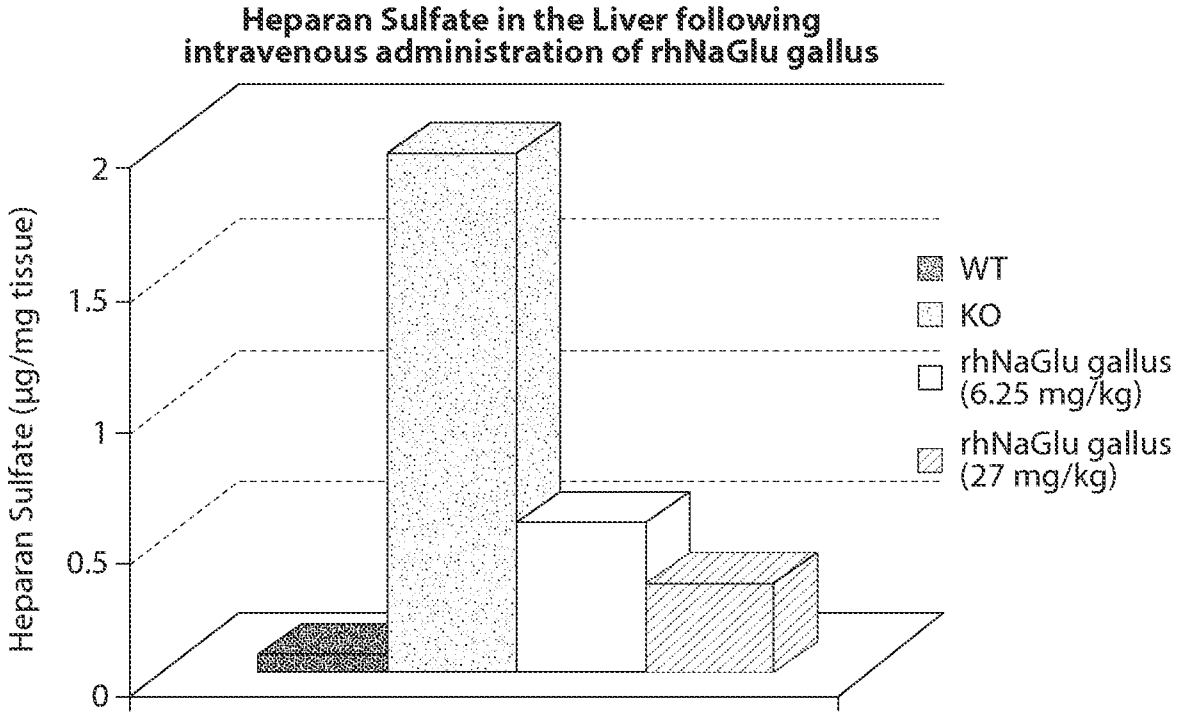
Heparan Sulfate in the Liver following intravenous administration of rhNaGlu gallus
Heparan Sulfate (µg/mg tissue)
2
1.5
1
0.5
0
WT
KO
rhNaGlu gallus (6.25 mg/kg)
rhNaGlu gallus (27 mg/kg)

Fig. 18

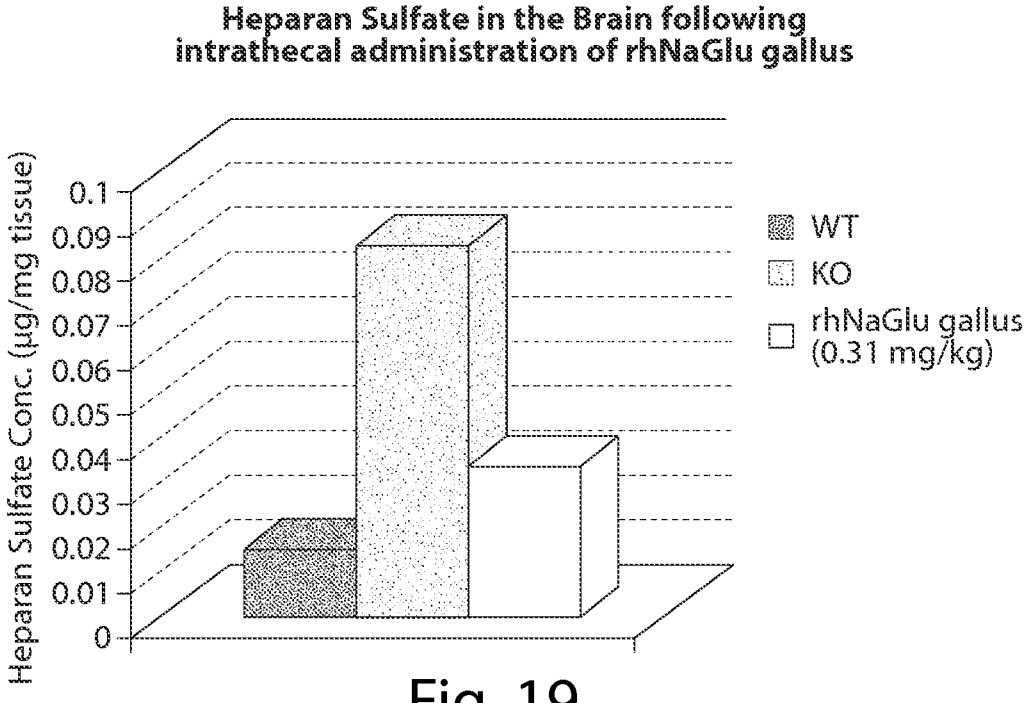
Heparan Sulfate in the Brain following intrathecal administration of rhNaGlu gallus
Heparan Sulfate Conc. (µg/mg tissue)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
WT
KO
rhNaGlu gallus (0.31 mg/kg)

Fig. 19

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 April 2013 (18.04.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/055888 A3

(51) **International Patent Classification**:
C12N 9/26 (2006.01) *A61K 38/47* (2006.01)
C12N 5/07 (2010.01)

(21) **International Application Number**:
PCT/US2012/059708

(22) **International Filing Date**:
11 October 2012 (11.10.2012)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/546,248 12 October 2011 (12.10.2011) US

(71) **Applicant**: **SYNAGEVA BIOPHARMA CORP.** [US/US]; 128 Spring Street, Suite 520, Lexington, MA 02421 (US).

(72) **Inventors**: **QUINN, Anthony**; 107 Manet Road, Chestnut Hill, MA 02467 (US). **LEAVITT, Markley, C.**; 1140 Latham Drive, Watkinsville, GA 30677 (US). **ZHINAN, Xia**; 29 Burke Lane, Wellesley, MA 02481 (US). **RUTKOWSKI, Joseph, Victor**; Synageva Biopharma Corp., 128 Spring St., Suite 520, Lexington, MA 02421 (US).

(74) **Agents**: **ZACHARAKIS, Maria, Laccotripe** et al.; McCarter & English, LLP, 265 Franklin Street, Boston, MA 02110 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) **Date of publication of the international search report**:
13 June 2013

(54) **Title**: RECOMBINANT HUMAN NAGLU PROTEIN AND USES THEREOF

(57) **Abstract**: The present invention provides compositions comprising an isolated mixture of recombinant human NaGlu proteins in which a substantial amount of the NaGlu proteins in the mixture has increased levels of phosphorylated mannose that confer the proteins to be efficiently internalized into human cells. The present invention also provides methods of producing such mixture of NaGlu proteins, vectors used in transgenesis and expression, host cells harboring such vectors, and methods of isolating and purifying the mixture of NaGlu proteins. The invention further provides methods of treating NaGlu associated diseases.

WO 2013/055888 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/59708

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 9/26; C12N 5/07; A61K 38/47 (2013.01)
USPC - 435/201, 435/349, 424/94.61
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)
USPC- 435/201, 435/349, 424/94.61

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/325; 435/85; 435/69.1; 435/68.1, 435/69.7, 435/195, 435/97, 530/350, 536/23.2, 536/23.5, 536/4.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); Google/ Patents/Scholar: Alpha-N-acetylglucosaminidase, NAGLU, EC 3.2.1.50, N-acetyl-alpha-glucosaminidase, NAG, transgenic avian. GenCore 6.3: SEQ ID NO: 1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2009/131698 A2 (Ellinwood, et al.) 29 October 2009 (29.10.2009) Abstract, SEQ ID NO: 1, pg 6, ln 13-14; pg 7, ln 6-11, 21-24; pg 8, ln 1-10; pg 9, ln 18-24; pg 16, ln 10; pg 17, ln 9-14, Fig 3B, 8, 9, 11; claim 7-9, 12, 19	1, 2, 11-17, 19-25, 32, 38-46, 51, 55-57, 72-76, 81-84 --- 3-10, 18, 26-31, 33-37, 47-50, 52-54, 87-88
Y	UniProtKB/Swiss-Prot Direct Submission P54802. ANAG_HUMAN (21 September 2011). [Retrieved from the Internet 21 February 2013: <http://www.uniprot.org/uniprot/P54802.txt?version=111>]; pg 5	3-10, 26, 28-31, 52, 54
Y	Kim, et al. Carbohydrate recognition by the mannose-6-phosphate receptors. Curr Opin Struct Biol. 2009, 19(5):534-42; pg 10, Fig 1 and its legend	4-10, 28-31, 33, 54
Y	WO 2009/088998 A2 (Harvey, et al.) 12 July 2009 (12.07.2009) Abstract, pg 3, ln 30-31; pg 5, ln 1-10, 28-31; pg 6, ln 1-4; pg 17, ln 5-14; pg 31, ln 18-36; pg 31, ln 25-36; pg 32, ln 1-3; Fig 8A; claim 1	18, 27, 34-37, 47-50, 53
Y	Ellinwood, et al. Safe, efficient, and reproducible gene therapy of the brain in the dog models of Sanfilippo and Hurler syndromes. Mol Ther. February 2011, 19(2):251-259; Abstract	87-88

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2013 (21.02.2013)	08 APR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/59708

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chen, et al. Glycoproteomics analysis of human liver tissue by combination of multiple enzyme digestion and hydrazide chemistry. J Proteome Res. 2009, 8(2):651-61	1-57, 72-76, 81-88
A	Liu, et al. Chemical synthesis of N-linked glycans carrying both mannose-6-phosphate and GlcNAc-mannose-6-phosphate motifs. J Org Chem. Epub 06 October 2011, 76(21):8682-8689	1-57, 72-76, 81-88
A	Bones, et al. Identification of N-glycans displaying mannose-6-phosphate and their site of attachment on therapeutic enzymes for lysosomal storage disorder treatment. Anal Chem. July 2011, 83(13):5344-52	1-57, 72-76, 81-88
A	Pohl, et al. Glycosylation- and phosphorylation-dependent intracellular transport of lysosomal hydrolases. Biol Chem. 2009, 390(7):521-7	1-57, 72-76, 81-88
A	Braulke, et al. Sorting of lysosomal proteins. Biochim Biophys Acta. 2009, 1793(4):605-14	1-57, 72-76, 81-88
A	Mizutani, et al. Genetic modification of a chicken expression system for the galactosylation of therapeutic proteins produced in egg white. Transgenic Res. Epub 13 April 2011, 21(1):63-75	1-57, 72-76, 81-88
A	US 2005/0142141 A1 (Pardridge) 30 June 2005 (30.06.2005) Abstract, claims 1-2	1-57, 72-76, 81-88
A	US 2010/0183577 A1 (Stern, et al.) 22 July 2010 (22.07.2010) Abstract, para [0011], [0067], [0096], claim 33, 34, 40 for fusion claims	1-57, 72-76, 81-88
A	US 7,001,994 B2 (Zhu) 21 February 2006 (21.02.2006) Abstract, claims 1, 4, 6,	1-57, 72-76, 81-88
A	US 2004/0248262 A1 (Koeberl, et al.) 09 December 2004 (09.12.2004) Abstract, para [0068], [0070], [0077], [0135], [0192], [0206]-[0207],	1-57, 72-76, 81-88
A	US 6,861,242 B2 (Canfield) 01 March 2005 (01.03.2005)	1-57, 72-76, 81-88
A	US 6,670,165 B2 (Canfield) 30 December 2003 (30.12.2003)	1-57, 72-76, 81-88
A	US 7,138,262 B1 (Daniel) 21 November 2006 (21.11.2006)	1-57, 72-76, 81-88
A	US 2009/0022702 A1 (Zhu) 22 January 2009 (22.01.2009)	1-57, 72-76, 81-88
A	US 7,001,994 B2 21 February 2006 (21.02.2006)	1-57, 72-76, 81-88
A	WO 2011/000958 A1 (Garcia, et al.) 01 June 2011 (01.06.2011)	1-57, 72-76, 81-88
A,P	US 2011/0318327 A1 (Concino, et al.) 29 December 2011 (29.12.2011)	1-57, 72-76, 81-88

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/59708

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

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

3. ☒ Claims Nos.: 77-80
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: claims 1-24, 72, 74, 81-88, drawn to a composition comprising an isolated mixture of recombinant human N-acetylalpha-D-glucosaminidase (rhNaGlu) comprising the amino acid sequence 24-743 of SEQ ID NO:I, wherein at least 10 % of said rhNaGlu in said mixture comprises at least one glycan structure having mannose-6-phosphate (M6P).

Group II: claims 25-57, 74, 81-88, drawn to a composition comprising an isolated mixture of rhNaGlu of the amino acid sequence 24-743 of SEQ ID NO:1, said mixture comprises a sufficient amount of rhNaGlu containing one or more glycan structures comprising M6P such that said rhNaGlu containing M6P is internalized into a mammalian cell having NaGlu deficiency via M6P receptor-mediated endocytosis and restores at least 50 % of NaGlu activity observed in a wild-type cell of the same type expressing endogenous NaGlu.

********** See Supplemental Sheet to continue **********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-57, 72-76, 81-88

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

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

********** Supplemental Sheet **********

In Continuation of Box III. Observations where unity of invention is lacking:

Group III: claims 58-70, drawn to a transgenic avian comprising a transgene containing a promoter operably linked to a nucleic acid sequence encoding a rhNaGlu, and a method of using said transgenic avian to produce said a rhNaGlu.

Group IV: claim 71, drawn to an isolated nucleic acid comprising the nucleic acid sequence of 5232-10248 of SEQID NO:4.

Group V: claims 73, 75, 76, drawn to a composition comprising recombinant human NaGlu protein that crosses the blood brain barrier of a mammal having NaGlu deficiency when administered intravenously, a method of delivering recombinant human NaGlu protein to the brain of a subject suffering from NaGlu deficiency by intravenously administering recombinant human NaGlu protein to the subject.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Groups I-III and V do not include the inventive concept of an isolated nucleic acid comprising the nucleic acid sequence of 5232-10248 of SEQID NO:4, as required by Group IV.

The inventions of Groups I-IV do not include the inventive concept of a composition comprising recombinant human NaGlu protein that crosses the blood brain barrier of a mammal having NaGlu deficiency when administered intravenously, a method of delivering recombinant human NaGlu protein to the brain of a subject suffering from NaGlu deficiency by intravenously administering recombinant human NaGlu protein to the subject, as required by Group V.

The inventions of Groups I, II, IV, and V do not include the inventive concept of a transgenic avian comprising a transgene containing a promoter operably linked to a nucleic acid sequence encoding a rhNaGlu, and a method of using said transgenic avian to produce said a rhNaGlu, as required by Group III.

The inventions of Groups III-V do not include the inventive concept of a composition comprising an isolated mixture of rhNaGlu comprising the amino acid sequence 24-743 of SEQ ID NO:1, wherein said rhNaGlu contains one or more glycan structures comprising M6P, as required by Groups I and II.

The inventions of Group I do not include the inventive concept of a composition comprising an isolated mixture of rhNaGlu comprising the amino acid sequence 24-743 of SEQ ID NO:1, wherein said mixture comprises a sufficient amount of rhNaGlu containing one or more glycan structures comprising M6P such that said rhNaGlu containing M6P is internalized into a mammalian cell having NaGlu deficiency via M6P receptor-mediated endocytosis and restores at least 50 % of NaGlu activity observed in a wild-type cell of the same type expressing endogenous NaGlu, as required by Group II.

The inventions of Group II do not include the inventive concept of a composition comprising an isolated mixture of recombinant human N -acetylalpha-D-glucosaminidase (rhNaGlu) comprising the amino acid sequence 24-743 of SEQ ID NO:I, wherein at least 10 % of said rhNaGlu in said mixture comprises at least one glycan structure having mannose-6-phosphate (M6P), as required by Group I.

In addition, a composition of claim 25 does not represent a contribution over prior art as being obvious over WO 2009/131698A2 to Ellinwood, et al. (hereinafter "Ellinwood") that discloses a composition (Ellinwood, claim 19, "A composition comprising the isolated recombinant mannose-6- phosphorylated N-acetyl-a-D-glucosaminidase (NaGlu) fusion protein of claims 12 and a pharmaceutically acceptable carrier") comprising an isolated mixture of rhNaGlu comprising the amino acid sequence 24-743 of SEQ ID NO:1 (Ellinwood, claim 12, "An isolated recombinant... NaGlu fusion protein, wherein the NaGlu protein comprises SEQ ID NO: 1", wherein SEQ ID NO: 1 is identical to the claimed SEQ ID NO: 1 and represents an amino acid sequence of human N-acetyl-alpha-D-glucosamidase (Ellinwood, Substituted Sheet 4, Fig 3B, showing that SEQ ID NO: 1 is prhNaGlu, wherein "p" stands for "protein", "r" stands for "recombinant", "h" stands for "human", as evidenced by the legend to the Fig 3B, "Figures 3 A-B depict the phNaGlu vector and cDNA sequences (SEQ ID NOs: 1-2) used to generate recombinant human NaGlu" (Ellinwood, pg 6, ln 13-14),
wherein said mixture comprises a sufficient amount of rhNaGlu containing one or more glycan structures comprising M6P (pg 17, ln 10; pg 7, ln 21-24, and Fig 11) such that said rhNaGlu containing M6P is internalized into a mammalian cell having NaGlu deficiency via M6P receptor-mediated endocytosis (pg 17, ln 11-14, pg 7, ln 6-11, "Figure 9 depicts the cellular uptake of NaGlu-ApoE. MPS HIB fibroblasts were incubated with 10 units of NaGlu- ApoE or native NaGlu for 5 hours. A. Depicts NaGlu activity in cells after uptake. B. Confocal microscopy of MPS IIIB fibroblasts incubated with NaGlu- ApoE or native NaGlu and stained with Anti-myc or Anti-Lamp 1 antibodies (third row is a merge of panels from columns one and two)").
Ellinwood does not specifically disclose restores at least 50% of NaGlu activity observed in a wild-type cell of the same type expressing endogenous NaGlu. However, Ellinwood does disclose that said NaGlu-ApoE has 120% activity of urine NaGlu when MPS IIIB fibroblasts were incubated with 10 units of NaGlu-ApoE or native urine NaGlu (see Fih 9 and its legend). Therefore, one of ordinary skill in the art would have a reasonable expectation that NaGlu-ApoE of Ellinwood restores at least 50% of NaGlu activity observed in a wild-type cell of the same type expressing endogenous NaGlu.

Groups I-V therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 103958676 A
(43)申请公布日 2014.07.30

(21)申请号 201280049876.4
(22)申请日 2012.10.11
(30)优先权数据
61/546,248 2011.10.12 US
(85)PCT国际申请进入国家阶段日
2014.04.11
(86)PCT国际申请的申请数据
PCT/US2012/059708 2012.10.11
(87)PCT国际申请的公布数据
WO2013/055888 EN 2013.04.18
(71)申请人 辛那杰瓦生物制药股份有限公司
地址 美国麻萨诸塞州
(72)发明人 A·奎恩 M·C·莱维特 X·芝南
J·V·鲁特科夫斯基
(74)专利代理机构 北京市铸成律师事务所 11313
代理人 孟锐
(51)Int.Cl.
C12N 9/26(2006.01)
C12N 5/07(2006.01)
A61K 38/47(2006.01)

权利要求书5页 说明书44页
序列表16页 附图19页

(54)发明名称

重组人NAGLU蛋白质及其用途

(57)摘要

本发明提供了包含重组人NaGlu蛋白质的分离混合物的组合物，其中所述混合物中大量NaGlu蛋白质具有增加水平的使所述蛋白质有效内化到人细胞中的磷酸化甘露糖。本发明还提供了生成此类NaGlu蛋白质混合物的方法、用于转基因和表达的载体、携带此类载体的宿主细胞及分离和纯化所述NaGlu蛋白质混合物的方法。本发明进一步提供了治疗NaGlu相关疾病的方法。

CN 103958676 A

1. 一种包含含有 SEQ ID NO:1 的氨基酸序列 24-743 的重组人 N- 乙酰基 -α-D- 氨基葡萄糖苷酶 (rhNaGlu) 的分离混合物的组合物，其中所述混合物中至少 10% 的所述 rhNaGlu 包含至少一个具有甘露糖 -6- 磷酸 (M6P) 的聚糖结构。

2. 根据权利要求 1 所述的组合物，其中具有 M6P 的所述 rhNaGlu 能够被吸收到缺乏 NaGlu 的哺乳动物细胞中，以致内化的 rhNaGlu 恢复了在相同类型的野生型哺乳动物细胞中观察到的正常 NaGlu 活性的至少 50%。

3. 根据权利要求 2 所述的组合物，其中所述聚糖结构为 N- 连接聚糖。

4. 根据权利要求 3 所述的组合物，其中所述 rhNaGlu 含有每摩尔蛋白质至少 1 摩尔的 M6P。

5. 根据权利要求 3 所述的组合物，其中所述 rhNaGlu 含有介于每摩尔蛋白质约 1 和约 6 摩尔之间的 M6P。

6. 根据权利要求 5 所述的组合物，其中所述 rhNaGlu 含有每摩尔蛋白质约 2 摩尔的 M6P。

7. 根据权利要求 5 所述的组合物，其中所述 rhNaGlu 含有每摩尔蛋白质约 3 摩尔的 M6P。

8. 根据权利要求 5 所述的组合物，其中所述 rhNaGlu 含有每摩尔蛋白质约 4 摩尔的 M6P。

9. 根据权利要求 5 所述的组合物，其中所述 rhNaGlu 含有每摩尔蛋白质约 5 摩尔的 M6P。

10. 根据权利要求 5 所述的组合物，其中所述 rhNaGlu 含有每摩尔蛋白质约 6 摩尔的 M6P。

11. 根据权利要求 2 所述的组合物，其中缺乏 NaGlu 的所述哺乳动物细胞为人细胞。

12. 根据权利要求 11 所述的组合物，其中缺乏 NaGlu 的所述人细胞为皮肤成纤维细胞、肝细胞或巨噬细胞。

13. 根据权利要求 11 所述的组合物，其中缺乏 NaGlu 的所述人细胞为神经元细胞。

14. 根据权利要求 13 所述的组合物，其中当全身施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

15. 根据权利要求 14 所述的组合物，其中当静脉内施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

16. 根据权利要求 13 所述的组合物，其中当鞘内施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

17. 根据权利要求 2 所述的组合物，其中具有 M6P 的所述 rhNaGlu 被 NaGlu 缺乏型细胞内化并且恢复了体内正常 NaGlu 活性的至少 100%。

18. 根据权利要求 2 所述的组合物，其中具有 M6P 的所述 rhNaGlu 含有每摩尔蛋白质至少 25 摩尔的甘露糖。

19. 根据权利要求 1 所述的组合物，其中所述混合物中至少 20%、30%、40%、50%、60%、70%、80%、90% 或 95% 的所述 rhNaGlu 含有 M6P。

20. 根据权利要求 19 所述的组合物，其中所述混合物中至少 20% 的所述 rhNaGlu 含有至少一个 M6P。

21. 根据权利要求 20 所述的组合物，其中所述混合物中至少 30% 的所述 rhNaGlu 含有至少一个 M6P。

22. 根据权利要求 21 所述的组合物，其中所述混合物中至少 40% 的所述 rhNaGlu 含有至少一个 M6P。

23. 根据权利要求 22 所述的组合物，其中所述混合物中至少 50% 的所述 rhNaGlu 含有至少一个 M6P。

24. 根据权利要求 23 所述的组合物，其中所述混合物中至少 60% 的所述 rhNaGlu 含有至少一个 M6P。

25. 一种包含含有 SEQ ID NO:1 的氨基酸序列 24-743 的重组人 N- 乙酰基 - α -D- 氨基葡萄糖苷酶 (rhNaGlu) 的分离混合物的组合物，其中所述混合物包含足够量的含有一个或多个聚糖结构的 rhNaGlu，所述聚糖结构包含甘露糖 -6- 磷酸 (M6P)，以致含有 M6P 的所述 rhNaGlu 经 M6P 受体介导的内吞作用被内化到有 NaGlu 缺乏症的哺乳动物细胞中并且恢复了在表达内源 NaGlu 的相同类型的野生型细胞中观察到的 NaGlu 活性的至少 50%。

26. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 经 N- 连接糖基化。

27. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 经 O- 连接糖基化。

28. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 包含每摩尔 rhNaGlu 至少 1 摩尔的 M6P。

29. 根据权利要求 26 所述的组合物，其中所述 rhNaGlu 包含每摩尔 rhNaGlu 约 1、2、3、4、5 或 6 摩尔的 M6P。

30. 根据权利要求 29 所述的组合物，其中所述 rhNaGlu 包含每摩尔 rhNaGlu 约 3 摩尔的 M6P。

31. 根据权利要求 29 所述的组合物，其中所述 rhNaGlu 包含每摩尔 rhNaGlu 约 4 摩尔的 M6P。

32. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 包含甘露糖。

33. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 包含 N- 乙酰葡萄糖胺 (GlcNAc)。

34. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 包含半乳糖。

35. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 包含 N- 乙酰半乳糖胺 (GalNAc)。

36. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 不含岩藻糖。

37. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 不含葡萄糖。

38. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 恢复了正常 NaGlu 酶活性的至少 60、70、80、90、95 或 100%。

39. 根据权利要求 25 所述的组合物，其中当全身施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

40. 根据权利要求 39 所述的组合物，其中当静脉内施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

41. 根据权利要求 25 所述的组合物，其中缺乏 NaGlu 的所述哺乳动物细胞为人细胞。

42. 根据权利要求 41 所述的组合物，其中所述人细胞为皮肤成纤维细胞、肝细胞或巨

噬细胞。

43. 根据权利要求 41 所述的组合物，其中缺乏 NaGlu 的所述人细胞为神经元细胞。

44. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 是包含第二部分的融合蛋白。

45. 根据权利要求 44 所述的组合物，其中所述第二部分为多肽。

46. 根据权利要求 45 所述的组合物，其中所述多肽选自转铁蛋白受体配体 (TfRL)、胰岛素样生长因子受体 (IGF2R) 配体、低密度脂蛋白 (LDL) 受体配体和酸性氨基酸 (AAA) 残基。

47. 根据权利要求 25 所述的组合物，其中所述 rhNaGlu 由转基因禽类生成。

48. 根据权利要求 47 所述的组合物，其中所述转基因禽类为鸡、火鸡、鸭或鹌鹑。

49. 根据权利要求 48 所述的组合物，其中所述转基因禽类为鸡。

50. 根据权利要求 49 所述的组合物，其中所述 rhNaGlu 由输卵管细胞生成。

51. 一种包含含有一个或多个聚糖结构的分离重组人 N- 乙酰基 -α-D- 氨基葡萄糖苷酶 (rhNaGlu) 的组合物，所述聚糖结构具有足够量的甘露糖 -6- 磷酸 (M6P)，所述 M6P 允许所述 rhNaGlu 经 M6P 受体介导的内吞作用内化到有 NaGlu 缺乏症的哺乳动物细胞中，以致当体内内化时，所述 rhNaGlu 恢复了在正常受试者相同类型的细胞中观察到的 NaGlu 活性的至少 50%。

52. 根据权利要求 51 所述的组合物，其中所述 rhNaGlu 蛋白质经 N- 连接糖基化。

53. 根据权利要求 51 所述的组合物，其中所述 rhNaGlu 蛋白质经 O- 连接糖基化。

54. 根据权利要求 51 所述的组合物，其中所述 rhNaGlu 包含每摩尔 rhNaGlu 约 2、3、4、5 或 6 摩尔的 M6P。

55. 根据权利要求 51 所述的组合物，其中当全身施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

56. 根据权利要求 55 所述的组合物，其中当静脉当内施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

57. 根据权利要求 51 所述的组合物，其中当鞘内施用时，所述 rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

58. 一种包含含有与编码重组人 NaGlu(rhNaGlu) 的核酸序列可操作地连接的启动子的转基因的转基因禽类，其中所述转基因包含在所述转基因禽类的基因组中并且在输卵管细胞中表达，以致所述 rhNaGlu 在所述转基因禽类的输卵管细胞中糖基化，分泌到输卵管腔内并且存积在所述转基因禽类蛋的蛋清中。

59. 根据权利要求 58 所述的转基因禽类，其中所述 rhNaGlu 包含每摩尔 rhNaGlu 约 2、3、4 或 6 摩尔的 M6P。

60. 根据权利要求 58 所述的转基因禽类，其中所述启动子组件是输卵管特异性启动子。

61. 根据权利要求 60 所述的转基因禽类，其中所述输卵管特异性启动子是卵白蛋白启动子。

62. 根据权利要求 58 所述的转基因禽类，其中所述转基因禽类选自鸡、火鸡、鸭和鹌鹑。

63. 一种由根据权利要求 58 所述的转基因禽类产出的蛋。

64. 一种生成重组人 NaGlu(rhNaGlu) 的方法，包括：

a) 生产包含具有与 SEQ ID NO:1 的 24-743 中所示的编码 rhNaGlu 的核酸序列可操作地连接的启动子组件的转基因的转基因禽类，其中所述转基因包含在所述转基因禽类的基因组中并且在输卵管细胞中表达，以致所述 rhNaGlu 在所述转基因禽类的输卵管细胞中糖基化，分泌到输卵管腔内并且存积在所述转基因禽类产的蛋的蛋清中；和

b) 从所述蛋清中分离所述 rhNaGlu。

65. 根据权利要求 64 所述的方法，其中所述启动子组件是输卵管特异性启动子。

66. 根据权利要求 65 所述的方法，其中所述输卵管特异性启动子是卵白蛋白启动子。

67. 根据权利要求 64 所述的方法，其中所述禽类选自鸡、火鸡、鸭和鹌鹑。

68. 根据权利要求 67 所述的方法，其中所述禽类为鸡。

69. 一种载体，其包含与卵白蛋白启动子可操作地连接的编码人 NaGlu 的核苷酸序列。

70. 一种宿主细胞，其包含根据权利要求 69 所述的载体。

71. 一种分离的核酸，其包含 SEQ ID NO:4 的 5232-10248 的核酸序列。

72. 一种药物制剂，其包含与药学上可接受的载体、稀释剂或赋形剂组合的根据权利要求 1 所述的组合物。

73. 一种组合物，其包含当静脉内施用时穿过有 NaGlu 缺乏症的哺乳动物的血脑屏障的重组人 NaGlu 蛋白质。

74. 一种治疗患有 NaGlu 缺乏症的受试者的方法，所述方法包括向所述受试者施用治疗有效量的根据权利要求 1、25 或 51 中任一项所述的组合物。

75. 一种将重组人 NaGlu 蛋白质递送到患有 NaGlu 缺乏症的受试者脑部的方法，所述方法包括向所述受试者静脉内施用重组人 NaGlu 蛋白质。

76. 一种以治疗有效量从循环中转运重组人 NaGlu 蛋白质穿过所述血脑屏障的方法，所述方法包括向有 NaGlu 缺乏症的受试者静脉施用重组人 NaGlu 蛋白质。

77. 根据权利要求 74-76 中任一项所述的方法，其中所述 NaGlu 缺乏症是桑菲列浦氏综合征 B 型。

78. 根据权利要求 74-76 中任一项所述的方法，其中所述受试者为人。

79. 根据权利要求 74-76 中任一项所述的方法，其中以约 1 至约 30mg/kg 体重的剂量向所述受试者静脉内施用所述重组人 NaGlu 蛋白质。

80. 根据权利要求 79 所述的方法，其中以约 6 至约 27mg/kg 体重的剂量向所述受试者静脉施用所述重组人 NaGlu 蛋白质。

81. 根据权利要求 74 所述的方法，其中向所述受试者鞘内施用所述重组人 NaGlu 蛋白质。

82. 根据权利要求 81 所述的方法，其中以至少约 0.3mg/kg 体重的剂量鞘内施用所述重组人 NaGlu 蛋白质。

83. 根据权利要求 82 所述的方法，其中以约 1、2、3、4、5、6、7、8、9 或 10mg/kg 体重的剂量鞘内施用所述重组人 NaGlu 蛋白质。

84. 根据权利要求 82 所述的方法，其中以约 10 至约 30mg/kg 体重的剂量鞘内施用所述重组人 NaGlu 蛋白质。

85. 根据权利要求 74 所述的方法，其中所述治疗有效量是有效降低所述受试者脑部、

肾脏或肝脏中的硫酸乙酰肝素水平的量。

86. 根据权利要求 74 所述的方法，其中所述治疗有效量是有效增加所述受试者脑部或肝脏中的 NaGlu 活性的量。

87. 根据权利要求 74 所述的方法，进一步包括施用第二治疗剂。

88. 根据权利要求 87 所述的方法，其中所述第二治疗剂是免疫抑制剂。

重组人 NAGLU 蛋白质及其用途

[0001] 相关申请的交叉引用

[0002] 本申请涉及 2011 年 10 月 12 日提交的美国临时申请序列号 61/546,248 并且要求其优先权，其全部内容通过引用明确并入本文。

[0003] 发明背景

[0004] 桑菲列浦氏综合征 B 型 (Sanfilippo Syndrome B) 是由称为 N- 乙酰基 -α -D- 氨基葡萄糖苷酶 (NaGlu) 的溶酶体酶缺乏引起的常染色体隐性溶酶体贮积病 (LSD)。作为溶酶体中糖胺聚糖 (GAG) 的逐步分解的一部分，降解硫酸乙酰肝素需要 NaGlu。缺乏或没有 NaGlu 导致硫酸乙酰肝素的积累和尿排泄。迄今为止鉴定了超过 70 种不同突变，桑菲列浦氏综合征 B 型表现出广泛的分子和遗传异质性。

[0005] 大约 200,000 个产儿中有 1 个患有桑菲列浦氏综合征 B 型并且缺乏症主要在幼儿中出现。在最初的无症状期后，患有桑菲列浦氏综合征 B 型的患者通常出现心理发展迟缓和行为问题，接着是智力逐渐下降，导致严重智力低下、痴呆和运动疾病。语言习得缓慢并且不完整。深受影响的患者可能早在 2 岁时就呈现精神运动和语言发展延迟。所述疾病通常发展为增加行为障碍和睡眠障碍。虽然临床特征主要是神经学上的，但是患者往往发展腹泻、龋齿、肝脾增大、关节强硬、脱发 (hirsteness) 和 / 或毛发粗糙并且可能表现出凝血问题。在疾病的最后阶段，患者变得不能移动和反应迟钝并且发展吞咽困难和癫痫。患病儿童的寿命通常不会超过少年晚期到二十岁出头。

[0006] 已经尝试了不同方法在患者中提供缺失的酶。为生成供酶替代疗法 (ERT) 的 NaGlu，已经在各种哺乳动物细胞培养系统中表达了人 NaGlu。然而，与细胞内天然存在的运输到溶酶体的 NaGlu 相反，发现由哺乳动物细胞生成和分泌的重组 NaGlu 蛋白质不含或仅含微量的甘露糖 6- 磷酸 (M6P)。已知分泌的 NaGlu 中 M6P 部分缺少或不足会妨碍其有效内化到在其质膜表面有 M6P 受体的靶细胞（例如，人皮肤成纤维细胞）中（见，Zhao 等，Protein Expression and Purification,19:202-211(2000)；和 Weber 等，Protein Expression and Purification,21:251-259(2001))。 在 CHO 细胞中表达的分泌型小鼠 NaGlu、HeLa 细胞中表达的分泌型人 NaGlu、人成纤维细胞中表达的分泌型人 NaGlu 和人胚肾 (HEK) 细胞系 293 中表达的分泌型人 NaGlu 中看到低程度的磷酸化（见，Zhao 等，Protein Expression and Purification,19:202-211(2000)；Yogalingam 等，Biochim Biophys.Acta1502:415-425；和 Weber 等，Protein Expression and Purification,21:251-259(2001))。从哺乳动物细胞分泌的 NaGlu 蛋白质中 N- 聚糖没有磷酸化或弱磷酸化对开发适合酶替代疗法的重组人 NaGlu 蛋白质造成了一个主要障碍，因为前述所有尝试均未能生成被靶细胞有效吸收的酶，因为如果完全可检测，内化蛋白质的浓度比野生型水平低近一千倍（见，Zhao 等，Protein Expression and Purification,19:202-211(2000))。迄今为止，没有经批准的产品可用于桑菲列浦氏综合征 B 型的治疗。

[0007] 已经尝试直接向 NaGlu 缺乏的小鼠的中枢神经系统 (CNS) 施用（例如，向脑脊髓液 (CSF) 鞘内施用）哺乳动物细胞生成的具有天然氨基酸序列的重组人 NaGlu 蛋白质

(rhNaGlu)，但是由于所述蛋白质在脑室室管膜衬里上过量积累以及缺乏有效细胞摄取的必需 M6P 残基，未能证明酶向脑部的成功生物分布。类似地，全身施用（即，静脉内 (IV) 注射）哺乳动物细胞生成的具有天然氨基酸序列的 rhNaGlu 也未能证明所述蛋白质向脑部的成功定位。除已知与高度侵入性鞘内施用相关的风险外，将 rhNaGlu 靶向脑部的这些障碍已经是获得用于治疗桑菲列浦氏综合征 B 型的有效疗法的太大挑战。

[0008] 因此，需要提供具酶活性并且具有允许蛋白质穿过血脑屏障 (BBB) 和允许蛋白质有效内化到靶细胞的溶酶体中的物理性质的稳定 NaGlu 蛋白质。还需要可提供有效穿过血脑屏障并有效内化到人靶细胞中的重组人 NaGlu 的高表达且稳健的蛋白质产生平台。

[0009] 发明概述

[0010] 本发明涉及包含对治疗有用，例如在桑菲列浦氏综合征 B 型的治疗中有用的重组人 NaGlu 蛋白质 (rhNaGlu) 的组合物。本发明基于惊人且出乎意料的发现，即本文所述 rhNaGlu 具有一个或多个糖基化模式，其允许 rhNaGlu 有效穿过血脑屏障 (BBB) 并且吸收到缺乏所述酶的动物的中枢神经系统 (CNS) 内的细胞中，导致脑部 α-N- 乙酰氨基葡萄糖苷酶活性显著增加，以及底物水平降低。而且，本文所述 rhNaGlu 被有效吸收到哺乳动物细胞（例如，人细胞）中，导致与由未设计成产生特定糖基化的未修饰哺乳动物细胞生成和分泌的 NaGlu 蛋白质相比，酶活性增加。NaGlu 蛋白质的细胞摄取增加还通过将对增加量和增加给药频率的需要减到最低，对用于患有桑菲列浦氏综合征 B 型的人患者的酶替代疗法提供了利益，并且从而大大降低了免疫原性的潜在风险。

[0011] 本文所述 rhNaGlu 蛋白质含有足够量的寡糖（例如，甘露糖和磷酸化甘露糖（即，M6P)），以允许经由甘露糖和 / 或 M6P 受体介导的内吞作用的有效细胞摄取，并且正确靶向人细胞中。在一个实施方案中，rhNaGlu 含有至少 1 摩尔蛋白质，例如每摩尔蛋白质 1、2、3、4、5 或 6 摩尔 M6P。在一个实施方案中，rhNaGlu 可内化到 NaGlu 缺乏的人细胞中，以致内化蛋白质完全 (100% 或更高）恢复了缺乏 NaGlu 的细胞中 NaGlu 活性的正常水平（即，野生型水平）。

[0012] 本文还公开了生产表达受益于甘露糖磷酸化的 rhNaGlu 的转基因禽类的方法。具体而言，在输卵管细胞中表达 rhNaGlu 蛋白质的转基因禽类向输卵管腔内分泌并将所述蛋白质存积在蛋清中。含有此类 rhNaGlu 的禽蛋也包括在本发明中。

[0013] 本发明还涵盖含有编码 rhNaGlu 的转基因的载体和宿主细胞以及包含用于应用此类 rhNaGlu 治疗桑菲列浦氏综合征 B 型的 rhNaGlu 的药物组合物。

[0014] 一方面，本发明提供了一种包含含有 SEQ ID NO:1 的氨基酸序列 24-743 的重组人 N- 乙酰基 -α-D- 氨基葡萄糖苷酶 (rhNaGlu) 的分离混合物的组合物，其中所述混合物中至少 10% 的 rhNaGlu 包含至少一个具有甘露糖 -6- 磷酸 (M6P) 的聚糖结构。在一个实施方案中，具有 M6P 的 rhNaGlu 能够被吸收到缺乏 NaGlu 的哺乳动物细胞中，以致内化的 rhNaGlu 恢复了在相同类型的野生型哺乳动物细胞中观察到的正常 NaGlu 活性的至少 50%、60%、70%、80%、90% 或 100%。在另一实施方案中，聚糖结构为 N- 连接聚糖。

[0015] 在一个实施方案中，rhNaGlu 含有每摩尔蛋白质至少 1 摩尔的 M6P。在另一实施方案中，rhNaGlu 含有介于每摩尔蛋白质约 1 摩尔和约 6 摩尔之间的 M6P。在另一实施方案中，rhNaGlu 含有每摩尔蛋白质约 2 摩尔的 M6P。在又一实施方案中，rhNaGlu 含有每摩尔蛋白质约 3 摩尔的 M6P。在另一实施方案中，rhNaGlu 含有每摩尔蛋白质约 4 摩尔的 M6P。在另

一实施方案中，rhNaGlu 含有每摩尔蛋白质约 5 摩尔的 M6P。在又一实施方案中，rhNaGlu 含有每摩尔蛋白质约 6 摩尔的 M6P。

[0016] 在一个实施方案中，缺乏 NaGlu 的哺乳动物细胞为人细胞。在另一实施方案中，缺乏 NaGlu 的人细胞为皮肤成纤维细胞、肝细胞或巨噬细胞。在一个实施方案中，缺乏 NaGlu 的人细胞为神经元细胞。

[0017] 在一个实施方案中，当全身施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。在一个特定实施方案中，当静脉内施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。在一个实施方案中，当鞘内施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

[0018] 在一个实施方案中，具有 M6P 的 rhNaGlu 被 NaGlu 缺乏型细胞内化并且恢复了体内正常 NaGlu 活性的至少 100%。在一个实施方案中，具有 M6P 的 rhNaGlu 含有每摩尔蛋白质至少 25 摩尔的甘露糖。

[0019] 在一个实施方案中，混合物中至少 20%、30%、40%、50%、60%、70%、80%、90% 或 95% 的 rhNaGlu 含有 M6P。在另一实施方案中，混合物中至少 20% 的 rhNaGlu 含有至少一个 M6P。在另一实施方案中，混合物中至少 30% 的 rhNaGlu 含有至少一个 M6P。在另一实施方案中，混合物中至少 40% 的 rhNaGlu 含有至少一个 M6P。在另一实施方案中，混合物中至少 50% 的 rhNaGlu 含有至少一个 M6P。在另一实施方案中，混合物中至少 60% 的 rhNaGlu 含有至少一个 M6P。

[0020] 另一方面，本发明提供了一种包含含有 SEQ ID NO:1 的氨基酸序列 24-743 的重组人 N- 乙酰基 -α-D- 氨基葡萄糖苷酶 (rhNaGlu) 的分离混合物的组合物，其中所述混合物包含足够量的含有一个或多个聚糖结构的 rhNaGlu，所述聚糖结构包含甘露糖 -6- 磷酸 (M6P)，以致含有 M6P 的 rhNaGlu 经 M6P 受体介导的内吞作用被内化到有 NaGlu 缺乏症的哺乳动物细胞中并且恢复了在表达内源 NaGlu 的相同类型的野生型细胞中观察到的 NaGlu 活性的至少 50%。在一个实施方案中，rhNaGlu 经 N- 连接糖基化。在另一实施方案中，rhNaGlu 经 O- 连接糖基化。

[0021] 在一个实施方案中，rhNaGlu 包含每摩尔 rhNaGlu 至少 1 摩尔的 M6P。在另一实施方案中，rhNaGlu 包含每摩尔 rhNaGlu 约 1、2、3、4、5 或 6 摩尔的 M6P。在另一实施方案中，rhNaGlu 包含每摩尔 rhNaGlu 约 3 摩尔的 M6P。在另一实施方案中，rhNaGlu 包含每摩尔 rhNaGlu 约 4 摩尔的 M6P。

[0022] 在一个实施方案中，rhNaGlu 包含甘露糖。在另一实施方案中，rhNaGlu 包含 N- 乙酰葡萄糖胺 (GlcNAc)。在另一实施方案中，rhNaGlu 包含半乳糖。在另一实施方案中，rhNaGlu 包含 N- 乙酰半乳糖胺 (GalNAc)。在另一实施方案中，rhNaGlu 不含岩藻糖。在另一实施方案中，rhNaGlu 不含葡萄糖。在一个实施方案中，rhNaGlu 恢复了正常 NaGlu 酶活性的至少 60、70、80、90、95 或 100%。

[0023] 在另一实施方案中，当全身施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。在一个实施方案中，当静脉内施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。在另一实施方案中，当鞘内施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

[0024] 在一个实施方案中，缺乏 NaGlu 的哺乳动物细胞为人细胞。在另一实施方案中，人

细胞为皮肤成纤维细胞、肝细胞或巨噬细胞。在一个实施方案中，缺乏 NaGlu 的所述人细胞为神经元细胞。

[0025] 在一个实施方案中，rhNaGlu 是包含第二部分的融合蛋白。在一个实施方案中，所述第二部分为多肽。在另一实施方案中，所述多肽选自转铁蛋白受体配体 (TfRL)、胰岛素样生长因子受体 (IGF2R) 配体、低密度脂蛋白 (LDL) 受体配体和酸性氨基酸 (AAA) 残基。

[0026] 在一个实施方案中，rhNaGlu 由转基因禽类生成。在一个实施方案中，所述转基因禽类为鸡、火鸡、鸭或鹌鹑。在一个实施方案中，所述转基因禽类为鸡。在一个实施方案中，rhNaGlu 由输卵管细胞生成。

[0027] 另一方面，本发明提供了一种包含含有一个或多个聚糖结构的分离重组人 N- 乙酰基 -α-D- 氨基葡萄糖苷酶 (rhNaGlu) 的组合物，所述聚糖结构具有足够量的甘露糖 -6- 磷酸 (M6P)，所述 M6P 允许所述 rhNaGlu 经 M6P 受体介导的内吞作用内化到有 NaGlu 缺乏症的哺乳动物细胞中，以致当体内内化时，所述 rhNaGlu 恢复了在表达内源 NaGlu 的相同类型的野生型细胞中观察到的 NaGlu 活性的至少 50%。

[0028] 在一个实施方案中，rhNaGlu 蛋白质经 N- 连接糖基化。在另一实施方案中，rhNaGlu 蛋白质经 O- 连接糖基化。在一个实施方案中，rhNaGlu 包含每摩尔 rhNaGlu 约 2、3、4、5 或 6 摩尔的 M6P。

[0029] 在一个实施方案中，当全身施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。在另一实施方案中，当静脉内施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。在另一实施方案中，当鞘内施用时，rhNaGlu 被有效递送到有 NaGlu 缺乏症的哺乳动物的脑部。

[0030] 另一方面，本发明提供了一种包含含有与编码重组人 NaGlu(rhNaGlu) 的核酸序列可操作地连接的启动子的转基因的转基因禽类，其中所述转基因包含在所述转基因禽类的基因组中并且在输卵管细胞中表达，以致所述 rhNaGlu 在所述转基因禽类的输卵管细胞中糖基化，分泌到输卵管腔内并且存积在所述转基因禽类蛋的蛋清中。

[0031] 在一个实施方案中，rhNaGlu 包含每摩尔 rhNaGlu 约 2、3、4 或 6 摩尔的 M6P。在另一实施方案中，所述启动子组件是输卵管特异性启动子。在另一实施方案中，所述输卵管特异性启动子是卵白蛋白启动子。在又一实施方案中，所述转基因禽类选自鸡、火鸡、鸭和鹌鹑。

[0032] 另一方面，本发明提供了一种由本发明的转基因禽类产出的蛋。

[0033] 再一方面，本发明提供了一种生成重组人 NaGlu(rhNaGlu) 的方法，包括 :a) 生产包含具有与 SEQ ID NO:1 的 24-743 中所示的编码 rhNaGlu 的核酸序列可操作地连接的启动子组件的转基因的转基因禽类，其中所述转基因包含在所述转基因禽类的基因组中并且在输卵管细胞中表达，以致所述 rhNaGlu 在所述转基因禽类的输卵管细胞中糖基化，分泌到输卵管腔内并且存积在所述转基因禽类产的蛋的蛋清中 ;和 b) 从所述蛋清中分离所述 rhNaGlu。

[0034] 在一个实施方案中，所述启动子组件是输卵管特异性启动子。在另一实施方案中，所述输卵管特异性启动子是卵白蛋白启动子。在一个实施方案中，所述禽类选自鸡、火鸡、鸭和鹌鹑。在一个实施方案中，所述禽类为鸡。

[0035] 另一方面，本发明提供了一种载体，其包含与卵白蛋白启动子可操作地连接的编

码人 NaGlu 的核苷酸序列。另一方面，本发明提供了一种包含本发明载体的宿主细胞。另一方面，本发明提供了一种包含 SEQ ID NO:4 的 5232-10248 的核酸序列的分离的核酸。

[0036] 一方面，本发明提供了一种药物制剂，其包含与药学上可接受的载体、稀释剂或赋形剂组合的本发明的组合物。

[0037] 另一方面，本发明提供了一种组合物，其包含当静脉内施用时穿过有 NaGlu 缺乏症的哺乳动物的血脑屏障的重组人 NaGlu 蛋白质。

[0038] 再一方面，本发明提供了一种治疗患有 NaGlu 缺乏症的受试者的方法，所述方法包括向所述受试者施用治疗有效量的本发明的组合物。

[0039] 再一方面，本发明提供了一种将重组人 NaGlu 蛋白质递送到患有 NaGlu 缺乏症的受试者脑部的方法，所述方法包括向所述受试者静脉内施用重组人 NaGlu 蛋白质。

[0040] 另一方面，本发明提供了一种以治疗有效量从循环中转运重组人 NaGlu 蛋白质穿过血脑屏障的方法，所述方法包括向有 NaGlu 缺乏症的受试者静脉内施用重组人 NaGlu 蛋白质。

[0041] 在一个实施方案中，NaGlu 缺乏症是桑菲列浦氏综合征 B 型。在另一实施方案中，所述受试者为人。

[0042] 在另一实施方案中，以约 0.5 至约 50mg/kg 体重的剂量向所述受试者静脉内施用所述重组人 NaGlu 蛋白质。在另一实施方案中，以约 1 至约 30mg/kg 体重的剂量向所述受试者静脉内施用所述重组人 NaGlu 蛋白质。在另一实施方案中，以约 6 至约 27mg/kg 体重的剂量向所述受试者静脉内施用所述重组人 NaGlu 蛋白质。

[0043] 又一实施方案中，向所述受试者鞘内施用所述重组人 NaGlu 蛋白质。在一个实施方案中，以至少约 0.3、0.4、0.5、0.6、0.7、0.8 或 0.9mg/kg 体重的剂量鞘内施用所述重组人 NaGlu 蛋白质。在另一实施方案中，以约 1、2、3、4、5、6、7、8、9 或 10mg/kg 体重的剂量鞘内施用所述重组人 NaGlu 蛋白质。在另一实施方案中，以约 10 至约 30mg/kg 体重的剂量鞘内施用所述重组人 NaGlu 蛋白质。

[0044] 在另一实施方案中，治疗有效量是有效降低所述受试者脑部、肾脏或肝脏中的硫酸乙酰肝素水平的量。在另一实施方案中，治疗有效量是有效增加所述受试者脑部或肝脏中的 NaGlu 活性的量。

[0045] 在另一实施方案中，所述方法进一步包括施用第二治疗剂。在一个实施方案中，所述第二治疗剂是免疫抑制剂。

[0046] 附图简述

[0047] 图 1 描绘了人重组 NaGlu 的氨基酸序列（氨基酸残基 1-23，信号肽）。

[0048] 图 2 描绘了人重组 NaGlu 的核酸序列 (cDNA)，包括编码信号肽的核酸序列。

[0049] 图 3 描绘了 1.1kb 卵白蛋白启动子的核酸序列。

[0050] 图 4A-D 描绘了用于禽类转基因的 pSIN-OV-1.1-I-rhNaGlu 载体的核酸序列。

[0051] 图 5 是 pSIN-OV-1.1-I-rhNaGlu 载体的示意图。

[0052] 图 6 描绘了从转基因原鸡 (Gallus) 的蛋清中分离和纯化的 rhNaGlu 的蛋白质印迹分析。

[0053] 图 7 描绘了存积在转基因原鸡蛋清中的 rhNaGlu 的平均浓度。

[0054] 图 8 描绘了使用 HPAEC-PAD 绘制的由转基因原鸡生成的 rhNaGlu 的寡糖图谱。

[0055] 图 9 描绘了人皮肤成纤维细胞对 rhNaGlu 的摄取分析 (MPS IIIB，NaGlu 缺乏型；正常，野生型人皮肤成纤维细胞；1U 酶活性 =nmol 蛋白质 /h)。

[0056] 图 10 描绘了使用不同浓度的 M6P 单糖对 rhNaGlu(原鸡) 做的摄取抑制分析 (1U 酶活性 =1μmol 蛋白质 /min)。

[0057] 图 11 描绘了含有重组人 NaGlu 融合构建体的 pTT22 载体的示意图 (AAA-NaGlu：与全长 NaGlu 的 N 端融合的酸性氨基酸残基)。

[0058] 图 12 描绘了含有重组人 NaGlu 融合构建体的 pTT22 载体的示意图 (NaGlu-TfRL：与全长 NaGlu 的 C 端融合的转铁蛋白受体配体)。

[0059] 图 13 描绘了与原鸡生成的 rhNaGlu 相比，HEK293 生成的 AAA-NaGlu 的酶活性。

[0060] 图 14 描绘了与 HEK293 生成的 AAA-NaGlu 相比，HEK293 生成的 NaGlu-TfRL 的酶活性。

[0061] 图 15 描绘了随时间推移 (48 小时)rhNaGlu(原鸡) 进入巨噬细胞系 (NR8383) 的摄取水平。按单位 /mg 蛋白质测量细胞 NaGlu 活性。

[0062] 图 16 描绘了静脉内施用媒介物 (KO)、剂量浓度为 6.25mg/kg 的原鸡 rhNaGlu 或剂量浓度为 27mg/kg 的原鸡 rhNaGlu 后，naglu$^{(-/-)}$ 小鼠肾脏中的硫酸乙酰肝素底物水平 (μg/mg 组织)。野生型 (WT) 小鼠未经处理。

[0063] 图 17 描绘了静脉内施用媒介物 (KO)、剂量浓度为 6.25mg/kg 的原鸡 rhNaGlu 或剂量浓度为 27mg/kg 的原鸡 rhNaGlu 后，naglu$^{(-/-)}$ 小鼠脑部中的硫酸乙酰肝素底物水平 (μg/mg 组织)。野生型 (WT) 小鼠未经处理。

[0064] 图 18 描绘了静脉内施用媒介物 (KO)、剂量浓度为 6.25mg/kg 的原鸡 rhNaGlu 或剂量浓度为 27mg/kg 的原鸡 rhNaGlu 后，naglu$^{(-/-)}$ 小鼠肝脏中的硫酸乙酰肝素底物水平 (μg/mg 组织)。野生型 (WT) 小鼠未经处理。

[0065] 图 19 描绘了鞘内施用媒介物 (KO) 或剂量浓度为 0.31mg/kg 的原鸡 rhNaGlu 后，naglu$^{(-/-)}$ 小鼠脑部中的硫酸乙酰肝素底物水平 (μg/mg 组织)。野生型 (WT) 小鼠未经处理。

[0066] 发明详述

[0067] 本发明提供包含对治疗有用，例如在 NaGlu 相关疾病 (例如桑菲列浦氏综合征 B 型) 的治疗中有用的重组人 NaGlu 蛋白质 (rhNaGlu) 的组合物。本发明基于以下发现，本文所述 rhNaGlu 蛋白质含有足够量的寡糖 (例如，甘露糖和磷酸化甘露糖 (即，M6P))，以允许经由甘露糖和 / 或 M6P 受体介导的内吞作用的有效细胞摄取，并且正确靶向人细胞中。因为本发明的 rhNaGlu 被更加有效地吸收到人细胞中，与由未设计成产生特定糖基化的未修饰哺乳动物细胞生成和分泌的 NaGlu 蛋白质相比，本发明的 rhNaGlu 表现增加的酶活性。另外，本文所述 rhNaGlu 具有一个或多个糖基化模式，其允许 rhNaGlu 在静脉内施用时有效穿过血脑屏障 (BBB)。本发明的 rhNaGlu 蛋白质的细胞摄取增加将对大量和频繁给药的需要减到最低，从而大大降低了免疫原性的潜在风险。

[0068] 本文使用的一些定义和缩写包括以下：aa，氨基酸；bp，碱基对；CDS，编码序列 cDNA，与 RNA 互补的 DNA；GalNac，N- 乙酰半乳糖胺；Gal，半乳糖；GlcNac，N- 乙酰葡萄糖胺；nt，核苷酸；kb，1,000 个碱基对；μg，微克；mL，毫升；ng，毫微克；和 nt，核苷酸。

[0069] 本文提出了某些定义来说明和定义本文用于描述本发明的各种术语的含义和范

围。

[0070] 如本文所使用的术语“禽类”指 ava 分类的生物的任何物种、亚种或品系，例如但不限于鸡、火鸡、鸭、鹅、鹌鹑、雉、鹦鹉、雀、鹰、乌鸦和平胸类鸟，包括鸵鸟、鸸鹋和食火鸡。术语包括各种已知品系的原鸡或鸡（例如，白色来亨鸡 (White Leghorn)、棕色来亨鸡 (Brown Leghorn)、横斑洛克鸡 (Barred-Rock)、苏塞克斯鸡 (Sussex)、新罕布什尔鸡 (New Hampshire)、罗得岛州鸡 (Rhode Island)、澳洲黑鸡 (Ausstralorp)、米诺卡鸡 (Minorca)、横斑芦花鸡 (Amrox)、加利福利亚灰鸡 (California Gray)、意大利花鹧鸪 (Italian Partridge-colored))，以及火鸡、雉、鹌鹑、鸭、鸵鸟的品系和常按商业数量繁殖的其它家禽。

[0071] 短语“基于”和“源自”通常指全部或部分从中获得。例如，逆转录病毒载体基于或源自特定逆转录病毒或基于特定逆转录病毒的核苷酸序列指，逆转录病毒载体的基因组含有特定逆转录病毒的基因组的很大一部分核苷酸序列。正如根据本领域技术人员的知识从说明书上下文中将显而易见，很大一部分可以是特定基因或核苷酸序列，例如编码 gag、pol 和 / 或 env 蛋白质的核苷酸序列或病毒基因组的其它结构或功能性核苷酸序列，例如编码长末端重复序列 (LTR) 的序列，或可大体上是完整逆转录病毒基因组，例如大部分（例如，超过 60% 或超过 70% 或超过 80% 或超过 90%) 或整个逆转录病毒基因组。基于或源自逆转录病毒的逆转录病毒载体的实例是如 Cosset 等，Journal of Virology(1991) 第 65 卷，第 3388-3394 页公开的源自禽白血病逆转录病毒（“ALV”）的 NL 逆转录病毒载体（例如，NLB)。

[0072] 如本文所使用的术语“编码序列”和“编码区域”指编码合成 RNA、蛋白质或 RNA 或蛋白质的任何部分的遗传信息的核苷酸序列和核酸序列，包括 RNA 和 DNA。

[0073] 并非天然是特定生物基因组的一部分或引入生物基因组的非天然位点的核苷酸序列称为“外来”核苷酸序列、“异源”核苷酸序列、“重组”核苷酸序列或“外源”核苷酸序列。另外，不将已经分离，然后重新引入相同类型（例如，相同物种）的生物中的核苷酸序列视为特定生物基因组的天然存在的部分并且因此视为外源或异源。“异源蛋白质”或“外源蛋白质”可以是由外来、异源或外源核苷酸序列编码的蛋白质，因此常常并非天然在宿主生物的细胞中表达。

[0074] 如本文所使用，关于核酸例如 DNA 和 RNA 的术语“外源”、“异源”和“外来”可交换使用并且指并非作为其所存在或发现在位置和 / 或数量上不同于其在自然界中存在的位置和 / 或数量的染色体、基因组或细胞的一部分而天然存在的核酸。其可以对基因组、染色体或细胞为非内源性的并且已经外源引入到基因组、染色体或细胞中的核酸。异源 DNA 的实例包括但不限于编码目标基因产物（例如）以生成编码蛋白质的 DNA。异源 DNA 的实例包括但不限于编码可追踪标记蛋白质的 DNA、编码治疗性蛋白质的 DNA。术语“异源”和“外源”可指生物分子，例如不常出现在由生物产生的某种细胞、组织或物质中或不常以与发现天然存在的相同数量或位置出现在由生物产生的某种细胞、组织或物质中的核酸或蛋白质。例如，对蛋异源或外源的蛋白质是一般不存在于蛋中的蛋白质。

[0075] 如本文所使用的术语“构建体”指由已经从天然来源分离或化学合成的核苷酸序列或其组合的一个以上片段组装的线性或环状核苷酸序列例如 DNA。

[0076] 如本文所使用的术语“互补”指可相互形成特定相互作用的两个核酸分子。在特

定相互作用中，当两条核酸链呈相反极性时，一条核酸链中的腺嘌呤碱基可与第二条核酸链中的胸腺嘧啶形成 2 个氢键。同样在特定相互作用中，当两条核酸链呈相反极性时，一条核酸链中的鸟嘌呤碱基可与第二条核酸链中的胞嘧啶形成 3 个氢键。

[0077] 本文所提到的互补核酸可进一步包括经修饰的碱基，其中经修饰的腺嘌呤可与胸腺嘧啶或经修饰的胸腺嘧啶形成氢键，并且经修饰的胞嘧啶可与鸟嘌呤或经修饰的鸟嘌呤形成氢键。

[0078] 如本文所使用的术语“被表达”或“表达”指转录编码序列以获得与编码序列两条核酸链之一的区域至少部分互补的 RNA 分子。如本文所使用的术语“被表达”或“表达”也可指翻译 mRNA 以生成蛋白质或肽。

[0079] 如本文所使用的术语“表达载体”指包含与编码至少一个多肽的核苷酸序列可操作地连接的基因表达控制区（例如启动子或启动子组件）的核酸载体。

[0080] 如本文所使用的术语“片段”可指（例如）已经人工构建（例如，通过化学合成）或通过使用限制性内切核酸酶或机械剪切将天然产物裂解成多片来构建，或酶促构建，例如通过 PCR 或本领域已知的任何其它聚合技术，或通过本领域技术人员已知的重组核酸技术在宿主细胞中表达的核酸的一个至少约 10、20、50、75、100、150、200、250、300、500、1000、2000、5000、6,000、8,000、10,000、20,000、30,000、40,000、50,000 或 60,000 个核苷酸长的部分。如本文所使用的术语“片段”还可指（例如）小于 NaGlu 的全长氨基酸序列（即，SEQ ID NO:1 的氨基酸序列 24-743）的至少约 5、10、15、20、25、30、40 或 50 个氨基酸残基，所述部分通过至少一种蛋白酶的蛋白水解性裂解从天然存在的氨基酸序列裂解，或是通过化学方法或使用本领域技术人员已知的重组 DNA 技术（例如，由编码天然存在的氨基酸序列的核苷酸序列的一部分表达）合成的天然存在的氨基酸序列的一部分。“片段”还可指（例如）特定核苷酸序列或氨基酸序列约 50%、约 60%、约 70%、约 80%、约 90%、约 95% 或约 99% 的一部分。

[0081] “功能部分”和“功能片段”可交换使用并且如本文所使用，指能够完全或部分进行整体功能的整体的一部分或片段。例如，分子的生物功能部分指进行整体或整个分子的生物功能的分子的一部分。功能部分可具任何有用尺寸。例如，功能片段尺寸范围可从长度约 20 个碱基到长度等于指定序列全长减去一个核苷酸。在另一个实例中，功能片段尺寸范围可从长度约 50 个碱基到长度等于指定序列全长减去一个核苷酸。在另一个实例中，功能片段尺寸范围可从长度约 50 个碱基到长度约 20kb。在另一个实例中，功能片段尺寸范围可从长度约 500 个碱基到长度约 20kb。在另一个实例中，功能片段尺寸范围可从长度约 1kb 到长度约 20kb。在另一个实例中，功能片段尺寸范围可从长度约 0.1kb 到长度约 10kb。在另一个实例中，功能片段尺寸范围可从长度约 20 个碱基 kb 到长度约 10kb。

[0082] 术语“完全转基因的”或“种系转基因的”指在其基本上所有细胞中含有至少一个拷贝的转基因的动物，例如禽类。

[0083] 如本文所使用的术语“基因表达控制区”指与编码序列相关并且完全或部分调控编码序列的表达，例如完全或部分调控编码序列的转录的核苷酸序列。基因表达控制区可从天然存在的来源分离或可化学合成并且可并入核酸载体中使得能够在适当细胞中受调控转录。“基因表达控制区”可能但不限于位于可转录为 mRNA 的编码序列 5’ 末端区域中的核酸序列区域之前。

[0084] 如本文所使用，“宿主细胞”指携带使用重组 DNA 技术构建而成并且编码至少一个异源基因的载体的细胞。

[0085] 如本文所使用的术语“分离的核酸”包括（例如）(a) 具有天然存在的基因组分子的一部分的序列，但是两侧不是在其天然存在的物种基因组中分子的该部分两侧的序列的至少一个的 DNA ；(b) 已经以使得所得载体或基因组 DNA 和从中获得核酸的天然存在的 DNA 不同的方式，并入载体或原核细胞或真核细胞基因组 DNA 中的核酸 ；(c) 单独分子，例如 cDNA、基因组片段和通过聚合酶链式反应 (PCR)、连接酶链式反应 (LCR) 或化学合成而生成的片段，或限制片段 ；(d) 是杂合基因，即编码融合蛋白的基因的一部分的重组核苷酸序列 ；和 (e) 是并非天然存在的杂交序列的一部分的重组核苷酸序列。本发明的分离的核酸分子可包括（例如）天然等位基因变体以及通过核苷酸缺失、插入、倒位或取代修饰的核酸分子。

[0086] 如本文所使用的术语“核酸”指任何线性或连续排列的核苷酸和核苷，例如 cDNA、基因组 DNA、mRNA、tRNA、寡核苷酸、寡核苷及其衍生物。为便于讨论，本文可将非天然存在的核酸称为构建体。核酸可包括细菌质粒载体，包括表达、克隆、粘粒和转化载体，例如动物病毒载体例如但不限于经修饰的腺病毒、流感病毒、脊髓灰质炎病毒、痘病毒、逆转录病毒（例如禽白血病病毒 (ALV) 逆转录病毒载体、鼠白血病病毒 (MLV) 逆转录病毒载体和慢病毒载体）等及其片段。另外，核酸可为禽白血病病毒 (ALV) 逆转录病毒载体、鼠白血病病毒 (MLV) 逆转录病毒载体或慢病毒载体及其片段的 LTR。核酸也可包括 NL 载体，例如 NLB、NLD 和 NLA 及其片段和合成寡核苷酸例如化学合成的 DNA 或 RNA。核酸可包括经修饰或衍生的核苷酸和核苷，例如但不限于卤化核苷酸（例如，但不仅是 5- 溴尿嘧啶）和衍生化核苷酸（例如生物素标记核苷酸）。

[0087] 如本文所使用，术语“聚糖”、“聚糖结构”、“聚糖部分”、“寡糖”、“寡糖结构”、“糖基化模式”、“糖基化图谱”和“糖基化结构”具有基本上相同的含义并且各自指由糖残基形成并且连接至糖基化蛋白质（例如人 NaG1u）的一个或多个结构。例如，“N- 聚糖”或“N- 连接聚糖”指连接至糖基化蛋白质的天门冬酰胺或精氨酸侧链的氮上的聚糖结构。O- 聚糖”或“O- 连接聚糖”指连接至糖基化蛋白质的丝氨酸、苏氨酸、酪氨酸、羟赖氨酸或羟脯氨酸侧链的羟基氧上的聚糖结构。

[0088] 如本文所使用，术语“载体”和“核酸载体”指可转染或转化到细胞中并独立于宿主细胞基因组或在宿主细胞基因组中复制的天然或合成单链或双链质粒或病毒核酸分子。可通过用适当的限制酶基于载体的核苷酸序列处理使环状双链载体线性化。如本领域所理解，可通过用限制酶切割载体并且将所需碎片连接到一起将核酸插入载体中。典型载体可由在适当距离可操作地连接，以允许功能基因表达的下列元件构成 ：复制起点、启动子、增强子、5’mRNA 前导序列、核醣体结合位点、核酸盒、终止和多腺苷酸化位点及可选标记序列。在具体应用中可省略这些元件中的一个或多个。核酸盒可包括插入待表达核酸序列的限制位点。在功能载体中，核酸盒含有待表达的核酸序列，包括翻译起始和终止位点。在构建体中可任选包括内含子，例如在编码序列的 5’。载体经构建，以致特定编码序列位于具有适当调控序列的载体中，编码序列关于控制序列的定位和方向是以便编码序列在控制或调控序列的“控制”下转录。为达到这一目的，修饰编码目标特定蛋白质的序列是可取的。例如，在一些情况下可能必须修饰序列，以便其可连接至具有适当方向的控制序列，或保持阅读框。

在插入载体之前，可将控制序列和其它调控序列连接至编码序列。可选地，可将编码序列直接克隆到已经含有控制序列和适当限制位点的表达载体中，所述限制位点在具有控制序列并且受控制序列调控的阅读框中。

[0089] 术语“可操作地连接”指元件的排列，其中所述组件配置成以便执行其一般功能。与编码序列可操作地连接的基因表达控制区或启动子（例如，启动子组件）能够实现编码序列的表达。控制序列或启动子无需与编码序列连续，只要它们起指示其表达的作用。因此，例如，在启动子序列和编码序列之间可存在尚未翻译但经转录的插入序列并且仍可认为启动子序列与编码序列“可操作地连接”。

[0090] 如本文所使用，“过表达”指在转基因生物中基因产物的生成超过正常或非转化生物中的生成水平。

[0091] 术语“输卵管”或“输卵管组织”指禽类输卵管的组织，例如输卵管膨大部 (magnum)（例如，管状腺细胞），其中生成的蛋白质具有 N- 连接寡糖，其相对于在禽类其它组织例如肝脏或肾脏组织中生成的蛋白质的 N- 连接寡糖，含有增加量的甘露糖和甘露糖 -6- 磷酸 (M6P) 及大幅减少量的半乳糖和 / 或唾液酸。

[0092] 如本文所使用的术语“输卵管特异性启动子”指功能性的，即在很大程度上，例如主要（即，动物中由输卵管细胞中生成的特定启动子类型生成的超过 50% 的转录产物）或专在鸟类的输卵管细胞中提供编码序列的转录的启动子或启动子组件。输卵管特异性启动子的实例包括但不限于卵白蛋白启动子、卵类粘蛋白启动子、卵抑制剂启动子、溶菌酶启动子和卵转铁蛋白启动子及这些启动子的功能部分，例如，启动子组件。通过使用输卵管特异性启动子将 NaGlu 蛋白质的表达限于输卵管膨大部，可将由于这些酶在鸟类其它组织中表达对鸟类产生的有害生理效应减到最少。

[0093] 术语“序列同一性百分比”、“同一性百分比”、“同一性 %”、“序列同源性百分比”、“同源性百分比”、“同源性 %”和“序列相似性百分比”可各自指两个核酸序列或两个氨基酸序列之间的序列匹配程度。可使用 Karlin&Altschul(1990)Proc.Natl.Acad.Sci.87:2264-2268 的算法，如 Karlin&Altschul(1993)Proc.Natl.Acad.Sci.90:5873-5877 中修改，测定此类序列匹配。将这种算法并入 Altschul 等 (1990)T.Mol.Biol.Q15:403-410 的 NBLAST 和 XBLAST 程序中。用 NBLAST 程序进行 BLAST 核苷酸搜索，得分 =100，字长 =12，以获得与本发明的核酸分子同源的核苷酸序列。用 XBLAST 程序进行 BLAST 蛋白质搜索，得分 =50，字长 =3，以获得与参考氨基酸序列同源的氨基酸序列。为了获得空位比对以进行比较，如 Altschul 等 (1997)Nucl.Acids Res.25:3389-3402 中所述利用空位 BLAST。利用 BLAST 和空位 BLAST 程序时，使用各程序（例如，NBLAST 和 XBLAST）的默认参数。其它算法、程序和默认设置也可能适合，例如但不仅是英国人类基因组计划资源中心 (U.K.Human Genome Mapping Project Resource Centre) 的 GCG 序列分析包，其包括用于核苷酸或氨基酸序列比较的程序。序列可能与另一序列，例如本文鉴定的 NaGlu 蛋白质序列具有至少 50%、60%、70%、75%、80%、85%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99% 或更多同一性。

[0094] 术语“禽来源的”指由鸟类、家禽或禽类生成或获得的组合物或物质。“禽类”指可作为家畜饲养的鸟类，包括但不限于鸡、鸭、火鸡、鹌鹑和平胸类鸟。例如，“禽来源的”可指鸡来源的、火鸡来源的和 / 或鹌鹑来源的。

[0095] 术语“多核苷酸”、“寡核苷酸”、“核苷酸序列”和“核酸序列”在本文中可交换使用

并且包括但不限于编码序列，即在置于适当调控或控制序列的控制下时，在体外或体内转录并翻译为多肽的多核苷酸或核酸序列；控制序列，例如翻译起始和终止密码子、启动子序列、核糖体结合位点、多聚腺苷酸化信号、转录因子结合位点、转录终止序列、上游和下游调控结构域、增强子、沉默子、转录因子与之结合并且正面（诱导）或负面（抑制）改变基因启动子活性的 DNA 序列等。本文所述术语表明对长度或合成起点没有限制。

[0096] 如本文所使用的术语“多肽”和“蛋白质”指氨基酸的聚合物，例如通过肽键呈连续排列连接的 3 个或更多个氨基酸。术语“多肽”包括蛋白质、蛋白质片段、蛋白质类似物、寡肽等。术语“多肽”包括如以上定义的由核酸编码，通过重组技术生成（例如，从转基因鸟类分离）或合成的多肽。术语“多肽”进一步涵盖如以上定义的包括经化学修饰的氨基酸或与标记配体共价或非共价连接的氨基酸的多肽。

[0097] 如本文所使用的术语“启动子”指禽类细胞中用于通过 RNA 聚合酶发起转录的 DNA 序列。“启动子组件”是可自身或与其它 DNA 序列组合实现或促进转录的 DNA 序列。启动子组件可为启动子的功能片段。

[0098] 如本文所使用的术语“重组核酸”和“重组 DNA”指真核或原核细胞中非天然存在的至少两个核酸序列的组合。核酸序列可包括但不限于核酸载体、基因表达调控元件、复制起点、表达时赋予抗生素抗性的适合基因序列、蛋白质编码序列等。术语“重组多肽”意在包括通过重组 DNA 技术生成，以致其在位置、纯度或结构上不同于天然存在的多肽的多肽。通常，这种重组多肽将以不同于一般在自然界中观察到的量存在于细胞中。

[0099] 如本文所使用，术语“调控”序列或元件包括启动子、增强子、终止子、终止密码子和可控制基因表达的其它元件。

[0100] “逆转录病毒”、“逆转录病毒颗粒”、“转导颗粒 (transducing particle)”或“转导颗粒 (transduction particle)”指能够将非病毒 DNA 或 RNA 转导至细胞中的复制缺陷型或能复制的病毒。

[0101] “SIN 载体”指自我失活型载体。具体而言，SIN 载体是基因组改变的逆转录病毒载体，以致整合到靶细胞（例如，禽类胚胎细胞）的基因组 DNA 后，被整合逆转录病毒载体的 5’LTR 将不起启动子的作用。例如，一经整合就产生逆转录病毒载体的 5’LTR 的 U3 区域的逆转录病毒载体的一部分或整个核苷酸序列可缺失或改变，以便降低或消除 5’LTR 的启动子活性。在某些实例中，如本领域所理解，从 5’LTR 的 U3 缺失 CAAT 盒和 / 或 TAATA 盒可产生 SIN 载体。

[0102] 如本文所使用的术语“有义链”指来自基因组 DNA，可转录成 RNA 并翻译成基因的天然多肽产物的单链 DNA 分子。如本文所使用的术语“反义链”指与基因的有义链互补的基因组 DNA 的单链 DNA 分子。

[0103] “治疗性蛋白质”或“药用蛋白质”是全部或部分构成药物的物质。具体而言，“治疗性蛋白质”和“药用蛋白质”包括全部或部分构成药物的氨基酸序列。

[0104] 如本文所使用的术语“启动子”、“转录调控序列”和“启动子组件”指调控编码序列的转录表达的核苷酸。示例性转录调控序列包括增强子元件、激素反应元件、类固醇反应元件、负调控元件等。“转录调控序列”可分离并且并入载体中，以使得部分载体 DNA 能够在适当细胞中受调控转录。“转录调控序列”可以但不限于位于转录为 mRNA 的蛋白质编码序列 5’端区域中的核酸序列区域之前。转录调控序列也可位于蛋白质编码区域内，例如位于

鉴定为“内含子”区域的基因区域内。

[0105] 如本文所使用的术语“转化”和“转染”指将核酸插入宿主的过程。本领域技术人员众所周知许多技术促进核酸向原核或真核生物中转化或转染。这些方法牵涉多种技术，例如用一定浓度的盐，例如但不限于钙盐或镁盐处理细胞，或将细胞暴露于电场、洗涤剂或脂质体材料，以使宿主细胞能够吸收核酸分子。

[0106] 如本文所使用，“转基因动物”是任何非人动物，例如禽类物种，包括鸡，其中动物的一种或多种细胞含有经过人为干预，例如通过本领域已知的转基因技术，包括本文公开的转基因技术引入的异源核酸（见，例如，2007 年 10 月 18 日公开的美国专利公开号 2007/0243165，其公开内容通过引用整体并入本文）。经过故意的遗传操纵，例如通过显微注射或通过感染重组病毒，通过直接或间接引入细胞（例如，蛋或胚胎细胞），将核酸引入动物中。术语遗传操纵不包括经典的杂交育种或体外受精，而是指向引入重组 DNA 分子。这种分子可整合在染色体中，或可能在染色体外复制的 DNA。在典型转基因动物中，转基因可使细胞表达重组形式的靶蛋白或多肽。术语“嵌合体动物 (chimeric animal)”或“镶嵌体动物 (mosaic animal)”在本文中用于指在其中发现转基因，或其中重组核苷酸序列在动物的一些而非全部细胞中表达的动物。种系嵌合体动物在其生殖细胞中含有转基因并且可产生子代转基因动物，其中子代的大多数或全部细胞将含有转基因。

[0107] 如本文所使用，“转基因”指核酸序列（例如，编码人 NaGlu 蛋白质），其对于引入其中的动物或细胞而言部分或完全异源，即外来，或对于引入其中的转基因动物或细胞的内源基因而言部分或完全同源，但是设计成以改变向其中插入的生物的基因组的方式待插入或插入动物或细胞基因组中（例如，在不同于天然基因的位置插入或其插入导致敲除）。

[0108] 如本文所使用，术语“酶替代疗法 (ERT)”指通过向受试者施用缺少的酶来用于矫正受试者的酶缺乏症的治疗策略。为使溶酶体酶替代疗法有效，必须将治疗性酶递送到表现出贮积缺陷的组织中适当细胞中的溶酶体。在一个实施方案中，酶可经静脉内、鞘内、大脑内、心室内或实质内向受试者施用。在一个实施方案中，酶能够穿过血脑屏障 (BBB)。不打算受机制的限制，据信血灌注患者组织，酶被细胞吸收并转运到溶酶体，在那里酶用以消除由于酶缺乏症在溶酶体中积累的物质。

[0109] I. NaGlu 的组合物

[0110] 本发明提供了具有糖基化模式的重组人 NaGlu(rhNaGlu 或 NaGlu)(SEQ ID NO:1 中所示氨基酸序列 24-743) 的新型组合物，所述糖基化模式赋予对治疗尤其有用，例如在桑菲列浦氏综合征 B 型（粘多糖贮积病 (MPS)IIIB) 的治疗中有用的增加的细胞摄取和增加的亚细胞活性。

[0111] 在一些方面，所述组合物可为包含 SEQ ID NO:1 的氨基酸序列 24-743 的 rhNaGlu 的分离的混合物。在一个实施方案中，所述混合物含有足够量的具有至少一个聚糖结构的 rhNaGlu，所述聚糖结构含有磷酸化甘露糖（例如，M6P) 或甘露糖，以致含 M6P 或甘露糖的 rhNaGlu 被内化到缺乏 NaGlu 的人细胞中并且恢复了在活跃表达内源 NaGlu 的相同类型的野生型人细胞中观察到的 NaGlu 活性的至少 50%。一方面，混合物中至少 10%、20%、30%、40%、50%、60%、70%、80%、90%、91%、92%、93%、94%、95%、96%、97%、98% 或 99% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。在一个实施方案中，混合物中至少 10% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。在一个实施方案中，混

合物中至少 20% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。在一个实施方案中，混合物中至少 30% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。在一个实施方案中，混合物中至少 30% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。在一个实施方案中，混合物中至少 40% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。在一个实施方案中，混合物中至少 50% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。在一个实施方案中，混合物中至少 60% 的 rhNaGlu 含有至少一个具有磷酸化甘露糖和 / 或甘露糖的聚糖结构。

[0112] 在一些方面，NaGlu 含有一个或多个 N- 连接的聚糖结构。NaGlu 含有至少一个磷酸化甘露糖（例如，M6P 或双 M6P），其允许蛋白质被甘露糖 6- 磷酸受体 (M6P 受体）识别，并且随后经 M6P 受体介导的内吞作用吸收到人细胞中，包括但不限于皮肤成纤维细胞、内皮细胞、神经元细胞、肝细胞、巨噬细胞或在细胞表面表达 M6P 受体的任何细胞。在一个实施方案中，NaGlu 含有至少一个甘露糖 (Man)。在另一实施方案中，NaGlu 含有至少一个 N- 乙酰葡萄糖胺 (GlcNAc)。

[0113] 在一些方面，NaGlu 含有包含磷酸化甘露糖 (M6P) 的聚糖结构。如本文所使用，M6P 可涵盖任何磷酸化甘露糖残基并且包括单和双 - 磷酸化甘露糖。在一个实施方案中，M6P 以每摩尔蛋白质约 1、约 2、约 3、约 4、约 5 或约 6 摩尔的浓度存在。在一个实施方案中，NaGlu 含有浓度为每摩尔蛋白质约 2、约 3、约 4 或约 5 摩尔的 M6P。在一个实施方案中，NaGlu 含有浓度为每摩尔蛋白质约 2 摩尔的 M6P。在一个实施方案中，NaGlu 含有浓度为每摩尔蛋白质约 3 摩尔的 M6P。在一个实施方案中，NaGlu 含有浓度为每摩尔蛋白质约 4 摩尔的 M6P。在一个实施方案中，NaGlu 含有浓度为每摩尔蛋白质约 5 摩尔的 M6P。在一个实施方案中，NaGlu 含有浓度为每摩尔蛋白质约 6 摩尔的 M6P。

[0114] 在一些方面，rhNaGlu 含有足够量的 M6P，以经 M6P 介导的内吞作用以供细胞摄取到在细胞表面具有 M6P 受体的人细胞中。在一个实施方案中，供摄取到人细胞的足够量的 M6P 为每摩尔蛋白质约 1、2、3、4、5 或 6 摩尔。rhNaGlu 可被内化到缺乏 NaGlu 的人细胞中，以致内化蛋白质完全 (100% 或更高）恢复了缺乏 NaGlu 的人细胞中 NaGlu 活性的正常水平。在一个实施方案中，在至少 0.5、0.6、0.7、0.8、0.9 或 1.0 μg/mL 的浓度下，内化的 rhNaGlu 蛋白质完全恢复了人细胞中 NaGlu 活性的正常水平。在一个实施方案中，在至少 2、3、4、5、6、7、8、9 或 10 μg/mL 的浓度下，内化蛋白质完全恢复了缺乏 NaGlu 的人细胞中 NaGlu 活性的正常水平。在一个实施方案中，在至少 20、30、40、50、60、70、80、90 或 100 μg/mL 的浓度下，内化蛋白质完全恢复了人细胞中 NaGlu 活性的正常水平。如本文所使用，NaGlu 活性的正常水平是在活跃表达正常 NaGlu 酶的相同类型的野生型人细胞中测量的 NaGlu 活性水平。

[0115] 在一些方面，rhNaGlu 可被内化到缺乏 NaGlu 的人细胞中，以致所述蛋白质恢复了相同类型的正常人细胞至少约 50%、约 60%、约 70%、约 80%、约 90% 或约 95% 的 NaGlu 活性。在一些实施方案中，rhNaGlu 可被内化到缺乏 NaGlu 的人细胞中，以致内化的 rhNaGlu 提供比在相同类型的正常人细胞中所观察到的更高的酶活性。在一个实施方案中，rhNaGlu 被内化到缺乏 NaGlu 的人细胞中，以致内化的 rhNaGlu 提供比在相同类型的正常人细胞中所观察到的高约 2、约 3、约 4、约 5、约 6、约 7、约 8、约 9 和约 10 倍的活性。在一个实施方案

中，rhNaGlu 被内化到缺乏 NaGlu 的人细胞中，以致内化的 rhNaGlu 提供比在正常人细胞中所观察到的高约 15、约 20、约 25、约 30、约 40、约 50、约 60、约 70、约 80、约 90 或约 100 倍的活性。

[0116] 在一个实施方案中，缺乏 NaGlu 的人细胞是在细胞表面表达一种或多种 M6P 受体，缺乏 NaGlu 的任何人细胞。在一个实施方案中，缺乏 NaGlu 的人细胞是积累硫酸乙酰肝素的人粘多糖贮积病 (MPS) IIIB 成纤维细胞。在一个实施方案中，缺乏 NaGlu 的人细胞是肝细胞。在一个实施方案中，缺乏 NaGlu 的人细胞是神经元细胞。在一个实施方案中，缺乏 NaGlu 的人细胞是内皮细胞。在一个实施方案中，缺乏 NaGlu 的人细胞是巨噬细胞。

[0117] 在一些方面，约 1、约 2、约 3、约 4、约 5、约 6、约 7、约 8、约 9 或约 10mM 的竞争性 M6P 单糖的存在抑制 rhNaGlu 向人细胞的摄取。在一些方面，约 0.1、约 0.2、约 0.3、约 0.4、约 0.5、约 0.6、约 0.7、约 0.8、约 0.9 或约 1.0mM 的 M6P 单糖的存在抑制细胞摄取 rhNaGlu。在一个实施方案中，约 0.01、约 0.02、约 0.03、约 0.04、约 0.05、约 0.06、约 0.07、约 0.08 或约 0.09 的 M6P 单糖的存在抑制细胞摄取 rhNaGlu。

[0118] 在一些方面，rhNaGlu 在其聚糖结构中含有浓度为每摩尔蛋白质约 17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34 或 35 摩尔的甘露糖。在一个实施方案中，rhNaGlu 具有浓度为每摩尔蛋白质约 20、21、22、23、24、25、26、27、28、29 或 30 摩尔的甘露糖。rhNaGlu 含有浓度为每摩尔蛋白质约 22、23、24、25、26、27 或 28 摩尔的甘露糖。rhNaGlu 含有浓度为每摩尔蛋白质约 24 摩尔的甘露糖。rhNaGlu 含有浓度为每摩尔蛋白质约 25 摩尔的甘露糖。rhNaGlu 含有浓度为每摩尔蛋白质约 26 摩尔的甘露糖。rhNaGlu 含有浓度为每摩尔蛋白质约 27 摩尔的甘露糖。在一个实施方案中，rhNaGlu 具有浓度介于每摩尔蛋白质约 20 和约 30 摩尔之间的甘露糖。

[0119] 在一些方面，rhNaGlu 包含 N- 乙酰葡萄糖胺 (GlcNAc)。在一个实施方案中，rhNaGlu 含有浓度介于每摩尔蛋白质约 28 和约 42 摩尔之间的 GlcNAc。在一个实施方案中，NaGlu 蛋白质具有浓度介于每摩尔蛋白质约 30 和约 40 摩尔之间的 GlcNAc。在一个实施方案中，NaGlu 蛋白质包含浓度介于每摩尔蛋白质约 32 和约 38 摩尔之间的 GlcNAc。在一个实施方案中，NaGlu 蛋白质包含浓度介于每摩尔蛋白质约 34 和约 36 摩尔之间的 GlcNAc。在一个实施方案中，NaGlu 蛋白质具有浓度为每摩尔蛋白质约 35 摩尔的 GlcNAc。在一个实施方案中，rhNaGlu 蛋白质含有浓度为每摩尔蛋白质约 30、31、32、33、34、35、36、37、38、39 或 40 摩尔的 GlcNAc。

[0120] 在一些方面，rhNaGlu 含有 N- 乙酰半乳糖胺 (GalNAc) 和 / 或半乳糖 (Gal)。GalNAc 和 Gal 的存在通常表明，NaGlu 可能含有添加到高尔基隔室中的蛋白质的一个或多个 O- 连接聚糖结构。相应地，本发明任选包括一种组合物，其包含含有一个或多个 O- 连接聚糖结构的重组人 NaGlu。

[0121] 在一个实施方案中，rhNaGlu 含有浓度为每摩尔蛋白质约 1、2、3、4、5、6 或 7 摩尔的半乳糖。在一个实施方案中，rhNaGlu 具有浓度为每摩尔蛋白质约 2、3、4、5 或 6 摩尔的半乳糖。在一个实施方案中，rhNaGlu 具有浓度为每摩尔蛋白质约 3 摩尔的半乳糖。在一个实施方案中，rhNaGlu 具有浓度为每摩尔蛋白质约 4 摩尔的半乳糖。

[0122] 在一个实施方案中，NaGlu 包含每摩尔蛋白质至少一个 GalNAc 分子。在一个实施方案中，NaGlu 包含浓度为每摩尔蛋白质约 1 或 2 摩尔的 GalNAc。

[0123] 在一个实施方案中，NaGlu 不含岩藻糖。在又一实施方案中，NaGlu 不含葡萄糖。在又一实施方案中，rhNaGlu 既不含岩藻糖也不含葡萄糖。

[0124] 本发明还涵盖了由 rhNaGlu 的经修饰核酸序列生成的经修饰 rhNaGlu 蛋白质的组合物。经修饰核酸序列包括不同核苷酸的缺失、插入或取代，形成编码功能等效的多核苷酸或多肽的多核苷酸。编码的蛋白质也可含有氨基酸残基的缺失、插入或取代，其产生沉默变化并形成功能等效的蛋白质或多肽。只要保持 NaGlu 的生物活性，可根据残基在极性、电荷、溶解性、疏水性、亲水性和 / 或两亲性性质上的相似性进行故意的氨基酸取代。例如，带负电的氨基酸可包括天冬氨酸和谷氨酸；带正电的氨基酸可包括赖氨酸和精氨酸；并且带具有相似亲水性值的不带电极性头基的氨基酸可包括亮氨酸、异亮氨酸和缬氨酸；甘氨酸和丙氨酸；天冬酰胺和谷氨酰胺；丝氨酸和苏氨酸；苯丙氨酸和酪氨酸。

[0125] 在其它方面，可修饰 rhNaGlu，以致其含有附加部分或第二个肽。虽然在高血清浓度下，未经修饰的 NaGlu 蛋白质可穿过血脑屏障，但是可进行蛋白质的修饰以增加中枢神经系统 (CNS) 靶向的效率。在一个实施方案中，转铁蛋白受体配体 (TfRL) 可在 NaGlu 蛋白质 N 或 C 端连接至人 NaGlu。TrRL 的非限制性实例为 THRPPMWSPVWP(SEQ ID NO:5)。在一个实施方案中，转铁蛋白受体配体可连接至 NaGlu 蛋白质的人 NaGlu C 端。在另一实施方案中，人 NaGlu 在 NaGlu 蛋白质 N 或 C 端与胰岛素样生长因子受体 (IGF2R) 配体融合。在又一实施方案中，NaGlu 蛋白质在 NaGlu 蛋白质 N 或 C 端与低密度脂蛋白 (LDL) 受体配体融合。在一个实施方案中，NaGlu 蛋白质与一段 5 至 10 个连续酸氨基酸残基融合。酸性氨基酸残基可包括天冬氨酸 (D) 或谷氨酸 (E)。

[0126] 在一个实施方案中，在含有编码 NaGlu 蛋白质的转基因的转基因禽类中生成 rhNaGlu。在一个实施方案中，在转基因禽类的（例如，鸡（原鸡））的输卵管细胞（例如，管状腺细胞）中生成 rhNaGlu。在一个实施方案中，rhNaGlu 在转基因禽类的输卵管细胞（例如，管状腺细胞）中糖基化。在一个实施方案中，rhNaGlu 具有由于在转基因禽类的输卵管细胞内生成 rhNaGlu 而产生的糖基化模式。在一个实施方案中，可从转基因禽类产的硬壳蛋的内含物中分离并纯化 rhNaGlu。在一个实施方案中，可从转基因禽类的蛋清中分离并纯化 rhNaGlu。

[0127] 本发明还包括 NaGlu 蛋白质的分离混合物，例如一个或多个片段与全长 rhNaGlu（例如，SEQ ID NO:1 中所示 24-743）的混合物的组合物。在一个实施方案中，大部分混合物含有磷酸化 M6P。在一个实施方案中，混合物中至少 10%、20%、30%、40%、50%、60%、70%、80%、85%、90%95%、97%、98% 或 99% 的 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 50% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 60% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 70% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 80% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 90% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 95% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 96% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 97% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 98% 的分离 rhNaGlu 含有 M6P。在又一实施方案中，混合物中至少 99% 的分离 rhNaGlu 含有 M6P。

[0128] 任选地，可进一步修饰由禽类或哺乳动物表达系统（例如，CHO、HEK293 或人皮肤成纤维细胞系）产生的 rhNaGlu 蛋白质以获得良好的糖基化模式（即，M6P 量增加），以在

保持生物活性的同时供细胞摄取。可通过如美国专利号 6,679,165、美国专利号 7,138,262 或美国公布号 2009/0022702 描述的，应用于其它水解酶的通用方法向 rhNaGlu 引入附加末端 M6P，所述专利各自的全部教义通过引用并入本文。例如，可用含有羰基反应基的化学化合物衍生化高度磷酸化的吡喃甘露糖基寡糖化合物，接着氧化 rhNaGlu 蛋白质以在蛋白质的一个聚糖结构上生成羰基（醛），并且使具有聚糖的氧化 NaGlu 蛋白质与衍生化的高度磷酸化吡喃甘露糖基寡糖化合物反应以形成具有肼键的新化合物。

[0129] II. 载体

[0130] 可使用本领域技术人员众所周知的方法构建含有编码 NaGlu 的序列和适当转录和翻译控制元件的表达载体。这些方法包括体外重组 DNA 技术、合成技术和体内基因重组。例如，在 Sambrook, J. 等 (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y. 和 Ausubel, F.M. 等 (1989) Current Protocols in Molecular Biology, John Wiley&Sons, New York, N.Y. 中描述了此类技术，所述参考文献的全部教义通过引用并入本文。

[0131] 可利用多种表达载体 / 宿主系统表达编码 rhNaGlu 的核酸序列。这些包括但不限于微生物，例如经重组噬菌体、质粒或粘粒 DNA 表达载体转化的细菌；经酵母表达载体转化的酵母；感染了病毒表达载体（例如，杆状病毒）或细菌表达载体（例如，Ti 或 pBR322 质粒）的昆虫细胞系统；或哺乳动物细胞培养系统（例如，pTT22 载体）。图 11 和 12 中示出了含有与编码酸性氨基酸残基和 TfRL 的核酸序列融合的人 NaGlu cDNA 的 pTT22 载体的非限制性实例。

[0132] 本发明的多核苷酸和核酸编码区可与编码分泌或信号肽的附加编码区相关联，这指导由本发明多核苷酸编码的多肽的分泌。根据信号假说，脊椎动物（例如，禽类或哺乳动物）细胞分泌的蛋白质具有一旦开始输出增长的蛋白质链穿过糙面内质网 (ER)，就从成熟蛋白质裂解的信号肽或分泌前导序列。本领域的普通技术人员意识到脊椎动物细胞在 ER 内生成的多肽通常具有与多肽 N 端融合的信号肽，所述信号肽从完整或“全长”多肽裂解以生成分泌或“成熟”形式的多肽。在某些实施方案中，使用天然信号肽，例如，人 NaGlu 的 MEAVAVAAAVGVLLLAGAGGAAG (SEQ ID NO:1 的 1-23) 信号肽，或保持了指导与之可操作地连接的多肽的分泌的能力的序列的功能衍生物。可选地，可使用异源信号肽（例如，异源哺乳动物或禽类信号肽）或其功能衍生物。例如，可用（例如）人组织纤溶酶原激活因子 (tPA) 或小鼠 β - 葡萄糖醛酸酶的前导序列取代野生型前导序列。

[0133] 控制元件或调控序列可包括载体的非翻译区 - 增强子、启动子、5’ 和 3’ 非翻译区，其与宿主细胞蛋白质相互作用以进行转录和翻译。此类元件在其强度和特异性上可改变。根据利用的载体系统和宿主细胞，可使用任何数量的适合转录和翻译元件。例如，当在细菌系统中克隆时，可使用诱导型启动子例如 Bluescript™ 噬菌粒 (Stratagene, LaJolla, California) 或 pSport1™ 质粒 (Gibco BRL) 的杂交 lac-Z 启动子等。在哺乳动物细胞系统中，优选来自哺乳动物基因或来自哺乳动物病毒的启动子。如果必须产生含有多个拷贝的编码 NaGlu 的序列的细胞系，基于 SV40 或 EBV 的载体也可与适当的可选标记例如嘌呤霉素 (puromycin) 和氨苄青霉素 (ampicillin) 一起使用（见，例如，图 11 和 12）。

[0134] 当在转基因禽类中生成 rhNaGlu 时，本发明涵盖 rhNaGlu 序列可置于启动子下游，

以致编码 rhNaGlu 的序列可在转基因禽类中以组织特异性方式表达。例如，启动子可为主要但非完全对输卵管膨大部有特异性的输卵管特异性启动子，例如输卵管特异性启动子，包括但不限于卵白蛋白、溶菌酶、伴白蛋白、卵类粘蛋白、卵类粘蛋白、卵粘蛋白和卵转铁蛋白启动子。在一个实施方案中，启动子是卵白蛋白启动子、溶菌酶启动子、伴白蛋白启动子、卵类粘蛋白启动子、卵粘蛋白启动子和 / 或卵转铁蛋白启动子或其任何功能部分。

[0135] 可选地，组成型启动子可用于在禽类中表达人 NaGlu 的编码序列。在这种情况下，表达不限于输卵管膨大部，在禽类的其它组织（例如，血液）中也发生表达。包括组成型启动子和 NaGlu 的编码序列的这种转基因的用途也适合实现或驱动蛋白质在输卵管中表达和蛋白质随后分泌到蛋中。在一个实施方案中，组成型启动子可为（例如）巨细胞病毒 (CMV) 启动子、劳氏肉瘤病毒 (RSV) 启动子、鼠白血病病毒 (MLV) 启动子和 β - 肌动蛋白启动子。在一个实施方案中，启动子是 CMV 启动子、MDOT 启动子、RSV 启动子、MLV 启动子或小鼠乳腺瘤病毒 (MMTV) 启动子或其任何功能部分。

[0136] 本发明还涵盖本文所述启动子的任何有用片段或组件。启动子可为启动子区的至少一个区段、片段或组件，例如卵白蛋白、溶菌酶、伴白蛋白、卵类粘蛋白、卵粘蛋白、卵转铁蛋白、CMV、RSV 或 MLV 启动子区的一个区段。在一个优选实施方案中，启动子是输卵管特异性启动子区的一个区段，其含有指导编码序列在管状腺细胞中表达的基本元件。例如，本发明范围内包括输卵管特异性启动子的一个区段、部分或片段和 / 或缩合输卵管特异性启动子的关键调控元件，以便其保留在输卵管的膨大部中的管状腺细胞中表达所需的序列。在一个实施方案中，使用卵白蛋白启动子区的一个区段。该区段包含卵白蛋白基因的 5' - 侧翼区。

[0137] 含有人 NaGlu 的编码序列的载体可用于转染禽类胚盘细胞或哺乳动物细胞以对禽类或哺乳动物基因组产生稳定整合并且创建种系转基因禽类或哺乳动物细胞系。图 4A-D 和 5 中示出了此类载体的非限制性实例。在禽类表达系统中，人 NaGlu 编码序列以在转基因禽类，特别是在禽类输卵管的膨大部的管状腺细胞中表达编码序列的位置关系与启动子可操作地连接，以致重组人 NaGlu 蛋白质在转基因禽类产的硬壳蛋的蛋清中表达并存积。在美国专利号 6,730,822、美国专利号 6,825,396、美国专利号 6,875,588、美国专利号 7,294,507、美国专利号 7,521,591、美国专利号 7,534,929、美国公布号 2008/0064862A1 和美国公布号 2006/0185024 中公开了另外的适合载体和制备用于在禽类系统中表达 rhNaGlu 的载体的方法，所述专利的全部教义通过引用并入本文。也可用于本发明的其它启动子的非限制性实例包括 Pol III 启动子（例如，1 型、2 型和 3 型 Pol III 启动子）例如 H1 启动子、U6 启动子、tRNA 启动子、RNA 酶 MPR 启动子和这些启动子各自的功能部分。通常，根据采用的启动子，选择功能终止序列用于本发明。

[0138] 在一个实施方案中，载体为逆转录病毒载体，其中编码序列和启动子均位于逆转录病毒载体的 5' 和 3' LTR 之间。在一个有用实施方案中，LTR 或逆转录病毒载体源自禽白血病病毒 (ALV)、鼠白血病病毒 (MLV) 或慢病毒。用于向禽类基因组随机引入转基因的一种有用的逆转录病毒是复制缺陷型 ALV、复制缺陷型 MLV 或复制缺陷型慢病毒。

[0139] 本发明还涵盖自我失活型 (SIN) 载体的用途。SIN 载体可用于增加在转基因禽类输卵管中生成的人 NaGlu 的量。当 SIN 载体不含具有功能启动子的任何可选标记盒 (SIN/SC 阴性载体）时，这种效应可进一步增强。在一个实施方案中，SIN 载体是基因组改变的逆

转录病毒载体，以致整合的逆转录病毒载体的 5’LTR 不起启动子的作用。在一个特定实施方案中，一经整合就产生逆转录病毒载体的 5’LTR 的 U3 区域的逆转录病毒载体的一部分或整个核苷酸序列可缺失或改变，以便降低或消除 5’LTR 的启动子活性。图 4A-D 和 5 中示出了含有与人 rhNaGlu 的编码序列融合的卵白蛋白启动子区的 SIN 载体的非限制性实例。表 1 中也列出了载体的功能组件。

[0140] 表 1. pSIN-OV-1.1kb-I-rhNaGlu 中的功能组件

[0141]

功能组件	SEQ ID NO:4中的核苷酸序列
聚腺苷酸位点	634-639
部分gag	692-945
LTR(RAV2)	1243-1588
部分LTR(RAV2)	4691-4863
ALV CTE	4899-4986
1.1kb卵白蛋白启动子	5232-6363
DHS II	5334-5714
DHS I	6064-6364
外显子L	6364-6410
内含子1	6411-7999
NaGlu	8017-10248

[0142] 本文所述任何载体均可包括编码信号肽的序列，信号肽指导由来自（例如）禽类输卵管的管状腺细胞的载体编码序列表达的蛋白质的分泌。不分泌重组人 NaGlu 蛋白质时，将含有编码序列的载体修饰为包含含有来自（例如）溶菌酶基因的编码信号肽的约 60bp 的 DNA 序列。将编码信号肽的 DNA 序列插入载体，以致其位于由 DNA 编码的 rhNaGlu 蛋白质的 N 端。

[0143] 进一步地，可为用于本发明任何方法中的载体的编码序列提供 3’非翻译区 (3’UTR) 以赋予生成的 RNA 稳定性。当向逆转录病毒载体添加 3’UTR 时，启动子、编码序列和 3’UTR 的方向优选相对于 3’UTR 的方向相反，以致添加 3’UTR 不会干扰全长基因组 RNA 的转录。在一个实施方案中，3’UTR 可能是卵白蛋白基因、溶菌酶基因的 3’UTR 或输卵管膨大部细胞中具功能性的任何 3’UTR，即 SV40 晚期区。

[0144] III. 转基因禽类

[0145] 可将本文所述转基因引入禽类胚胎胚盘细胞以产生在其种系组织的基因组中携带编码重组人 NaGlu 的转基因的转基因鸡、转基因火鸡、转基因鹌鹑或其它禽类物种。在本发明的一个方面，通过用在逆转录病毒载体的 5’和 3’LTR 之间携带转基因的复制缺陷型或能复制的逆转录病毒颗粒转导胚胎胚盘细胞产生生成 rhNaGlu 的转基因禽类。例如，可使用禽白血病病毒 (ALV) 逆转录病毒载体或鼠白血病病毒 (MLV) 逆转录病毒载体。可使用包装成病毒颗粒的经修饰逆转录病毒载体的 RNA 拷贝感染发育成转基因禽类的胚胎胚盘。

[0146] 通过本发明的方法，可将转基因引入各种禽类物种的胚胎胚盘细胞中。例如，可应用所述方法产生在其种系组织的基因组中携带转基因的转基因鸡、转基因火鸡、转基因鹌鹑、转基因鸭和其它禽类物种，以便生成本发明的蛋白质。胚盘细胞通常是由 Eyal-Giladi 和 Kochav(1976) 定义的 VII-XII 期细胞或其等效物。在一个优选实施方案中，胚盘细胞处于或接近 X 期。

[0147] 在转染胚盘细胞的一种方法中，可使用基于包装逆转录病毒的载体将载体递送到胚胎胚盘细胞中，以便载体整合到禽类基因组中。为载体生成此类病毒颗粒（即，转导颗粒）并且滴定以测定可用于注射胚胎的适当浓度。在一个实施方案中，根据美国专利号 5,897,998 中描述的程序为禽蛋装窗 (window)，所述美国专利的公开内容通过引用整体并入本文，并且在或接近 X 期时为蛋注射转导颗粒。

[0148] 由已经引入了载体的胚盘细胞发育成本发明生成 rhNaGlu 的转基因禽类。使所得胚胎发育并且使小鸡成熟。在这个阶段，由胚盘细胞产生的转基因禽类称为创始者 (founder) 并且相对于携带转基因的细胞为嵌合体且称为 G0。G0 创始者禽类通常对于每个插入转基因而言是嵌合体。即，仅 G0 转基因鸟类的一些细胞含有转基因。一些创始者在其输卵管的膨大部中的管状腺细胞中携带转基因。这些禽类在其输卵管中表达由转基因编码的 rhNaGlu 蛋白质。除输卵管外，NaGlu 蛋白质也可在其它组织（例如，血液）中表达。一些创始者是在种系组织的基因组中携带转基因的种系创始者，并且也可在表达外源蛋白质的输卵管膨大部管状腺细胞中携带转基因。

[0149] 转基因禽类可在其以孟德尔 (Mendelian) 形式稳定地为禽类子代提供外源转基因传播的种系中携带转基因。G0 代对于编码 rhNaGlu 的转基因通常是半合子的。可使 G0 代繁育成非转基因动物，以产生对于转基因也是半合子的并且在基本上所有鸟类细胞中含有转基因的 G1 转基因子代。可使 G1 半合子子代繁育成产生 G2 半合子子代的非转基因动物或可一起繁育以产生对于转基因为纯合子的 G2 子代。大体上所有对源自 G1 子代的转基因呈阳性的禽类细胞均含有转基因。在一个实施方案中，可使来自同一系的半合子 G2 子代繁育生成对于转基因为纯合子的 G3 子代。在另一实施方案中，例如，使半合子 G0 或 G1 动物一起繁育以产生在动物每个细胞中含 2 个拷贝的转基因的纯合子 G1 子代。这些仅仅是某些有用的育种方法的实例并且本发明涵盖采用任何有用的育种方法，例如本领域的普通技术人员已知的育种方法。

[0150] IV. rhNaGlu 的生成

[0151] 可使用基因组中含有编码 rhNaGlu 的转基因的转基因禽类生成 rhNaGlu。在一个实施方案中，转基因禽类是种系转基因鸡、鹌鹑、鸭或火鸡。在一个特别有用的实施方案中，本发明涉及生成可在鸡的输卵管中生成的 NaGlu。

[0152] 在禽类系统（例如，在禽类输卵管）中生成有或无修饰的 rhNaGlu 在本发明范围内。在一个实施方案中，未经修饰的 rhNaGlu 包含具有使得能够被人细胞有效摄取的糖基化结构（即，M6P）的野生型氨基酸序列 (SEQ ID NO:1 的 24-743)。在另一实施方案中，经修饰的蛋白质可为具有使得能够被人细胞有效摄取的糖基化模式（即，M6P）的 rhNaGlu 融合蛋白。

[0153] 如本文所述，在或接近 X 期将含有编码 NaGlu 蛋白质，与驱动编码序列在鸡输卵管中表达的组织特异性或组成型启动子可操作地连接的核酸序列的适合禽类载体引入鸡胚

细胞中。在有利于孵化活的小鸡的条件下培育经转化的胚细胞。将活的小鸡养成成熟的嵌合体鸡，使成熟的嵌合体鸡自然或经人工受精与非转基因鸡交配。通过筛选种系并入了蛋白质编码序列的后代来鉴定转基因鸡。转基因后代可与另一转基因或非转基因鸡交配以产生产蛋的完全种系转基因母鸡。

[0154] 可按组织特异性方式生成 rhNaGlu。例如，rhNaGlu 可在转基因禽类的输卵管、血液和 / 或其它细胞或组织中表达。在一个实施方案中，NaGlu 在转基因禽类的输卵管膨大部的管状腺细胞中表达，分泌到输卵管腔内并且存积在蛋清中。在一个实施方案中，收获含 rhNaGlu 的蛋清并散装储存在范围为 4℃至 -20℃的温度下。然后使用本领域已知的各种方法从蛋的内含物中分离并纯化 NaGlu。

[0155] 本发明的一个方面涉及含有 rhNaGlu 蛋白质的禽类硬壳蛋（例如，硬壳鸡蛋）。转基因禽类生成和分泌的 rhNaGlu 以利于人细胞的细胞摄取的方式被糖基化。蛋白质可以任何有用量存在。在一个实施方案中，蛋白质以范围介于每个硬壳蛋约 0.01μg 和每个硬壳蛋约 1g 之间的量存在。在另一实施方案中，蛋白质以范围介于每个硬壳蛋约 1μg 和每个硬壳蛋约 1g 之间的量存在。例如，蛋白质可能以范围介于每个硬壳蛋约 10μg 和每个硬壳蛋约 1g 之间（例如，介于每个硬壳蛋约 10μg 和每个硬壳蛋约 400mg 之间）的量存在。

[0156] 在一个实施方案中，rhNaGlu 存在于蛋的蛋清中。在一个实施方案中，rhNaGlu 以范围介于每毫升蛋清约 1ng 和每毫升蛋清约 0.2g 之间的量存在。例如，rhNaGlu 可能以范围介于每毫升蛋清约 0.1μg 和每毫升蛋清约 0.2g 之间（例如，rhNaGlu 可能以范围介于每毫升蛋清约 1μg 和每毫升蛋清约 100mg 之间的量存在）的量存在。在一个实施方案中，rhNaGlu 以范围介于每毫升蛋清约 1μg 和每毫升蛋清约 50mg 之间的量存在。例如，rhNaGlu 可能以范围介于每毫升蛋清约 1μg 和每毫升蛋清约 10mg 之间（例如，rhNaGlu 可能以范围介于每毫升蛋清约 1μg 和每毫升蛋清约 1mg 之间存在的量）的量存在。在一个实施方案中，rhNaGlu 以大于每毫升蛋清 0.1μg 的量存在。在一个实施方案中，rhNaGlu 以大于每毫升蛋清 0.5μg 的量存在。在一个实施方案中，rhNaGlu 以大于每毫升蛋清 1μg 的量存在。在一个实施方案中，所述蛋白质以大于每毫升蛋清 1.5μg 的量存在。在一个实施方案中，rhNaGlu 以大于每毫升蛋清 0.5μg 的量存在。在一个实施方案中，所述蛋白质以大于每毫升蛋清 0.1μg 的量存在。

[0157] 在一个实施方案中，rhNaGlu 以 20mg/L、30mg/L、40mg/L、50mg/L、60mg/L、70mg/L、80mg/L、90mg/L、100mg/L、120mg/L、130mg/L、140mg/L、150mg/L、160mg/L、170mg/L、200mg/L、300mg/L、400mg/L、500mg/L、600mg/L、700mg/L、800mg/L、900mg/L 或 1,000mg/L 蛋清的量存在。在一个实施方案中，rhNaGlu 以约 100mg/L 蛋清的量存在。在一个实施方案中，rhNaGlu 以约 200mg/L 蛋清的量存在。

[0158] V. 宿主细胞

[0159] 本发明还涵盖在任何有用蛋白质表达系统，包括但不限于细胞培养物（例如，禽类细胞、CHO 细胞、HEK293 细胞和 COS 细胞）、酵母、细菌和植物中生成的 rhNaGlu。

[0160] 可选择宿主细胞菌株调节插入序列的表达或以所需方式加工表达的 NaGlu 的能力。NaGlu 多肽的这种修饰包括但不限于糖基化、磷酸化或脂化。可选择具有进行此类翻译后活动的特定细胞机器和特征机制的不同宿主细胞例如 CHO、COS、HeLa、MDCK、HEK293 和 W138，以确保正确修饰和加工本发明的融合蛋白。将禽类肿瘤细胞系也视为表达本发明多

肽的宿主细胞。在美国专利公开号 2009/0253176 中描述了有用禽类细胞系（例如，禽类输卵管肿瘤细胞系）的实例，所述专利公开的全部教义通过引用并入本文。

[0161] VI. 药物组合物

[0162] 本发明还特写了包含经分离和大体纯化的 rhNaGlu 或其药学上可接受的盐的药物组合物。可使用一种或多种载体，例如作为药物制剂的一部分，或不用载体施用重组人 NaGlu 蛋白质。从与制剂的其它成分相容并且对其受者无害的意义上说，载体必须“可接受”。包含此类载体的组合物，包括复合分子，通过众所周知的常规方法配制（见，例如，Remington's Pharmaceutical Sciences，第 14 版，Mack Publishing Co.，Easton，Pa.），其全部教义通过引用并入本文。载体可包含稀释剂。在一个实施方案中，药物载体可为液体并且所述蛋白质可呈溶液形式。药物载体可为蜡、脂肪或醇。在另一实施方案中，药学上可接受的载体可能是呈粉末、冻干粉或片剂形式的固体。在一个实施方案中，载体可包含脂质体或微胶囊。

[0163] 在一些实施方案中，包含重组人 NaGlu 蛋白质的药物组合物进一步包含缓冲剂。示例性缓冲剂包括醋酸盐、磷酸盐、柠檬酸盐和谷氨酸盐缓冲剂。示例性缓冲剂还包括柠檬酸锂、柠檬酸钠、柠檬酸钾、柠檬酸钙、乳酸锂、乳酸钠、乳酸钾、乳酸钙、磷酸锂、磷酸钠、磷酸钾、磷酸钙、马来酸锂、马来酸钠、马来酸钾、马来酸钙、酒石酸锂、酒石酸钠、酒石酸钾、酒石酸钙、琥珀酸锂、琥珀酸钠、琥珀酸钾、琥珀酸钙、醋酸锂、醋酸钠、醋酸钾、醋酸钙及其混合物。在一些实施方案中，缓冲剂是柠檬酸三钠二水合物。在一些实施方案中，缓冲剂是柠檬酸一水合物。在一些实施方案中，药物组合物包含柠檬酸三钠二水合物和柠檬酸一水合物。

[0164] 在一些实施方案中，包含重组人 NaGlu 蛋白质的药物组合物进一步包含稳定剂。示例性稳定剂包括白蛋白、海藻糖、糖、氨基酸、多元醇、环糊精、盐（例如氯化钠、氯化镁和氯化钙）、冻干保护剂及其混合物。在一些实施方案中，药物组合物包含人血清白蛋白。

[0165] 在一些实施方案中，需要向药物组合物添加表面活性剂。示例性表面活性剂包括非离子型表面活性剂，例如聚山梨醇酯（例如，聚山梨醇酯 20 或 80）；泊洛沙姆（例如，泊洛沙姆 188）；Triton；十二烷基硫酸钠 (SDS)；月桂基硫酸钠；辛基葡萄糖苷钠；月桂基 -、肉豆蔻基 -、亚油基 - 或硬脂酰基 - 磺基三甲铵乙内酯；月桂基 -、肉豆蔻基 -、亚油基 - 或硬脂酰基 - 肌氨酸；亚油基 -、肉豆蔻基 - 或鲸蜡基 - 甜菜碱；月桂酰胺基丙基 -、椰油酰胺基丙基 -、亚油酰胺基丙基 -、肉豆蔻酰胺基丙基 -、棕榈酰胺基丙基 - 或异硬脂酰胺基丙基 - 甜菜碱（例如，月桂酰胺基丙基甜菜碱）；肉豆蔻酰胺基丙基 -、棕榈酰胺基丙基 - 或异硬脂酰胺基丙基 - 二甲胺；甲基椰油酰基牛磺酸钠或甲基油酰基牛磺酸二钠；和 MONAQUAT™ 系列 (Mona Industries，Inc.，Paterson，N.J.)，聚乙二醇、聚丙二醇及乙二醇和丙二醇的共聚物（例如，Pluronics、PF68 等）。通常，添加的表面活性剂的量是以致其减少蛋白质的聚集并且将颗粒的形成或起泡减到最少。例如，制剂中可存在浓度约 0.001-0.5%（例如，约 0.005-0.05% 或 0.005-0.01%）的表面活性剂。具体而言，制剂中可存在浓度约 0.005%、0.01%、0.02%、0.1%、0.2%、0.3%、0.4% 或 0.5% 等的表面活性剂。上述范围和值中间的范围和值也视为本发明的一部分。

[0166] 在一些实施方案中，本发明的适合药物组合物可进一步包括一种或多种疏松剂，特别是对于冻干制剂而言。“疏松剂”是增加冻干混合物的质量并且有助于冻干块状物的物

理结构的化合物。例如，疏松剂可改善冻干块状物（例如，基本均匀的冻干块状物）的外观。适合的疏松剂包括但不限于氯化钠、乳糖、甘露糖醇、甘氨酸、蔗糖、海藻糖、羟乙基淀粉。疏松剂的示例性浓度从约 1% 到约 10%（例如，1.0%、1.5%、2.0%、2.5%、3.0%、3.5%、4.0%、4.5%、5.0%、5.5%、6.0%、6.5%、7.0%、7.5%、8.0%、8.5%、9.0%、9.5% 和 10.0%）。上述范围和值中间的范围和值也视为本发明的一部分。药物组合物可呈无菌冻干粉形式，以在用稀释剂复水后注射。稀释剂可为注射用水、抑菌性注射用水或无菌盐水。可通过冷冻干燥融合蛋白质溶液生产冻干粉，以生产呈干燥形式的蛋白质。如本领域所知，冻干蛋白质通常具有比液体蛋白质溶液更高的稳定性和更长的保质期。

[0167] 药物制剂包括适合口服、直肠、鼻内、局部（包括口腔或舌下）、阴道或肠胃外施用的药物制剂。优选地，本发明的药物制剂包括适合通过注射施用，包括鞘内、实质内、大脑内、心室内、肌肉内、皮下和静脉内施用的药物制剂。在一个实施方案中，本发明的制剂适合静脉内施用。在另一实施方案中，本发明的制剂适合鞘内施用。本发明的药物制剂还包括适合通过吸入或吹入施用的药物制剂。在适当情况下，所述制剂可方便地以离散剂量单位呈现并且可通过药学领域众所周知的任何方法制备。生产药物制剂的方法通常包括使治疗性蛋白质与液体载体或磨碎的固体载体或二者缔合，然后如有必要，使产品定形为所需制剂的步骤。

[0168] 本发明的重组人 NaGlu 蛋白质也可配制用于肠胃外施用（例如，通过注射，例如团注或连续输注）并且可在安瓿、预填充注射器、小体积输注或多剂量容器中与添加的防腐剂一起以单位剂量形式呈现。例如，可通过皮下注射、肌肉内注射、鞘内注射、大脑内注射、实质内注射、心室内注射和静脉内 (IV) 输注或注射，注射治疗性蛋白质。

[0169] 在一个实施方案中，可通过任何有用方法 IV 输注，经静脉施用重组人 NaGlu 蛋白质。在一个实例中，可通过外周管道静脉内输注施用重组人 NaGlu 蛋白质。在另一个实例中，可通过外周导入中心静脉置管静脉输注施用重组人 NaGlu 蛋白质。在另一个实例中，可通过与静脉血管入口连接的流动输液机促进的静脉内输注施用重组人 NaGlu 蛋白质。在静脉输注的一个实施方案中，根据要输注的药物的量和患者先前的输注相关反应史，由本领域的技术医师确定，在 1 至 8 小时的时期内施用药物。在另一实施方案中，通过 IV 注射经静脉内施用重组人 NaGlu 蛋白质。在另一实施方案中，可经腹腔内或鞘内注射施用重组人 NaGlu 蛋白质。

[0170] 在一些实施方案中，可通过输注施用治疗性蛋白质，并且输注可在更长时间段发生，例如 30 分钟至 10 小时。因此，输注可在例如，约 1 小时、约 2 小时、约 3 小时、约 4 小时或约 5 小时的时期发生。输注也可以不同速率发生。因此，例如，输注速率可为约 1mL/h 至约 20mL/h。在一些实施方案中，输注速率为 5mL 至 10mL/h。在一个实施方案中，输注速率为 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19 或 20mL/h。在一个实施方案中，输注速率为 0.1 至 5mg/kg/h。在一个实施方案中，输注速率为约 0.1、约 0.2、约 0.3、约 0.5、约 1.0、约 1.5、约 2.0 或约 3mg/kg/h。上述范围和值中间的范围和值也视为本发明的一部分。

[0171] 治疗性蛋白质可在油或水媒介物中呈诸如悬浮液、溶液或乳液的形式，并且可含有配方剂，例如助悬剂、稳定剂和 / 或分散剂。重组人 NaGlu 蛋白质可呈粉末形式，通过无菌分离无菌固体或由溶液冻干获得，以在使用之前用适合媒介物，例如无菌、无热源的水复

水。

[0172] 可基于产品质量分析、复水时间（如果冻干）、复水质量（如果冻干）、高分子量、湿度和玻璃态转化温度评估根据本发明所述的制剂。通常，蛋白质质量和产品分析包括使用下列方法进行的产品降解率分析，包括但不限于尺寸排阻 HPLC(SE-HPLC)、阳离子交换 HPLC(CEX-HPLC)、X 射线衍射 (XRD)、调制示差扫描量热法 (mDSC)、反相 HPLC(RP-HPLC)、多角度光散射 (MALS)、荧光、紫外线吸收、浊度测定法、毛细管电泳 (CE)、SDS-PAGE 及其组合。在一些实施方案中，评价根据本发明所述的产品可包括评价外观（液体或块状物外观）的步骤。

[0173] 通常，制剂（冻干或含水）可在室温下储存更长时间。储存温度范围可能通常从 0℃到 45℃（例如，4℃、20℃、25℃或 45℃）。制剂可储存几个月的时期到几年的时期。储存时间将通常为 24 个月、12 个月、6 个月、4.5 个月、3 个月、2 个月或 1 个月。制剂可直接储存在用于施用的容器中，取消了转移步骤。上述范围和值中间的范围和值也视为本发明的一部分。

[0174] 制剂可直接储存在冻干容器中（如果冻干），冻干容器也起复水器皿的作用，取消了转移步骤。可选地，可将冻干产品制剂测量成较小增量以便储存。储存通常应避免导致蛋白质降解的环境，包括但不限于暴露于阳光、紫外辐射、其它形式的电磁辐射、过热或过冷、快速热冲击和机械冲击。根据本发明所述的药物组合物还含有以下更加详细讨论的其它活性成分，例如免疫抑制剂、抗菌剂或防腐剂。

[0175] VII. 治疗方法

[0176] 本发明还提供了治疗 NaGlu 相关疾病，例如桑菲列浦氏综合征 B 型的方法。根据本发明采用的重组 NaGlu 包括可在任何有用的蛋白质表达系统中生成的重组 NaGlu，所述有用的蛋白质表达系统包括但不限于细胞培养物（例如，CHO 细胞、COS 细胞）、细菌（例如，大肠杆菌 (E.coli)）、转基因动物（例如哺乳动物和禽类（例如，鸡、鸭和火鸡））和植物系统（例如，浮萍和烟草）。在一个实施方案中，在转基因动物，例如禽类中生成重组 NaGlu。

[0177] 在一个实施方案中，所述方法包括向受试者施用重组人 NaGlu 蛋白质 (rhNaGlu)，例如含有足够量的寡糖（例如，甘露糖和磷酸化甘露糖（即，M6P））的重组人 NaGlu 蛋白质，其量足以治疗（例如，减轻、改善）或预防 NaGlu 缺乏症或 NaGlu 相关疾病的一种或多种症状。重组人 NaGlu 蛋白质可治疗性或预防性施用，或二者。重组人 NaGlu 蛋白质 (rhNaGlu) 可单独或与本文所述其它治疗方式组合施用给受试者。

[0178] 术语“治疗”指缓解、缓和或改善疾病或症状，预防额外症状，改善或预防症状的根本原因，抑制疾病或病状，阻止疾病或病状发展，减轻疾病或病状，引起疾病或病状恢复，减轻所述疾病或病状引起的状况，或预防性地和 / 或在症状出现之后终止所述疾病或病状的症状的方法。

[0179] 如本文所使用的“治疗有效剂量”指产生预期治疗反应（例如，靶组织中硫酸乙酰肝素水平降低和 / 或 NaGlu 活性增加）所需的药物的剂量（例如，量和 / 或间隔）。治疗有效剂量指，与尚未接受此类剂量的相应受试者相比，导致对疾病、病症或副作用的治疗、治愈、预防或改善提高，或疾病或病症的发生或增进率降低的剂量。术语还包括，在其范围内有效增强生理功能的剂量。

[0180] 如本文所使用，术语“受试者”或“患者”旨在包括人和非人动物。非人动物包括

所有脊椎动物，例如哺乳动物和非哺乳动物，例如非人灵长类动物、羊、狗、猫、牛、马、鸡、两栖动物和爬行动物。优选的受试者包括有 NaGlu 缺乏症或 NaGlu 相关疾病的人受试者。

[0181] 如本文所使用，“NaGlu 相关疾病”是由 NaGlu 活性介导或与 NaGlu 表达或活性异常相关的疾病或病状。NaGlu 相关疾病的实例包括但不限于 NaGlu 缺乏症例如桑菲列浦氏综合征 B 型（也称为粘多糖贮积病 IIIB 型）。

[0182] 本发明的治疗方法涵盖利于重组人 NaGlu 蛋白质摄取或转运到有关器官和组织的任何施用途径。在一个实施方案中，本发明的方法包括将本发明的重组人 NaGlu 蛋白质递送到受试者的 CNS（中枢神经系统）、肾脏或肝脏，以用于治疗 NaGlu 相关疾病（例如，NaGlu 缺乏症）。例如，重组人 NaGlu 蛋白质可经静脉内（例如，经静脉内注射或静脉内输注）向患者施用并且惊人地穿过有 NaGlu 缺乏症的受试者的血脑屏障 (BBB)。在本发明的另一实施方案中，经鞘内向患者施用重组人 NaGlu 蛋白质。

[0183] A. 用于鞘内递送的装置

[0184] 各种装置均可用于根据本发明的鞘内递送。在一些实施方案中，用于鞘内施用的装置含有流体入口（例如，注射口）；空心体（例如，导管），其具有与流体入口流体连通的第一流孔和配置为插入脊髓的第二流孔；和紧固机构，用于紧固空心体插入脊髓。作为非限制性实例，适合的紧固机构含有固定在空心体表面的一个或多个冒口 (nob) 和在所述一个或多个冒口上方可调节的缝合环，以防止空心体（例如，导管）从脊髓中滑出。在各实施方案中，流体入口包括贮器。在一些实施方案中，流体入口包括机械泵（例如，输注泵）。在一些实施方案中，植入导管与贮器（例如，对于团注递送而言）或输注泵连接。流体入口可植入或外置。

[0185] 在一些实施方案中，可通过腰椎穿刺（即，缓慢团注）或经由入口 - 导管递送系统（即，输注或团注）进行鞘内施用。在一些实施方案中，导管插在腰椎板之间并且尖端在鞘间隙上紧螺纹至所需水平（通常 L3-L4）。

[0186] 相对于静脉施用，适合鞘内施用的单剂量体积通常很小。通常，根据本发明的鞘内递送维持 CSF 组合物以及受试者颅内压的平衡。在一些实施方案中，进行鞘内递送，没有从受试者相应去除 CSF。在一些实施方案中，适合的单剂量体积可（例如）小于约 10mL、8mL、6mL、5mL、4mL、3mL、2mL、1.5mL、1mL 或 0.5mL。在一些实施方案中，适合的单剂量体积可为约 0.5-5mL、0.5-4mL、0.5-3mL、0.5-2mL、0.5-1mL、1-3mL、1-5mL、1.5-3mL、1-4mL 或 0.5-1.5mL。在一些实施方案中，根据本发明的鞘内递送牵涉首先去除所需量的 CSF 的步骤。在一些实施方案中，在鞘内施用之前，首先去除小于约 10mL（例如，小于约 9mL、8mL、7mL、6mL、5mL、4mL、3mL、2mL、1mL）的 CSF。在那些情况下，适合的单剂量体积可（例如）大于约 3mL、4mL、5mL、6mL、7mL、8mL、9mL、10mL、15mL 或 20mL。上述范围和值中间的范围和值也视为本发明的一部分。

[0187] 各种其它装置可用于实现治疗组合物的鞘内施用。例如，可使用常用于鞘内施用脑膜性癌病药物的 Ommaya 储器供给含所需酶类的制剂 (Lancet2:983-84, 1963)。更具体地，在这种方法中，脑室管通过前角中形成的孔插入并且连接到安装在头皮下的 Ommaya 储器，并且皮下穿刺储器以在鞘内递送更换的注入储器中的特定酶。在美国专利号 6,217,552 中描述了用于向个体鞘内施用治疗组合物或制剂的其它装置，因为其涉及这些装置，所以其全部内容通过引用并入本文。可选地，例如可通过单次注射或连续输注经鞘内给药。应

理解，剂量治疗可呈单剂量施用或多剂量形式。

[0188] 对于注射，可将本发明的制剂配制成液体溶液。另外，可将 NaGlu 酶配制成固体形式并且在使用之前立即重新溶解或悬浮。也包括冻干形式。例如，注射可呈 NaGlu 酶的团注或连续输注形式（例如，使用输注泵）。

[0189] 在本发明的一个实施方案中，通过侧脑室注射到受试者脑部来施用 NaGlu 酶。例如，可通过在受试者颅骨上产生的钻孔进行注射。在另一实施方案中，通过向受试者脑室手术插入的分流器来施用酶和 / 或其它药物制剂。例如，可向更大的侧脑室进行注射。在一些实施方案中，也可向第三和第四较小脑室进行注射。

[0190] 又一实施方案中，通过向受试者的小脑延髓池或腰部注射来施用本发明中使用的药物组合物。

[0191] 在本发明方法的另一实施方案中，在向受试者施用药学上可接受的制剂后，药学上可接受的制剂向受试者提供至少 1、2、3、4 周或更长时间的酶或本发明中使用的其它药物组合物的持续递送，例如“缓释”。

[0192] 如本文所使用，术语“持续递送”指在施用后的一段时间内，优选至少几天、一周或几周，在体内连续递送本发明的药物制剂。例如，通过随时间推移，酶的连续疗效可证明组合物的持续递送（例如，通过受试者中储存颗粒的量连续减少可证明酶的持续递送）。可选地，可通过检测随时间推移体内酶的存在证明酶的持续递送。

[0193] B. 静脉内递送

[0194] 如以上所讨论，本发明其中一个令人惊讶的特征是，当静脉内施用时，本发明的重组人 NaGlu 蛋白质能够有效且广泛地扩散穿过血脑屏障 (BBB) 和脑部表面并且渗透脑部的各个层或区域，包括脑部深区。本发明的方法有效地将 rhNaGlu 蛋白质递送到难以通过现有 CNS 递送方法靶向的中枢神经系统 (CNS) 的各种组织、神经元或细胞。此外，本发明的方法将足够量的重组人 NaGlu 蛋白质递送到血流及各种外周器官和组织中。

[0195] “静脉内注射”，在医学上常称为 IV 推注或团注，指其中注射器与 IV 接入装置连接并且直接注射药物，通常很快而且如果可能引起静脉刺激或过快效应，有时候长达 15 分钟的施用途径。一旦将药品注射到 IV 管的液体流中，就必须有一些方式确保其从管流到患者体内。通常这通过使液体流正常流动并从而将药品带入血流中实现。然而，在一些情况下在第一次注射后使用第二次液体注射，有时称为“冲洗”，以促进药品进入血流中。

[0196] “静脉内输注”指其中在更长时间段内递送药物的施用途径。例如，可在介于 1 和 8 小时的时期内向患者递送药物。也可在约 1、约 2、约 3、约 4、约 5、约 6、约 7 或约 8 小时的时期内向患者递送药药物。为实现静脉内输注，可使用 IV 重力滴注或 IV 泵。当患者仅在某些时候需要药物而不需要额外的静脉内液体（例如，含有钠、氯、葡萄糖或其任何组合的水溶液），例如恢复电解质、血糖和水损失的液体时，通常使用 IV 输注。

[0197] C. 靶组织

[0198] 在一些实施方案中，向受试者的中枢神经系统 (CNS) 递送本发明的 rhNaGlu。在一些实施方案中，向脑部、脊髓和 / 或外周器官的一个或多个靶组织递送本发明的 rhNaGlu。如本文所使用，术语“靶组织”指受待治疗的 NaGlu 相关疾病影响的任何组织或缺乏的 NaGlu 在其中正常表达的任何组织。在一些实施方案中，靶组织包括其中在患有或易患 NaGlu 相关疾病的患者中，存在可检测量或异常高的量的酶底物（例如储存在组织的细胞

溶酶体中）的组织。在一些实施方案中，靶组织包括展现出疾病相关病理、症状或特征的那些组织。在一些实施方案中，靶组织包括其中缺乏的 NaGlu 以升高水平正常表达的那些组织。如本文所使用，靶组织可为脑部靶组织、脊髓靶组织和 / 或外周靶组织。下面详细描述了示例性靶组织。

[0199] D. 脑部靶组织

[0200] 一般而言，脑部可分为不同区域、层和组织。例如，脑膜组织是包裹中枢神经系统（包括脑部）的膜系统。脑膜含有 3 层，包括硬脑膜、蛛网膜和软脑膜。一般而言，脑膜和脑脊髓液的主要功能是保护中枢神经系统。在一些实施方案中，根据本发明的治疗性蛋白质递送到脑膜的一层或多层。

[0201] 脑部具有 3 个主要分区，包括大脑、小脑和脑干。大脑半球，位于大多数其它脑部结构上方并且为皮层所覆盖。脑干位于大脑下面，其类似于大脑在其上附着的茎。在脑部的后面，在大脑下面和脑干后面是小脑。

[0202] 位于脑部中间附近和中脑上方的间脑含有丘脑、后丘脑、下丘脑、上丘脑、前丘脑和前顶盖。中脑 (mesencephalon)，也成为中脑 (midbrain)，含有顶盖、大脑脚盖、脑室中脑腔和大脑脚，红核和第三脑神经核。中脑与视力、听力、运动控制、睡 / 醒、警惕性和温度调节相关联。

[0203] 可根据组织的深度表征中枢神经系统，包括脑部的组织区域。例如，CNS（例如，脑部）组织可表征为表面或浅层组织、中深组织和 / 或深部组织。

[0204] 根据本发明，本发明的 rhNaGlu 可递送到与受试者中待治疗特定疾病相关的任何适当脑部靶组织。在一些实施方案中，本发明的 rhNaGlu 递送到表面或浅层脑部靶组织。在一些实施方案中，本发明的 rhNaGlu 递送到中深脑部靶组织。在一些实施方案中，本发明的 rhNaGlu 递送到深部脑部靶组织。在一些实施方案中，本发明的 rhNaGlu 递送到表面或浅层脑部靶组织、中深脑部靶组织和 / 或深部脑部靶组织的组合。在一些实施方案中，本发明的 rhNaGlu 递送到脑部外表面下面（或内部）至少 4mm、5mm、6mm、7mm、8mm、9mm、10mm 或更多的深部脑组织。上述范围和值中间的范围和值也视为本发明的一部分。

[0205] 在一些实施方案中，本发明的 rhNaGlu 递送到大脑的一个或多个表面或浅层组织。在一些实施方案中，大脑的靶向表面或浅层组织位于大脑表面 4mm 内。在一些实施方案中，大脑的靶向表面或浅层组织选自软脑膜组织、大脑皮质带组织、海马、Virchow Robin 间隙、VR 间隙中的血管、海马、脑部内表面上的下丘脑部分、视神经和视束、嗅球和突出及其组合。

[0206] 在一些实施方案中，本发明的 rhNaGlu 递送到大脑的一个或多个深部组织。在一些实施方案中，大脑的靶向表面或浅层组织位于大脑表面下面（或内部）至少 4mm（例如，5mm、6mm、7mm、8mm、9mm 或 10mm）。在一些实施方案中，大脑的靶向深部组织包括大脑皮质带。在一些实施方案中，大脑的靶向深部组织包括间脑（例如，下丘脑、丘脑、前丘脑或丘脑底部）、中脑、豆状核、基底神经节、尾状核、壳核、杏仁核、苍白球及其组合中的一者或多者。

[0207] 在一些实施方案中，本发明的 rhNaGlu 递送到小脑的一个或多个组织。在某些实施方案中，小脑的一个或多个靶向组织选自分子层的组织、蒲肯野细胞层 (Purkinje cell layer) 的组织、粒细胞层的组织、小脑脚及其组合。在一些实施方案中，治疗剂（例如，酶）递送到小脑的一个或多个深部组织，包括但不限于蒲肯野细胞层的组织、粒细胞层的组织、

小脑白质深部组织（例如，相对于粒细胞层较深）和小脑核深部组织。

[0208] 在一些实施方案中，本发明的 rhNaGlu 递送到脑干的一个或多个组织。在某些实施方案中，脑干的一个或多个靶向组织包括脑干白质组织和 / 或脑干核组织。

[0209] 在一些实施方案中，本发明的 rhNaGlu 递送到各种脑组织，包括但不限于灰质、白质、运动前区、软蛛网膜、脑膜、新皮质、小脑、大脑皮质中的深部组织、分子层、尾状核 / 壳核区、中脑、脑桥或髓质的深区及其组合。

[0210] 在一些实施方案中，本发明的 rhNaGlu 递送到脑部中的各种细胞，包括但不限于神经元、胶质细胞、血管周细胞和 / 或脑膜细胞。在一些实施方案中，治疗性蛋白质递送到深部白质的少突胶质细胞。

[0211] E. 脊髓靶组织

[0212] 一般而言，可根据组织的深度表征脊髓的区域或组织。例如，脊髓组织可表征为表面或浅层组织、中深组织和 / 或深部组织。

[0213] 在一些实施方案中，本发明的 rhNaGlu 递送到脊髓的一个或多个表面或浅层组织。在一些实施方案中，脊髓的靶向表面或浅层组织位于脊髓表面 4mm 内。在一些实施方案中，脊髓的靶向表面或浅层组织含有软脑膜和 / 或白质束。

[0214] 在一些实施方案中，本发明的 rhNaGlu 递送到脊髓的一个或多个深部组织。在一些实施方案中，脊髓的靶向深部组织位于脊髓表面 4mm 内。在一些实施方案中，脊髓的靶向深部组织含有脊髓灰质和 / 或室管膜细胞。

[0215] 在一些实施方案中，替代酶（例如，NaGlu 融合蛋白）递送到脊髓的神经元。

[0216] F. 外周靶组织

[0217] 如本文所使用，外周器官或组织指并非中枢神经系统 (CNS) 的一部分的任何器官或组织。外周靶组织可包括但不限于血液系统、肝脏、肾脏、心脏、内皮、骨髓和骨髓来源的细胞、脾脏、肺部、淋巴结、骨骼、软骨、卵巢和睾丸。在一些实施方案中，本发明的 rhNaGlu 递送到一个或多个外周靶组织。

[0218] G. 生物分布和生物利用率

[0219] 在各实施方案中，一旦递送到靶组织，本发明的 rhNaGlu 就在细胞内定位。例如，本发明的 rhNaGlu 可定位于靶细胞（例如，神经元（例如蒲肯野细胞））的内含子、外显子、溶酶体、线粒体或空泡。例如，在一些实施方案中，本发明的 rhNaGlu 展示出易位动力学，以致 rhNaGlu 在血管周隙内移动（例如，通过搏动辅助的对流机制）。另外，与施用的蛋白质或酶和神经纤维细丝缔合有关的主动轴突运输机制也可能有助于或利于本发明的 rhNaGlu 蛋白质分布到中枢神经系统更深的组织中。

[0220] 在一些实施方案中，根据本发明递送的本发明的 rhNaGlu 可在本文所述各种靶组织中达到治疗或临床有效水平或活性。如本文所使用，治疗或临床有效水平或活性是足以在靶组织中赋予疗效的水平或活性。疗效可能是客观的（即，可通过某种试验或标志物测量）或主观的（即，受试者给出效果指征或感觉有效）。例如，治疗或临床有效水平或活性可为足以在靶组织中改善疾病（例如，GAG 贮积）相关症状的酶水平或活性。

[0221] 在一些实施方案中，根据本发明递送的本发明的 rhNaGlu 可在靶组织中达到相应 NaGlu 酶的正常水平或活性的至少 5%、10%、20%、30%、40%、50%、60%、70%、80%、90%、91%、92%、93%、94%、95%、96%、97%、98% 或 99% 的酶水平或活性。在一些实施方案中，根据本发明

递送的本发明的 rhNaGlu 可达到与对照（例如，未经处理的内源水平或活性）相比增加至少 1 倍、2 倍、3 倍、4 倍、5 倍、6 倍、7 倍、8 倍、9 倍或 10 倍的酶水平或活性。在一些实施方案中，根据本发明递送的 rhNaGlu 可在靶组织中达到增加的酶水平或活性，至少约 10nmol/h/mg、20nmol/h/mg、40nmol/h/mg、50nmol/h/mg、60nmol/h/mg、70nmol/h/mg、80nmol/h/mg、90nmol/h/mg、100nmol/h/mg、150nmol/h/mg、200nmol/h/mg、250nmol/h/mg、300nmol/h/mg、350nmol/h/mg、400nmol/h/mg、450nmol/h/mg、500nmol/h/mg、550nmol/h/mg 或 600nmol/h/mg。上述范围和值中间的范围和值也视为本发明的一部分。

[0222] 在一些实施方案中，根据本发明的发明方法对靶向腰部特别有用。在一些实施方案中，根据本发明递送的 rhNaGlu 可在腰部达到增加的酶水平或活性，至少约 500nmol/h/mg、600nmol/h/mg、700nmol/h/mg、800nmol/h/mg、900nmol/h/mg、1000nmol/h/mg、1500nmol/h/mg、2000nmol/h/mg、3000nmol/h/mg、4000nmol/h/mg、5000nmol/h/mg、6000nmol/h/mg、7000nmol/h/mg、8000nmol/h/mg、9000nmol/h/mg 或 10,000nmol/h/mg。上述范围和值中间的范围和值也视为本发明的一部分。

[0223] 一般而言，根据本发明递送的治疗剂（例如，rhNaGlu）在脑部的 CSF 和靶组织、脊髓和外周器官中具有足够长的半衰期。在一些实施方案中，根据本发明递送的 rhNaGlu 的半衰期可能至少约 30 分钟、45 分钟、60 分钟、90 分钟、2 小时、3 小时、4 小时、5 小时、6 小时、7 小时、8 小时、9 小时、10 小时、12 小时、16 小时、18 小时、20 小时、25 小时、30 小时、35 小时、40 小时、长达 3 天、长达 7 天、长达 14 天、长达 21 天或长达 1 个月。在一些实施方案中，在一些实施方案中，根据本发明递送的 rhNaGlu 在施用 12 小时、24 小时、30 小时、36 小时、42 小时、48 小时、54 小时、60 小时、66 小时、72 小时、78 小时、84 小时、90 小时、96 小时、102 小时或 1 周后，可在 CSF 或血流中保持可检测的水平或活性。可使用本领域已知的各种方法测定可检测的水平或活性。上述范围和值中间的范围和值也视为本发明的一部分。

[0224] 在某些实施方案中，根据本发明递送的 rhNaGlu 在施用后（例如，向受试者施用药物组合物 1 周、3 天、48 小时、36 小时、24 小时、18 小时、12 小时、8 小时、6 小时、4 小时、3 小时、2 小时、1 小时、30 分钟或更短时间后），在受试者的 CNS 组织和细胞中达到至少 30μg/mL 的浓度。在某些实施方案中，根据本发明递送的 rhNaGlu 在施用给受试者后（例如，向受试者施用此类药物组合物 1 周、3 天、48 小时、36 小时、24 小时、18 小时、12 小时、8 小时、6 小时、4 小时、3 小时、2 小时、1 小时、30 分钟或更短时间后），在此类受试者的靶组织或细胞（例如，脑组织或神经元）中达到至少 2μg/mL、至少 15μg/mL、至少 1μg/mL、至少 7μg/mL、至少 5μg/mL、至少 2μg/mL、至少 1μg/mL 或至少 0.5μg/mL 的浓度。上述范围和值中间的范围和值也视为本发明的一部分。

[0225] H. 桑菲列浦氏综合征的治疗

[0226] 桑菲列浦氏综合征或粘多糖贮积病 III 型（MPS III）是特征在于糖胺聚糖（GAG）降解中牵涉的酶缺乏的罕见遗传病症。缺少酶时，部分降解的 GAG 分子不能从体内清除并且在各种组织的溶酶体中积累，导致普遍躯体功能逐渐失调（Neufeld 和 Muenzer, 2001）。

[0227] 已经鉴定了 4 种不同形式的 MPS III，指定为 MPS IIIA、B、C 和 D。各自代表 GAG 硫酸乙酰肝素降解中牵涉的 4 种酶之一的缺乏。所有形式均包括不同程度的相同临床症状，包括面部特征粗糙、肝脾肿大、角膜浑浊和骨骼畸形。然而，最显著的是认知能力严重且逐步丧失，这不但与神经元中硫酸乙酰肝素的积累相关联，而且与主要 GAG 积累引起的神

经节苷脂 GM2、GM3 和 GD2 随后升高相关联 (Walkley1998)。

[0228] 粘多糖贮积病 IIIB 型 (MPS IIIB ;桑菲列浦氏综合征 B 型) 是常染色体隐性病症，其特征在于酶 α -N- 乙酰基 - 氨基葡萄糖苷酶 (NaGlu) 缺乏。在缺少这种酶时，GAG 硫酸乙酰肝素在神经元和胶质细胞的溶酶体中积累，较少在脑部外积累。

[0229] 这种病症的最典型的临床特征是中枢神经系统 (CNS) 退化，这导致丧失或不能获得主要发育里程碑。进行性认知减退在痴呆和过早死亡中达到最高。疾病本身通常在幼儿中表现，并且患病个体的寿命通常不超过少年晚期到二十岁出头。

[0230] 本发明的组合物和方法可用于有效治疗患有或易患桑菲列浦氏综合征 B 型的个体。如本文所使用，术语“治疗”指改善与疾病相关的一种或多种症状，预防或延迟疾病的一种或多种症状的发作，和 / 或降低疾病的一种或多种症状的严重程度或频率。

[0231] 在一些实施方案中，治疗指部分或完全缓解、改善、减轻、抑制、延迟发作，降低桑菲列浦氏综合征 B 型患者中的神经损伤的严重程度和 / 或发生率。如本文所使用，术语“神经损伤”包括与中枢神经系统 (例如，脑部和脊髓) 损伤相关的各种症状。神经损伤的症状可包括 (例如) 发育延迟、进行性认知损伤、听力丧失、语言发展受损、运动技能不足、极度活跃、攻击性和 / 或睡眠障碍等。

[0232] 因此，在一些实施方案中，治疗指各种组织中溶酶体贮积 (例如，GAG) 减少。在一些实施方案中，治疗指脑部靶组织、脊髓神经元和 / 或外周靶组织中的溶酶体贮积减少。在某些实施方案中，与对照相比，溶酶体贮积减少约 5%、10%、15%、20%、25%、30%、35%、40%、45%、50%、55%、60%、65%、70%、75%、80%、85%、90%、95%、100% 或更多。在一些实施方案中，与对照相比，溶酶体贮积减少至少 1 倍、2 倍、3 倍、4 倍、5 倍、6 倍、7 倍、8 倍、9 倍或 10 倍。在一些实施方案中，通过 LAMP-1 染色确定溶酶体贮积。上述范围和值中间的范围和值也视为本发明的一部分。

[0233] 在一些实施方案中，治疗指神经元 (例如，含蒲肯野细胞的神经元) 中的液泡化减少。在某些实施方案中，与对照相比，神经元中的液泡化减少约 5%、10%、15%、20%、25%、30%、35%、40%、45%、50%、55%、60%、65%、70%、75%、80%、85%、90%、95%、100% 或更多。在一些实施方案中，与对照相比，液泡化减少至少 1 倍、2 倍、3 倍、4 倍、5 倍、6 倍、7 倍、8 倍、9 倍或 10 倍。上述范围和值中间的范围和值也视为本发明的一部分。

[0234] 在一些实施方案中，治疗指各种组织中的 NaGlu 酶活性增加。在一些实施方案中，治疗指脑部靶组织、脊髓神经元和 / 或外周靶组织中的 NaGlu 酶活性增加。在一些实施方案中，与对照相比，NaGlu 酶活性增加约 5%、10%、15%、20%、25%、30%、35%、40%、45%、50%、55%、60%、65%、70%、75%、80%、85%、90%、95%、100%、200%、300%、400%、500%、600%、700%、800%、900%、1000% 或更多。在一些实施方案中，与对照相比，NaGlu 酶活性增加至少 1 倍、2 倍、3 倍、4 倍、5 倍、6 倍、7 倍、8 倍、9 倍或 10 倍。在一些实施方案中，增加的 NaGlu 酶活性为至少约 10nmol/h/mg、20nmol/h/mg、40nmol/h/mg、50nmol/h/mg、60nmol/h/mg、70nmol/h/mg、80nmol/h/mg、90nmol/h/mg、100nmol/h/mg、150nmol/h/mg、200nmol/h/mg、250nmol/h/mg、300nmol/h/mg、350nmol/h/mg、400nmol/h/mg、450nmol/h/mg、500nmol/h/mg、550nmol/h/mg、600nmol/h/mg 或更多。在一些实施方案中，腰部中 NaGlu 酶活性增加。在一些实施方案中，腰部中增加的 NaGlu 酶活性为至少约 2000nmol/h/mg、3000nmol/h/mg、4000nmol/h/mg、5000nmol/h/mg、6000nmol/h/mg、7000nmol/h/mg、8000nmol/h/mg、9000nmol/h/mg、

10,000nmol/h/mg 或更多。上述范围和值中间的范围和值也视为本发明的一部分。

[0235] 在某些实施方案中，根据本发明的治疗导致减少（例如，减少约 5%、10%、15%、20%、25%、30%、40%、50%、55%、60%、65%、70%、75%、80%、90%、95%、97.5%、99% 或更多）或完全消除与 NaGlu 相关疾病相关的一种或多种病理或生物标志物的存在（或可选地积累）。此类减少或消除在 CNS 的细胞和组织（例如，神经元和少突胶质细胞）中可能特别显而易见。例如，在一些实施方案中，向受试者施用后，本发明的药物组合物展示或实现受试者的 CNS 细胞和组织（例如，在大脑皮质、小脑、尾状核和壳核、白质和 / 或丘脑）中，生物标志物溶酶体相关膜蛋白 1(LAMP1) 的积累减少。LAMP1 是在溶酶体膜中高度表达的糖蛋白并且其存在在患有溶酶体贮积症的许多患者中增加 (Meikle 等，Clin. Chem. (1997)43:1325-1335)。因此患有溶酶体贮积症的患者体内 LAMP1 的存在和缺乏（例如，通过 LAMP 染色确定）可提供有用的溶酶体活性指示及诊断和监测溶酶体贮积症的标志。

[0236] 相应地，本发明的一些实施方案涉及减少或消除与 NaGlu 相关疾病相关的一种或多种病理或生物标志的存在或积累的方法。类似地，本发明的一些实施方案涉及增加与溶酶体贮积症相关的一种或多种病理或生物标志（例如，LAMP1）的降解（降解速率）的方法。

[0237] 在一些实施方案中，治疗指减弱认知能力丧失的进展。在某些实施方案中，与对照相比，认知能力丧失的进展减弱约 5%、10%、15%、20%、25%、30%、35%、40%、45%、50%、55%、60%、65%、70%、75%、80%、85%、90%、95%、100% 或更多。在一些实施方案中，治疗指减弱发育延迟。在某些实施方案中，与对照相比，发育延迟减弱约 5%、10%、15%、20%、25%、30%、35%、40%、45%、50%、55%、60%、65%、70%、75%、80%、85%、90%、95%、100% 或更多。上述范围和值中间的范围和值也视为本发明的一部分。

[0238] 在一些实施方案中，治疗指存活率（例如，存活时间）增加。例如，治疗可导致患者的预期寿命增加。在一些实施方案中，与患有类似疾病、未经治疗的一个或多个对照个体的平均预期寿命相比，根据本发明的治疗导致患者的预期寿命增加约 5%、约 10%、约 15%、约 20%、约 25%、约 30%、约 35%、约 40%、约 45%、约 50%、约 55%、约 60%、约 65%、约 70%、约 75%、约 80%、约 85%、约 90%、约 95%、约 100%、约 105%、约 110%、约 115%、约 120%、约 125%、约 130%、约 135%、约 140%、约 145%、约 150%、约 155%、约 160%、约 165%、约 170%、约 175%、约 180%、约 185%、约 190%、约 195%、约 200% 或更多。在一些实施方案中，与患有类似疾病、未经治疗的一个或多个对照个体的平均预期寿命相比，根据本发明的治疗导致患者的预期寿命增加大于约 6 个月、约 7 个月、约 8 个月、约 9 个月、约 10 个月、约 11 个月、约 12 个月、约 2 年、约 3 年、约 4 年、约 5 年、约 6 年、约 7 年、约 8 年、约 9 年、约 10 年或更多。在一些实施方案中，根据本发明的治疗导致患者长期存活。如本文所使用，术语“长期存活”指存活时间或预期寿命长于约 40 年、45 年、50 年、55 年、60 年或更长。上述范围和值中间的范围和值也视为本发明的一部分。

[0239] 如本文所使用，术语“提高”、“增加”或“减少”指示相对于对照的值。在一些实施方案中，适合的对照为基线测量，例如在开始本文所述治疗之前对同一个体测量或在缺乏本文所述治疗时对对照个体（或多个对照个体）测量。“对照个体”是患有桑菲列浦氏综合征 B 型，年龄和 / 或性别与正在治疗的个体大致相同的个体（以确保受治个体和对照个体的疾病阶段可比）。

[0240] 正在治疗的个体（也称为“患者”或“受试者”）是患有桑菲列浦氏综合征 B 型或

有可能发展桑菲列浦氏综合征 B 型的个体（胎儿、婴儿、儿童、青少年或成年人）。个体可具有残留内源 NaGlu 表达和 / 或活性或没有可测量的活性。例如，患有桑菲列浦氏综合征 B 型的个体的 NaGlu 表达水平可能是正常 NaGlu 表达水平的小于约 30-50%、小于约 25-30%、小于约 20-25%、小于约 15-20%、小于约 10-15%、小于约 5-10%、小于约 0.1-5%。上述范围和值中间的范围和值也视为本发明的一部分。

[0241] 在一些实施方案中，个体是最近已经诊断出患有所述疾病的个体。通常，早期治疗（诊断后尽快开始治疗）对将疾病影响减到最低和将治疗益处增到最大很重要。

[0242] I. 联合疗法

[0243] 可单独或组合使用重组人 NaGlu 蛋白质，例如含足够量的寡糖（例如，甘露糖和磷酸化甘露糖（即，M6P））的重组人 NaGlu 蛋白质，治疗 NaGlu 相关疾病（例如，桑菲列浦氏综合征 B 型）。应理解，本发明的重组人 NaGlu 蛋白质可单独使用或与附加程序组合使用，例如手术程序或试剂（例如治疗剂），附加程序或试剂由技术人员选择用于其预期目的。例如，附加程序或试剂可为本领域公认对治疗用本发明的重组人 NaGlu 蛋白质治疗的疾病或病状有用的治疗程序或试剂。附加程序或试剂也可为赋予治疗组合物有益属性的试剂，例如，影响组合物粘度的试剂。

[0244] 还应理解，包括在本发明内的组合是对其预期目的有用的那些组合。下述试剂和程序是为了说明的目的而非旨在限于本发明。为本发明的一部分的组合可为本发明的重组人 NaGlu 蛋白质和选自下面列表的至少一种附加试剂或程序。如果组合是这样的，形成的组合物可执行其预期功能，则组合也可包括一种以上的附加试剂或程序，例如两种或三种附加试剂。

[0245] 联合疗法可包括手术程序、基因疗法或酶替代疗法。另外，重组人 NaGlu 蛋白质可与一种或多种附加治疗剂共配制，例如能够预防或减少未降解底物的积累的其它重组蛋白质或抗体或药物（例如，底物减少疗法）。

[0246] 在一个或多个实施方案中，联合疗法可包括与如以下更加详细描讨论的免疫抑制剂一起共施用。例如，如果患者预料到或经历了过敏反应或不良免疫反应，则在施用重组人蛋白质，例如重组人 NaGlu 蛋白质之前、期间或之后，也可施用免疫抑制剂例如但不限于抗组胺、皮质类固醇、西罗莫司 (sirolimus)、伏环孢素 (voclosporin)、环孢素 (ciclosporin)、甲氨蝶呤 (methotrexate)、IL-2 受体定向抗体、T 细胞受体定向抗体、TNF-α 定向抗体或融合蛋白（例如，英夫利昔 (infliximab)、依那西普 (etanercept) 或阿达木单抗 (adalimumab))、CTLA-4-Ig（例如，阿巴西普 (abatacept))、抗 OX-40 抗体。

[0247] J. 免疫原性

[0248] 本发明的药物组合物特征在于其耐受性。如本文所使用，术语“耐受的”和“耐受性”指本发明药物组合物在施用了此类组合物的受试者中不会引起不良反应，或可选地在施用了此类组合物的受试者中不会引起严重不良反应的能力。在一些实施方案中，施用了此类组合物的受试者良好耐受本发明的药物组合物。

[0249] 通常，施用根据本发明的 rhNaGlu 蛋白质不会在受试者中导致严重的副作用。如本文所使用，严重的副作用包括但不限于大量免疫反应、毒性或死亡。如本文所使用，术语“大量免疫反应”指严重或重度免疫反应，例如适应性 T 细胞免疫反应。

[0250] 因此，在许多实施方案中，根据本发明的发明方法不牵涉同步免疫抑制剂疗法

(即,用作预治疗/预处理或与所述方法并行的任何免疫抑制剂疗法)。在一些实施方案中,根据本发明的发明方法不牵涉对受治疗的受试者的免疫耐受诱导。在一些实施方案中,根据本发明的发明方法不牵涉使用T细胞免疫抑制剂对受试者的预治疗/预处理。

[0251] 然而,在一些实施方案中,受试者在施用本发明的rhNaGlu之后出现免疫反应。因此,在一些实施方案中,使接受本发明的rhNaGlu的受试者耐受酶替代疗法可能有用。可使用本领域已知的各种方法诱导免疫耐受。例如,可使用T细胞免疫抑制剂(例如环孢菌素A(CsA))和抗增殖剂(例如硫唑嘌呤(Aza))联合每周鞘内输注低剂量的所需替代酶的最初30-60天方案。

[0252] 技术人员已知的任何免疫抑制剂均可与本发明的联合疗法一起采用。此类免疫抑制剂包括但不限于环孢霉素(cyclosporine)、FK506、雷帕霉素(rapamycin)、CTLA4-Ig和抗TNF试剂(例如依那西普)(见例如,Moder,2000,Ann.Allergy Asthma Immunol.84,280-284;Nevins,2000,Curr.Opin.Pediatr.12,146-150;Kurlberg 等,2000,Scand.J.Immunol.51,224-230;Ideguchi 等,2000,Neuroscience95,217-226;Potter 等,1999,Ann.N.Y.Acad.Sci.875,159-174;Slavik 等,1999,Immunol.Res.19,1-24;Gaziev 等,1999,Bone Marrow Transplant.25,689-696;Henry,1999,Clin.Transplant.13,209-220;Gummert 等,1999,J.Am.Soc.Nephrol.10,1366-1380;Qi等,2000,Transplantation69,1275-1283)。已经在移植患者中证明有效的抗IL2受体(α-亚基)抗体达利珠单抗(daclizumab)(例如,Zenapax™)也可用作免疫抑制剂(见例如,Wiseman 等,1999,Drugs58,1029-1042;Beniaminovitz 等,2000,N.Engl J.Med.342,613-619;Ponticelli 等,1999,Drugs R.D.1,55-60;Berard 等,1999,Pharmacotherapy19,1127-1137;Eckhoff 等,2000,Transplantation69,1867-1872;Ekberg等,2000,Transpl.Int.13,151-159)。另外的免疫抑制剂包括但不限于抗CD2(Branco等,1999,Transplantation68,1588-1596;Przepiorka 等,1998,Blood92,4066-4071)、抗CD4(Marinova-Mutafchieva 等,2000,Arthritis Rheum.43,638-644;Fishwild等,1999,Clin.Immunol.92,138-152)和抗CD40配体(Hong 等,2000,Semin.Nephrol.20,108-125;Chirmule 等,2000,J.Virol.74,3345-3352;Ito 等,2000,J.Immunol.164,1230-1235)。

[0253] 在其它实施方案中,本发明包括以下方法,包括与降低或抑制对NaGlu蛋白质的免疫反应的试剂,例如免疫抑制剂一起共施用本发明的NaGlu蛋白质。例如,如果患者预料到或经历了过敏反应或不良免疫反应,则在施用重组人蛋白质,例如重组人NaGlu蛋白质之前、期间或之后,也可施用免疫抑制剂例如但不限于抗组胺、皮质类固醇、西罗莫司、伏环孢素、环孢素、甲氨蝶呤、IL-2受体定向抗体、T细胞受体定向抗体、TNF-α定向抗体或融合蛋白(例如,英夫利昔、依那西普或阿达木单抗)、CTLA-4-Ig(例如,阿巴西普)、抗OX-40抗体。

[0254] 在一个实施方案中,本发明提供了预治疗程序以最大程度减少或预防施用根据本发明的重组蛋白质可引起的任何潜在过敏反应。在一个实施方案中,为预防潜在过敏反应,向患者施用H-1受体拮抗剂,也称为抗组胺(例如,苯海拉明(diphenhydramine))。在一个实施方案中,按约1mg至约10mg/kg体重的剂量施用H-1受体拮抗剂。例如,按约5mg/kg的剂量施用抗组胺。在一个实施方案中,按介于约0.1mg和约10mg/kg体重的剂量施用抗

组胺。在一个实施方案中，按介于约 1mg 和约 5mg/kg 体重的剂量施用抗组胺。例如，剂量可为 1mg、2mg、3mg、4mg 或 5mg/kg 体重。可通过任何有用方法施用抗组胺。在一个实施方案中，静脉内施用抗组胺。在另一实施方案中，在药学上可接受的胶囊内施用抗组胺。

[0255] 可在施用根据本发明的重组 NaGlu 蛋白质之前施用抗组胺。在一个实施方案中，在施用重组 NaGlu 约 10 至约 90 分钟，例如约 30 至约 60 分钟之前施用 H-1 受体拮抗剂。可使用与血管入口连接的流动系统施用 H-1 受体拮抗剂。在一个实施方案中，在施用重组 NaGlu 约 90 分钟之前施用抗组胺。在一个实施方案中，在施用重组 NaGlu 约 10 至约 60 分钟之前施用抗组胺。在另一实施方案中，在施用重组 NaGlu 约 20 至约 40 分钟之前施用抗组胺。例如，在施用重组 NaGlu 前 20、25、30、35 或 40 分钟之前施用抗组胺。

[0256] 在一个实施方案中，施用的抗组胺是苯海拉明。可使用任何有用的抗组胺。此类抗组胺包括但不限于氯马斯丁 (clemastine)、抗敏安 (doxylamine)、克敏能 (loratidine)、地氯雷他定 (desloratidine)、非索非那定 (fexofenadine)、非尼拉敏 (pheniramine)、西替利嗪 (cetirizine)、依巴斯汀 (ebastine)、异丙嗪 (promethazine)、扑尔敏 (chlorpheniramine)、左西替利嗪 (levocetirizine)、奥洛他定 (olopatadine)、喹硫平 (quetiapine)、敏克静 (meclizine)、乘晕宁 (dimenhydrinate)、恩布拉敏 (embramine)、dimethidene 和右氯苯那敏 (dexchloropheniramine)。

[0257] 在另一实施方案中，关于静脉内输注，可通过使用爬坡方案 (ramp-up protocol) 施用输注来降低过敏反应的可能。关于这一点，爬坡方案指在输注过程中缓慢增加输注速率以便患者对药物输注脱敏。

[0258] K. 施用

[0259] 本发明的方法涵盖了单次以及多次施用治疗有效量的本文所述本发明的 rhNaGlu。可根据受试者病状的性质、严重程度和范围，每隔一定时间施用本发明的 rhNaGlu。在一些实施方案中，可每隔一定时间（例如，每年一次、每 6 个月一次、每 5 个月一次、每 3 个月一次、两月一次（每 2 个月一次）、每月一次（每个月一次）、两周一次（每 2 周一次）或每周一次），定期经静脉内或鞘内施用治疗有效量的本发明的 rhNaGlu 蛋白质。

[0260] 在一些实施方案中，可连同其它施用途径（例如，静脉内、皮下、肌肉内、肠胃外、经皮或透粘膜(例如，口服或鼻内)）一起使用鞘内施用。在一些实施方案中，可进行那些其它施用途径（例如，静脉内施用），不能比两周一次、每月一次、每 2 个月一次、每 3 个月一次、每 4 个月一次、每 5 个月一次、每 6 个月一次、一年一次施用更频繁。

[0261] 如本文所使用，术语“治疗有效量”主要基于本发明药物组合物中所含的治疗剂的总量确定。通常，治疗有效量足以对患者实现有意义的益处（例如，治疗、调节、治愈、预防和 / 或缓解原发疾病或病状）。例如，治疗有效量可能是足以实现期望治疗和 / 或预防效果的量，例如足以调节溶酶体酶受体或其活性，从而治疗此类溶酶体贮积病或其症状（例如，向受试者施用本发明的组合物后，减少或消除“斑马体 (zebra body)”或细胞液泡化的存在或发生率）的量。通常，向有需要的受试者施用的治疗剂（例如，本发明的 rhNaGlu) 的量将取决于受试者的特征。此类特征包括受试者的病状、疾病严重程度、总体健康状况、年龄、性别和体重。本领域的技术人员将能够容易地根据这些和其它相关因素确定适当的剂量。另外，可任选地采用客观和主观测定以鉴定最佳的剂量范围。

[0262] 通常在可包含多个单位剂量的给药方案中施用治疗有效量。对于任何特定的治疗

性蛋白质而言，治疗有效量（和/或有效给药方案中的适当单位剂量）可（例如）根据施用途径、根据与其它药物试剂的组合而改变。同样，对任何特定患者的具体治疗有效量（和/或单位剂量）可取决于多种因素，包括受治疗的病症和病症的严重程度；所采用的具体药物试剂的活性；所采用的具体组合物；患者的年龄、体重、总体健康状况、性别和饮食；施用时间、施用途径和/或采用的具体融合蛋白的排泄率或代谢率；治疗持续时间；和医学领域众所周知的相似因素。

[0263] 在一些实施方案中，治疗有效剂量范围从约0.005mg/kg体重到500mg/kg体重，例如从约0.005mg/kg体重到400mg/kg体重、从约0.005mg/kg体重到300mg/kg体重、从约0.005mg/kg体重到200mg/kg体重、从约0.005mg/kg体重到100mg/kg体重、从约0.005mg/kg体重到90mg/kg体重、从约0.005mg/kg体重到80mg/kg体重、从约0.005mg/kg体重到70mg/kg体重、从约0.005mg/kg体重到60mg/kg体重、从约0.005mg/kg体重到50mg/kg体重、从约0.005mg/kg体重到40mg/kg体重、从约0.005mg/kg体重到30mg/kg体重、从约0.005mg/kg体重到25mg/kg体重、从约0.005mg/kg体重到20mg/kg体重、从约0.005mg/kg体重到15mg/kg体重、从约0.005mg/kg体重到10mg/kg体重。上述范围和值中间的范围和值（例如，10-50mg/kg、1-5mg/kg、2-8mg/kg、5-10mg/kg、0.1-10mg/kg、0.3-30mg/kg、0.3-50mg/kg、0.5-10mg/kg、5-30mg/kg或6-27mg/kg）也视为本发明的一部分。

[0264] 在一些实施方案中，治疗有效剂量大于或至少约0.1mg/kg体重、大于或至少约0.2mg/kg体重、大于或至少约0.3mg/kg体重、大于或至少约0.4mg/kg体重、大于或至少约0.5mg/kg体重、大于或至少约1.0mg/kg体重、大于或至少约3mg/kg体重、大于或至少约5mg/kg体重、大于或至少约6mg/kg体重、大于或至少约7mg/kg体重、大于或至少约10mg/kg体重、大于或至少约15mg/kg体重、大于或至少约20mg/kg体重、大于或至少约30mg/kg体重、大于或至少约40mg/kg体重、大于或至少约50mg/kg体重、大于或至少约60mg/kg体重、大于或至少约70mg/kg体重、大于或至少约80mg/kg体重、大于或至少约90mg/kg体重、大于或至少约100mg/kg体重。上述范围和值中间的范围和值也视为本发明的一部分。

[0265] 在一些实施方案中，也可用mg/kg脑重定义治疗有效剂量。正如本领域技术人员将认识到那样，可将脑重和体重联系起来（见，例如，Dekaban AS."Changes in brain weights during the span of human life:relation of brain weights to body heights and body weights,"Ann Neurol 1978;4:345-56）。

[0266] 在一些实施方案中，也可用mg/15cc CSF定义治疗有效剂量。正如本领域技术人员将认识到那样，可将基于脑重和体重的治疗有效剂量转化为mg/15cc CSF。例如，成年人中CSF的体积为约150mL(Johanson CE等"Multiplicity of cerebrospinal fluid functions:New challenges in health and disease,"Cerebrospinal Fluid Res.2008年5月14日;5:10)。因此，向成人单剂量注射0.1mg至50mg蛋白质在成人中将为约0.01mg/15cc CSF(0.1mg)至5.0mg/15cc CSF(50mg)剂量。

[0267] 应进一步理解，对于任何特定受试者而言，应随时间推移根据个体需要和施用或监管酶替代疗法施用的人员的专业判断调整具体剂量方案，并且本文提出的剂量范围仅为示例性并非旨在限制要求保护的发明的范围或实践。

[0268] VIII.试剂盒

[0269] 本发明进一步提供了含有本发明的重组人NaGlu并且提供其复水（如果冻干）和

/ 或使用说明书的试剂盒或其它制品。试剂盒或其它制品可包括容器、导管及用于鞘内施用和相关手术的任何其它物品、装置或设备。适合的容器包括（例如）瓶子、小瓶、注射器（例如，预填充注射器）、安瓿、药筒、储器或二室注射器给药系统（lyo-ject）。容器可由多种材料形成，例如玻璃或塑料。在一些实施方案中，容器为预填充注射器。适合的预填充注射器包括但不限于有烘干硅酮涂层的硼硅玻璃注射器、有喷涂硅酮的硼硅玻璃注射器或没有硅酮的塑料树脂注射器。

[0270] 通常，容器上或容器随附的标签可指示使用和 / 或复水说明。例如，标签可指示制剂经复水成如上所述的蛋白质浓度。标签可进一步指示制剂用于或预期用于（例如）静脉内或鞘内施用。在一些实施方案中，容器可装有单剂量的含有替代酶（例如，重组 NaGlu 蛋白质）的稳定制剂。在各实施方案中，单剂量的稳定制剂以小于约 15mL、10mL、5.0mL、4.0mL、3.5mL、3.0mL、2.5mL、2.0mL、1.5mL、1.0mL 或 0.5mL 的体积存在。可选地，容纳制剂的容器可为多用小瓶，其允许重复施用（例如，施用 2-6 次）制剂。试剂盒或其它制品可进一步包括含适合稀释剂（例如，BWFI、盐水、缓冲盐水）的第二容器。稀释剂和制剂混合后，复水制剂中的最终蛋白质浓度将通常为至少 1mg/mL（例如，至少 5mg/mL、至少 10mg/mL、至少 25mg/mL、至少 50mg/mL、至少 75mg/mL、至少 100mg/mL）。

[0271] 试剂盒或其它制品可进一步包括从商业和用户角度看可取的其它材料，包括其它缓冲液、稀释剂、填料、针头、导管、注射器和带使用说明的包装说明书。上述范围和值中间的范围和值也视为本发明的一部分。

实施例

[0272] 下列具体实施例旨在说明本发明而不得视为限制本发明的范围。整篇申请中引用的所有图和所有参考文献、专利和公开专利申请的内容以及附图通过引用整体明确地并入本文。

[0273] 实施例 1

[0274] rhNaGlu 的纯化

[0275] 通过使用本领域已知的方法纯化 rhNaGlu 蛋白质。在 pH6 下使含有 rhNaGlu 的蛋清 (EW) 溶解过夜并通过离心和 / 或深层过滤澄清。用 1M NaOAc 缓冲液 (pH4) 将 EW 调节为 pH6。对于深层过滤过程，使用 T2600 过滤器 (Pall™，40um) 作为第一次过滤，然后使用 PDF1(Pall™，K200P，15um+EKS，0.22um) 作为第二次过滤步骤。过滤器是一次性使用膜，其中对于每个过滤器而言最佳容量为 60LEW/m^2。对 T2600 而言，膜的容量为 2L/m^2 而对 PDF1 而言，为 4-5L/m^2。在所述过程中，在收集过滤的 EW 之前丢弃容量。使用与膜容量相当的缓冲液 (20mM 磷酸盐 /137mM NaCl，pH6) 冲走留在过滤器上的 EW。

[0276] 应用苯基 -HIC（疏水相互作用色谱）柱作为捕获步骤。因为大部分蛋清蛋白为亲水性的，所以 99% 的蛋清蛋白通过 HIC 柱进入流过液 (flow through)。rhNaGlu 与苯基 -HIC 有较高的疏水性结合。

[0277] 按 30:1 的比例将含有 rhNaGlu 的蛋清上样柱中。完成上样后，用平衡缓冲液、5mM 磷酸盐缓冲液 (pH6) 和 5mM Tris 缓冲液 (pH7.2) 洗涤所述柱。用 30% 丙二醇 (pH7.2) 洗脱 rhNaGlu。完成上样后，用平衡缓冲液和 5mM 磷酸盐缓冲液 (pH6) 洗涤所述柱。用 30% 丙二醇和 5mM Tris 缓冲液 (pH7.2) 洗脱 rhNaGlu。柱结合容量约为 4.5mg/mL。通过苯基 -HIC

柱的 rhNaGlu 的纯度可达到 >95%(增加 950 倍)。用 30% 丙二醇洗脱,回收率约为 80%。

[0278] 用 1M 醋酸将洗脱的 rhNaGlu 组分调节为 pH5,然后上样到 GigaCap S 柱 (EW: 柱尺寸 =10:1) 上。用 50mM NaOAc 缓冲液 (pH5) 使所述柱平衡。完成上样后,用平衡缓冲液洗涤所述柱。用 50mMNaOAc/60mM NaCl(pH5) 洗脱 rhNaGlu。

[0279] 使用纯化的 rhNaGlu 进行蛋白质表征。在 SDS-PAGE 上分析从蛋清中纯化的 rhNaGlu 的分子量 (约 90kDa)(图 6)。图 7 中示出了蛋清中 rhNaGlu 的平均表达水平。表 2 中总结了从转基因禽类生成的 rhNaGlu 的特征。

[0280] 表 2.

[0281]

	rhNaGlu(原鸡)
表观分子量	约 90kDa
pI	6.1-6.9
pH 稳定性	pH5-8
在蛋清中的稳定性	>50 天

[0282] 实施例 2

[0283] rhNaGlu 在蛋清中的稳定性

[0284] 一个蛋在产下 7 天后破裂并分析其活性。将内容物分为两半并且使每一半都进行标准蛋清澄清。等分未经处理和经澄清的蛋清并储存在 4℃和 -20℃下以使酶活性稳定。蛋清中的 rhNaGlu 显示稳定酶活性至少长达 50 天。

[0285] 评估冻 / 融循环稳定性。纯化的 rhNaGlu 在液氮中冷冻 10 秒并且在 37℃下解冻 2min。10 次循环,酶活性显示无变化。

[0286] 将纯化的 rhNaGlu 透析到不同 pH 缓冲液中以测量纯酶的稳定性。结果显示,在 pH5-8 之间纯 rhNaGlu 稳定 12 天。

[0287] 实施例 3

[0288] 寡糖图谱

[0289] 甘露糖 -6- 磷酸 (M6P) 是 N- 连接寡糖的末端单糖,其是糖蛋白的三级结构的重要部分并且,在并入糖蛋白的最后一个寡糖中时,被细胞表面存在的 M6P 受体识别并结合,随后允许内化到溶酶体中。因此,M6P 是将糖蛋白靶向溶酶体的有效表位。

[0290] 对蛋白质糖基化的分析是糖蛋白表征的重要部分。寡糖可通过丝氨酸或苏氨酸与蛋白质连接呈 O- 连接聚糖或通过天冬酰胺连接呈 N- 连接聚糖。

[0291] 为分析寡糖的结构,进行了各种色谱和光谱技术。采用高效阴离子交换色谱法和脉冲安培检测 (HPAEC-PAD)。使用这种技术,基于电荷 (即,中性、带单电荷或带多电荷) 使寡糖迅速分成一般群并且通过与纯标准品比较确定其结构。

[0292] 所有方法均基于 Hardy 和 Townsend 描述的方案 (Hardy, M. R. and Townsend, R. R., "High-pH anion-exchange chromatography of glycoprotein-derived carbohydrates", 1994, Methods Enzymol. 230:208-225)。在 4℃下使用 Tube-O-Dialyzer,

用超纯水透析转基因禽类来源的 rhNaGlu 的纯化样品约 24 小时，以去除盐和其它污染物。整个透析过程中更换超纯水 4 次。透析后，将每个样品分为 3 个等分试样。在 100℃下用 2N 三氟乙酸 (TFA) 水解预期用于中性和氨基糖类分析的等分试样 4 小时并且在 100℃下用 6.75N TFA 水解用于甘露糖 -6- 磷酸分析的等分试样 1.5 小时。然后在 N2 下干燥水解产物，用 50μLH2O 重新溶解，于冰中超声处理 7min 并转移到注射小瓶中。

[0293] 以和样品相同的方式同时水解已知摩尔数的中性和氨基糖类及甘露糖 -6- 磷酸的标准品混合物。制备了 4 个不同浓度的中性和氨基糖标准品混合物及甘露糖 -6- 磷酸以建立校准方程式。通过线性插值由校准方程式量化样品中每种糖的摩尔数。

[0294] 分别用 HPAEC-PAD 分析寡糖图谱和甘露糖 -6- 磷酸图谱。使用 Dionex chromeleon 软件实现仪器控制和数据采集。对水解 rhNaGlu 的 HPAEC-PAD 分析检测到 M6P。M6P 的平均测得量为每 210μg 水解蛋白 3.8μg(CV3.7%)。转换为摩尔产生每 2.8nmol 蛋白质 13.4nmol 的 M6P，这与每摩尔蛋白质 3.2 摩尔 M6P 的比例相当。

[0295] 也使用 HPAEC-PAD 获得 rhNaGlu（原鸡）的寡糖图谱（见图 8）。图谱展示了单个样品上 PNG 酶 F 反应的良好重复性。在对应于中性寡糖的区域中观察到峰丛（约 10min 至约 20min）。也观察到在约 25 至约 35min 洗脱的一组明显更小的峰，这可能归因于带单电荷的种类。

[0296] 表 3 中总结了由转基因禽类（原鸡）生成的 rhNaGlu 样品获得的单糖组成分析结果，其列出了对 rhNaGlu 分析的每种单糖的平均摩尔比。

[0297] 表 3. rhNaGlu 中的单糖摩尔比（原鸡）

[0298]

N- 乙酰半乳糖胺 (GalNAc)	1.1*
N- 乙酰葡萄糖胺 (GlcNAc)	35.6*
半乳糖 (Gal)	4*
甘露糖 (Man)	25.5*
甘露糖 -6- 磷酸 (M6P)	3.2*
岩藻糖	未检测到
葡萄糖	未检测到

[0299] * 每摩尔蛋白质的单糖摩尔数

[0300] 实施例 4

[0301] 向成纤维细胞内的细胞摄取

[0302] 将野生型人成纤维细胞和粘多糖贮积病 III B 型 (NaGlu 缺陷型）人成纤维细胞置于 24 孔板 (2.5×10^4 个细胞 / 孔）并且在 37℃下于 5%CO_2 中孵育过夜。使用含成纤维细胞基础培养基的条件培养基和具有低血清的成纤维细胞生长试剂盒。在 37℃下用 5%CO_2 共同孵育不同量的 rhNaGlu(30、10、3.0、1.0、0.3 和 0μg/mL)24 小时以测定人成纤维细胞的细胞摄取水平（见图 9）。用 PBS 洗涤孔 3 次。每个孔加 100μL 裂解缓冲液并且在

37℃下孵育所述板 10min。将细胞裂解物转移到 1.5mL 离心管中。进行一个冻融循环。在 10,000rpm 下离心细胞裂解物 10min。使用 25μL 上清液进行测定。测定时间为 2 小时。使用本领域已知的方法并且根据 Marsh 等，Clinical Genetics(1985)27:258-262,Chow 等，Carbohydrate Research(1981)96:87-93 ;Weber 等，Protein Expression and Purification,(2001)21:251-259) 描述的方法测量酶活性。

[0303] 如图 9 所示，阴性对照（即，MPS IIIB) 不会表现出任何 NaGlu 活性，而阳性对照（即，野生型人成纤维细胞）显示出 NaGlu 活性。用 0.3μg/mL 的 rhNaGlu 处理的 MPS IIIB 细胞表现出在野生型成纤维细胞中观察到的正常活性水平的约 50%。用 1μg/mL 的 rhNaGlu 处理的 MPS IIIB 细胞展示出比在野生型细胞中观察到的高约 4 倍的 NaGlu 活性。令人惊讶的是，用 30μg/mL 的 rhNaGlu 处理的 MPS IIIB 细胞显示出比在野生型细胞中观察到的高至少 40 倍的 NaGlu 活性。该结果表明，由转基因禽类（原鸡）生成的 rhNaGlu 以高水平有效内化到人成纤维细胞中。

[0304] 为确定 rhNaGlu 的内化是否经由 M6P 受体介导的内吞作用，进行了 M6P 抑制测定。对于 M6P 抑制测定而言，向用 30μg/mL 的 rhNaGlu 处理的人 MPS IIIB 成纤维细胞添加不同浓度的游离 M6P 并且如上所述测量酶活性。如图 10 所示，人 MPS IIIB 成纤维细胞未表现出任何 NaGlu 活性，表明 NaGlu 摄取被游离 M6P 有效抑制。相反，在缺乏游离 M6P 时用 30μg/mL 的 rhNaGlu 处理的 MPSIII 成纤维细胞表现出高水平的酶活性，表明蛋白质有效内化到缺乏 NaGlu 的成纤维细胞中并且保持活性。这种酶活性受培养基中 0.03mM 和更高浓度的 M6P 单糖的存在抑制。条件培养基中 1mM M6P 单糖的存在抑制了 90% 以上的蛋白质的细胞摄取。

[0305] 这些结果表明，由转基因禽类生成的 rhNaGlu 经 M6P 受体介导的内吞作用有效内化到 MPS IIIB 成纤维细胞中并且 rhNaGlu 与 M6P 单糖竞争受体识别。结果与揭示由转基因禽类生成的 rhNaGlu 上存在 M6P 结构的聚糖分析一致。

[0306] 实施例 5

[0307] 活性 NaGlu 融合蛋白的生成

[0308] 设计两种不同的 rhNaGlu 融合构建体以验证在禽类表达系统中表达 rhNaGlu 融合蛋白的可行性。

[0309] 在一种构建体中，使用常规 PCR 和 DNA 重组技术使编码 8 个连续天冬氨酸残基 (DDDDDDDD) 的核酸序列与在全长 NaGlu cDNA 序列 (SEQ ID NO:2) 的 5’末端，编码 NaGlu 蛋白质的核酸序列融合。在另一种构建体中，使编码 TfRL 的核酸序列（即，THRPPMWSPVWP ; SEQ ID NO:5) 与在全长 NaGlu cDNA 序列的 3’末端，编码 NaGlu 的核酸序列融合。使用 EcoRI 和 HindIII 限制位点将所述每种构建体插入 pTT22 表达载体中。所得载体各自转染到人胚肾 (HEK)293 细胞中并且获得表达高水平的融合 NaGlu 蛋白质的稳定克隆。从条件培养基中分离出在 N 端与一段 8 个连续天冬氨酸残基融合的 rhNaGlu 蛋白质 (AAA-NaGlu) 及在 C 端与转铁蛋白受体配体 (TfRL) 融合的 rhNaGlu 蛋白质 (NaGlu-TfRL)。

[0310] 使用本领域已知的方法测量 AAA-NaGlu 和 NaGlu-TfRL 的酶活性（见，例如，Marsh 等，Clinical Genetics(1985)27:258-262 ;Chow 等，Carbohydrate Research,(1981)96:87-93 ;Weber 等，Protein Expression and Purification(2001)21:251-259 ;Neufeld 等，Protein Expression

and Purification(2000)19:202-211；和 Weber 等，Human Molecular Genetics(1996)5:771-777。

[0311] 如图 13 和 14 所示，由 HEK293 细胞生成的 AAA-NaGlu 和 NaGlu-TfRL 融合蛋白显示出高水平的酶活性。这些结果确认了这些构建体可用于生成具有更高水平的磷酸化甘露糖，同时保持来自转基因禽类表达系统的酶活性的 NaGlu 融合蛋白的可能性。

[0312] 实施例 6

[0313] 向巨噬细胞内的细胞摄取

[0314] 还测量了由原鸡生成的 rhNaGlu 向人巨噬细胞中的内化。在 37℃下用 5%CO_2，在 F12 生长培养基中使 NR8383 巨噬细胞与 10μg/mL 的 rhNaGlu 孵育 0、4、8、24、32 和 48 小时。裂解之前回收样品并用 PBS 洗涤。在 1mL 裂解缓冲液 (10mM 磷酸钠 (pH6.0)，0.05%NP40) 中裂解 2.5×10^5 个细胞，并且将裂解物转移到 1.5mL 离心管中并在 10,000rpm 下离心 10min。通过 Bradford 测定法测定蛋白质浓度并且冷冻等分试样以进行 NaGlu 酶测定。

[0315] 使用标准方法测量酶活性。在超纯水中将 25mM 的底物 (4- 甲基伞形酮基 2- 乙酰胺基 -2- 脱氧 -a-D- 吡喃葡萄糖苷）稀释为 2mM 以形成工作底物储液。在测定缓冲液 (1% 牛血清白蛋白）中制备样品的稀释液。将 25μL 的 200mM 醋酸钠分布到多孔板的孔中。向指定孔中添加 25μL 标准品和 25μL 样品。向每个孔添加 50μL 工作底物储液并且轻轻敲打所述板以混合。用粘性膜密封所述板并且在 37℃下孵育 30 分钟。然后通过添加 50μL 停止液 (1M 甘氨酸 pH12.5) 终止反应。使用荧光底部将所述板置于酶标仪上并且在 360nm 激发波长和 460nm 发射波长下测量强度。通过在 0.25mM、0.125mM、0.0625mM、0.0312mM、0.0156mM 和 0.0078mM 下，与 4-MU 标准品比较来测量释出的 4- 甲基伞形酮 (4-MU) 的水平。

[0316] 如图 15 所示，与 10μg/mL 的 rhNaGlu 孵育的巨噬细胞中 NaGlu 活性的水平在 48 小时内几乎呈线性增加：巨噬细胞摄取 rhNaGlu 相当缓慢，但是在测量的整个时间段内很稳定。相对缓慢、长期的 NaGlu 活性摄取（与在其糖基化结构中含有 M6P 和 / 或甘露糖的其它溶酶体酶相比）是出乎意料且令人惊讶的。同样令人惊讶且出乎意料的是，在更长时间段内有大量 rhNaGlu 蛋白质被吸收到巨噬细胞中，导致细胞内酶活性水平比在未暴露于 rhNaGlu 的野生型巨噬细胞中观察到的基础水平高至少 10、50、100、200、300、500 或甚至 1,000 倍。结果证明，rhNaGlu 在细胞外以及细胞内环境中极其稳定。进一步地，这些结果表明，rhNaGlu 可能具有在体内允许更长血清半衰期（例如，更长时间循环）和高血清浓度的物理化学特征，对增强向中枢神经系统 (CNS) 的摄取而言理想的性质。

[0317] 表 4. NaGlu 特征的总结

[0318]

	禽类(原鸡)生成的rhNaGlu	天然人NaGlu	CHO生成的人NaGlu
表观分子质量(kDa)	约85-约90	约86	约79-约89
酶活性(nmol/min/mg)	> 1,000	约500	约1,057
甘露糖-6-磷酸	高	高	没有或非常低

[0319] 实施例 7

[0320] 向缺少 NaGlu 的小鼠施用 rhNaGlu

[0321] 由繁殖配对的 B6.129S6-NaGlu^{tm1Efn}/J 菌株产生纯合子裸鼠。以相同方式产生对照野生型小鼠。根据标准 PCR 方案进行基因分型。在本领域中描述了，出生时虽然在所有组织中未展示出 NaGlu，但是纯合子 naglu$^{(-/-)}$ 裸鼠能活，大小正常，并未展现出任何显著的身体或行为异常（见，Li 等，(1999)Proc,Natl.Acad.Sci.USA96:14505-14510）。一月龄时，在大多数组织中发现液泡化巨噬细胞。肾脏中的表皮细胞和脑部一些部分中的神经元也受了影响。液泡化随年龄增长变得更加显著。在 4-5 个月时，小鼠在旷场试验中显示出行为异常。年长的动物可能有尿潴留和行走困难。纯合子 naglu$^{-/-}$ 裸鼠的通常寿命为 8-12 个月（见，Li 等，(1999)Proc,Natl.Acad.Sci.USA96:14505-14510）。

[0322] 静脉内 (IV) 施用

[0323] 如下所述完成试验物品和媒介物通过尾静脉注射的静脉内施用。注射之前，通过用白炽灯温热动物或将尾部浸入约 43℃的温水中实现血管舒张。然后将动物放在限制装置中。注射之前用 70% 异丙醇为尾部表面消毒。使尾部侧静脉正好位于皮肤下方并且在尾部远端部分施加张力鉴定。将 27G 斜面针头插入静脉 3-4mm。因为施用的液体瞬间占据血管间隙，如通过观察到静脉清除证明，然后试验物品或媒介物在 10 秒时期内作为缓慢团注施用。移出针头后，向穿刺部位施加轻微压力以供止血。在程序后立即监测动物以确保正常活动。

[0324] 鞘内 (IT) 施用

[0325] 如下所述完成试验物品和媒介物通过腰椎穿刺注射的鞘内施用。注射之前，使用在整个程序中经前锥体 (nose cone) 保持的异氟烷麻醉动物。必要时，在每次注射前，通过刮毛准备好注射部位。以伏卧位将动物放在平台上，确保后肢跨在平台上，形成动物背部的凸曲线。注射之前，用 70% 异丙醇擦洗背部表面并使其干燥。触摸脊柱和髋骨以定位 L4-L5 或 L5-L6 边缘。将斜面面向颅侧的 30G 针头插入椎间隙。通过观察尾部拍打确认放置。然后试验物品或媒介物作为推注施用。使动物从麻醉中恢复并且在程序后立即监测动物以确保肢体的正常活动和使用。

[0326] 结果

[0327] 经尾静脉注射 (IV 施用)，按 6.75 或 27mg/kg 的剂量水平为 12 周龄的 naglu$^{-/-}$ 小

鼠 (B6.129S6-NaGlu^{tm1Efn}/J) 施用 rhNaGlu(原鸡),每隔一天一次,总共 5 个剂量 (rhNaGlu 浓度分别为 1.125 或 4.5mg/mL)。类似地,经腰椎穿刺注射 (IT 施用) 按 0.31mg/kg 的剂量水平为 12 周龄的 naglu$^{-/-}$ 小鼠施用 rhNaGlu(原鸡),每隔一天一次,总共 5 个剂量,NaGlu 浓度为 1.54mg/mL。以相同剂量浓度每隔一天为 naglu$^{-/-}$ 敲除小鼠施用媒介物 (10mM 磷酸盐缓冲液、150mM NaCl 和 0.02% 吐温 80(Tween80),pH5.5-5.8),5 个剂量。在研究持续时间内还喂养了未经处理的野生型和 naglu$^{-/-}$ 敲除小鼠。

[0328] 在第 5 次和最后一次注射 4 小时后处死动物。对所有动物进行尸检并且切除肝脏、脑部、脾脏、心脏、肺部和肾脏。矢状分隔每个器官,从而提供冷冻 (-80℃) 和福尔马林固定储存的样品。

[0329] 分析组织样品:(1) 基于对硫酸乙酰肝素二糖的 SAX-HPLC 分析,使用分析方法分析硫酸乙酰肝素浓度;和 (2) 使用基于细胞的酶活性测定分析 α-N- 乙酰氨基葡萄糖苷酶酶活性。

[0330] 使用经福尔马林固定的组织样品,包埋在石蜡中、以 4μm 切片、固定在载玻片上并用苏木精和伊红 (H&E) 染色进行脑部、肝脏、肾脏、脾脏、心脏和肺部组织的组织病理学评估。

[0331] 按 6.25 和 27mg/kg 体重的剂量水平,向 naglu$^{-/-}$ 小鼠重复静脉施用 (10 天时期内 5 个剂量)rhNaGlu(原鸡) 后,naglu$^{-/-}$ 小鼠脑部、肝脏和肾脏内硫酸乙酰肝素的浓度存在明显的剂量依赖性降低 (表 5;图 16-18)。静脉内施用后在脑部和肝脏中,相对 α-N- 乙酰氨基葡萄糖苷酶活性增加 (表 6)。这些结果是出人意料且令人惊讶的,因为在经 IV 施用处理的 naglu$^{-/-}$ 小鼠的脑部观察到 NaGlu 酶活性和引起的底物清除,表明全身施用的 rhNaGlu(原鸡) 分布到 naglu$^{-/-}$ 小鼠的脑部并且甚至在血脑屏障 (BBB) 的存在下,有效产生功效。

[0332] 按 0.31mg/kg 的剂量水平,向 naglu$^{-/-}$ 小鼠鞘内施用 (10 天时期内 5 个剂量) rhNaGlu(原鸡) 后,naglu$^{-/-}$ 小鼠脑部内硫酸乙酰肝素的浓度降低 (表 5;图 19),表明 rhNaGlu(原鸡) 靶向脑部并且对减少 naglu$^{-/-}$ 小鼠脑部中积累的底物有效。

[0333] 表 5. 组织底物水平 (原鸡 rhNaGlu)

[0334]

组织	动物数量	基因型	处死时的年龄(周)	处理	剂量(mg/kg)	途径	硫酸乙酰肝素ug/mg组织	平均值	标准偏差
肾脏	253	野生型	4	na	-	-	0.1		
	155	野生型	12	na	-	-	0.045	0.0725	0.038891
	178	敲除型	12	na	-	-	1.882		

[0335]

	242	敲除型	4	na	-	-	1.687		
	145	敲除型	13	na	-	-	1.904		
	474	敲除型	13	媒介物	0	IV	1.501		
	479	敲除型	13	媒介物	0	IV	1.983		
	484	敲除型	13	媒介物	0	IV	1.839	1.799333	0.175908
	487	敲除型	13	rhNaGlu	6.25	IV	0.928		
	492	敲除型	13	rhNaGlu	6.25	IV	0.737	0.8325	0.135057
	481	敲除型	13	rhNaGlu	27	IV	0.591		
	485	敲除型	13	rhNaGlu	27	IV	0.311		
	490	敲除型	13	rhNaGlu	27	IV	0.585	0.495667	0.159954
	86	敲除型	15	媒介物	0	IT	2.105		
	91	敲除型	14	媒介物	0	IT	1.704	1.9045	0.28355
	94	敲除型	14	rhNaGlu	0.31	IT	1.324		
	101	敲除型	14	rhNaGlu	0.31	IT	2.233	1.7785	0.64276
肝脏	253	野生型	4	na	-	-	0.045		
	155	野生型	12	na	-	-	0.092	0.0685	0.033234
	243	野生型	4	na	-	-	0.045		
	178	敲除型	12	na	-	-	1.85		
	242	敲除型	4	na	-	-	2.263	2.0565	0.292035
	255	敲除型	4	na	-	-	1.85		
	474	敲除型	13	媒介物	0	IV	1.822		
	479	敲除型	13	媒介物	0	IV	1.981		
	484	敲除型	13	媒介物	0	IV	2.004	1.961667	0.165779
	487	敲除型	13	rhNaGlu	6.25	IV	0.748		
	492	敲除型	13	rhNaGlu	6.25	IV	0.444		
	504	敲除型	13	rhNaGlu	6.25	IV	0.494	0.562	0.163009
	481	敲除型	13	rhNaGlu	27	IV	0.491		
	485	敲除型	13	rhNaGlu	27	IV	0.172	0.3315	0.225567
脑部	253	野生型	4	na	-	-	0.021		
	155	野生型	12	na	-	-	0.013		
	243	野生型	4	na	-	-	0.014308		
	10	野生型	36	na	-	-	0.012649	0.015239	0.003906
	239	敲除型	4	na	-	-	0.095		
	178	敲除型	12	na	-	-	0.084		
	242	敲除型	4	na	-	-	0.099		
	255	敲除型	4	na	-	-	0.094538		
	165	敲除型	24	na	-	-	0.084015		
	474	敲除型	13	媒介物	0	IV	0.085447		
	479	敲除型	13	媒介物	0	IV	0.072		

[0336]

	484	敲除型	13	媒介物	0	IV	0.073	0.085875	0.009972
	487	敲除型	13	rhNaGlu	6.25	IV	0.045		
	492	敲除型	13	rhNaGlu	6.25	IV	0.044119		
	504	敲除型	13	rhNaGlu	6.25	IV	0.044	0.044373	0.000546
	481	敲除型	13	rhNaGlu	27	IV	0.017796		
	485	敲除型	13	rhNaGlu	27	IV	0.016668		
	490	敲除型	13	rhNaGlu	27	IV	0.028	0.020821	0.006242
	86	敲除型	15	媒介物	0	IT	0.094521		
	91	敲除型	14	媒介物	0	IT	0.072623	0.083572	0.015484
	94	敲除型	14	rhNaGlu	0.31	IT	0.038866		
	101	敲除型	14	rhNaGlu	0.31	IT	0.028229	0.033548	0.007521

[0337] na :不适用（小鼠未经处理）。

[0338] 表 6 :组织酶活性（原鸡 rhNaGlu ;U/ng 蛋白质）

[0339]

组织	动物数量	基因型	处死时的年龄(周)	处理	剂量(mg/kg)	途径	酶活性(U/ug蛋白质)
脑部	253	野生型	4	na	-	-	7.7
	178	敲除型	12	na	-	-	0
	474	敲除型	13	媒介物	0	IV	0
	479	敲除型	13	媒介物	0	IV	0
	484	敲除型	13	媒介物	0	IV	0.575
	487	敲除型	13	rhNaGlu	6.25	IV	10.58
	492	敲除型	13	rhNaGlu	6.25	IV	5.066666667
	504	敲除型	13	rhNaGlu	6.25	IV	4.033333333
	481	敲除型	13	rhNaglu	27	IV	87.91666667
	485	敲除型	13	rhNaGlu	27	IV	90.15
	490	敲除型	13	rhNaGlu	27	IV	17.35
肝脏	253	野生型	4	na	-	-	36.69
	178	敲除型	12	na	-	-	0
	474	敲除型	13	媒介物	0	IV	0
	479	敲除型	13	媒介物	0	IV	0
	484	敲除型	13	媒介物	0	IV	0
	487	敲除型	13	rhNaGlu	6.25	IV	512.92
	492	敲除型	13	rhNaGlu	6.25	IV	378.805
	504	敲除型	13	rhNaGlu	6.25	IV	607.9225
	481	敲除型	13	rhNaGlu	27	IV	659.6825
	485	敲除型	13	rhNaGlu	27	IV	654.2475
	490	敲除型	13	rhNaGlu	27	IV	677.8725

[0340] na :不适用（小鼠未经处理）。

[0341] ***

[0342] 以上说明中的每个实施例通过解释本发明，而非限制本发明的方式提供。事实上，对本领域技术人员显而易见的是，在不背离本发明范围或精神的前提下可对本发明进行各

种修改、组合、添加、删除和改变。例如，作为一个实施方案的一部分说明或描述的特征可用于另一实施方案中以产生又一实施方案。意图是，本发明涵盖此类修改、组合、添加、删除和改变。

[0343] 出于种种目的，本文引用的所有出版物、专利、专利申请、互联网网站和登记号／数据库序列（包括多核苷酸和多肽序列）据此通过引用整体并入，程度与特别且单独地指出将每个独立出版物、专利、专利申请、互联网网站或登记号／数据库序列通过引用这样并入相同。

[0001]

序列表

<110> 辛那杰瓦生物制药公司

<120> 重组人 NAGLU 蛋白质及其用途

<130> 121424-00520

<140> 尚未转让
<141> 2012-10-12

<150> US 61/546,248
<151> 2011-10-12

<160> 5

<170> PatentIn 版本 3.5

<210> 1
<211> 743
<212> PRT
<213> 人

<400> 1

Met Glu Ala Val Ala Val Ala Ala Ala Val Gly Val Leu Leu Leu Ala
1 5 10 15

Gly Ala Gly Gly Ala Ala Gly Asp Glu Ala Arg Glu Ala Ala Ala Val
20 25 30

Arg Ala Leu Val Ala Arg Leu Leu Gly Pro Gly Pro Ala Ala Asp Phe
35 40 45

Ser Val Ser Val Glu Arg Ala Leu Ala Ala Lys Pro Gly Leu Asp Thr
50 55 60

Tyr Ser Leu Gly Gly Gly Gly Ala Ala Arg Val Arg Val Arg Gly Ser
65 70 75 80

[0002]

Thr Gly Val Ala Ala Ala Ala Gly Leu His Arg Tyr Leu Arg Asp Phe
85 90 95

Cys Gly Cys His Val Ala Trp Ser Gly Ser Gln Leu Arg Leu Pro Arg
100 105 110

Pro Leu Pro Ala Val Pro Gly Glu Leu Thr Glu Ala Thr Pro Asn Arg
115 120 125

Tyr Arg Tyr Tyr Gln Asn Val Cys Thr Gln Ser Tyr Ser Phe Val Trp
130 135 140

Trp Asp Trp Ala Arg Trp Glu Arg Glu Ile Asp Trp Met Ala Leu Asn
145 150 155 160

Gly Ile Asn Leu Ala Leu Ala Trp Ser Gly Gln Glu Ala Ile Trp Gln
165 170 175

Arg Val Tyr Leu Ala Leu Gly Leu Thr Gln Ala Glu Ile Asn Glu Phe
180 185 190

Phe Thr Gly Pro Ala Phe Leu Ala Trp Gly Arg Met Gly Asn Leu His
195 200 205

Thr Trp Asp Gly Pro Leu Pro Pro Ser Trp His Ile Lys Gln Leu Tyr
210 215 220

Leu Gln His Arg Val Leu Asp Gln Met Arg Ser Phe Gly Met Thr Pro
225 230 235 240

Val Leu Pro Ala Phe Ala Gly His Val Pro Glu Ala Val Thr Arg Val
245 250 255

Phe Pro Gln Val Asn Val Thr Lys Met Gly Ser Trp Gly His Phe Asn

[0003]

260 265 270

Cys Ser Tyr Ser Cys Ser Phe Leu Leu Ala Pro Glu Asp Pro Ile Phe
275 280 285

Pro Ile Ile Gly Ser Leu Phe Leu Arg Glu Leu Ile Lys Glu Phe Gly
290 295 300

Thr Asp His Ile Tyr Gly Ala Asp Thr Phe Asn Glu Met Gln Pro Pro
305 310 315 320

Ser Ser Glu Pro Ser Tyr Leu Ala Ala Ala Thr Thr Ala Val Tyr Glu
325 330 335

Ala Met Thr Ala Val Asp Thr Glu Ala Val Trp Leu Leu Gln Gly Trp
340 345 350

Leu Phe Gln His Gln Pro Gln Phe Trp Gly Pro Ala Gln Ile Arg Ala
355 360 365

Val Leu Gly Ala Val Pro Arg Gly Arg Leu Leu Val Leu Asp Leu Phe
370 375 380

Ala Glu Ser Gln Pro Val Tyr Thr Arg Thr Ala Ser Phe Gln Gly Gln
385 390 395 400

Pro Phe Ile Trp Cys Met Leu His Asn Phe Gly Gly Asn His Gly Leu
405 410 415

Phe Gly Ala Leu Glu Ala Val Asn Gly Gly Pro Glu Ala Ala Arg Leu
420 425 430

Phe Pro Asn Ser Thr Met Val Gly Thr Gly Met Ala Pro Glu Gly Ile
435 440 445

[0004]

Ser Gln Asn Glu Val Val Tyr Ser Leu Met Ala Glu Leu Gly Trp Arg
450 455 460

Lys Asp Pro Val Pro Asp Leu Ala Ala Trp Val Thr Ser Phe Ala Ala
465 470 475 480

Arg Arg Tyr Gly Val Ser His Pro Asp Ala Gly Ala Ala Trp Arg Leu
485 490 495

Leu Leu Arg Ser Val Tyr Asn Cys Ser Gly Glu Ala Cys Arg Gly His
500 505 510

Asn Arg Ser Pro Leu Val Arg Arg Pro Ser Leu Gln Met Asn Thr Ser
515 520 525

Ile Trp Tyr Asn Arg Ser Asp Val Phe Glu Ala Trp Arg Leu Leu Leu
530 535 540

Thr Ser Ala Pro Ser Leu Ala Thr Ser Pro Ala Phe Arg Tyr Asp Leu
545 550 555 560

Leu Asp Leu Thr Arg Gln Ala Val Gln Glu Leu Val Ser Leu Tyr Tyr
565 570 575

Glu Glu Ala Arg Ser Ala Tyr Leu Ser Lys Glu Leu Ala Ser Leu Leu
580 585 590

Arg Ala Gly Gly Val Leu Ala Tyr Glu Leu Leu Pro Ala Leu Asp Glu
595 600 605

Val Leu Ala Ser Asp Ser Arg Phe Leu Leu Gly Ser Trp Leu Glu Gln
610 615 620

[0005]

Ala Arg Ala Ala Ala Val Ser Glu Ala Glu Ala Asp Phe Tyr Glu Gln
625 630 635 640

Asn Ser Arg Tyr Gln Leu Thr Leu Trp Gly Pro Glu Gly Asn Ile Leu
645 650 655

Asp Tyr Ala Asn Lys Gln Leu Ala Gly Leu Val Ala Asn Tyr Tyr Thr
660 665 670

Pro Arg Trp Arg Leu Phe Leu Glu Ala Leu Val Asp Ser Val Ala Gln
675 680 685

Gly Ile Pro Phe Gln Gln His Gln Phe Asp Lys Asn Val Phe Gln Leu
690 695 700

Glu Gln Ala Phe Val Leu Ser Lys Gln Arg Tyr Pro Ser Gln Pro Arg
705 710 715 720

Gly Asp Thr Val Asp Leu Ala Lys Lys Ile Phe Leu Lys Tyr Tyr Pro
725 730 735

Arg Trp Val Ala Gly Ser Trp
740

<210> 2
<211> 2234
<212> DNA
<213> 人

<400> 2
atggaggcgg tggcggtggc cgcggcggtg ggggtccttc tcctggccgg ggccgggggc 60

gcggcaggcg acgaggcccg ggaggcggcg gccgtgcggg cgctcgtggc ccggctgctg 120

gggccaggcc ccgcggccga cttctccgtg tcggtggagc gcgctctggc tgccaagccg 180

[0006]

ggcttggaca cctacagcct gggcggcggc ggcgcggcgc gcgtgcgggt gcgcggctcc 240

acgggcgtgg cagccgccgc ggggctgcac cgctacctgc gcgacttctg tggctgccac 300

gtggcctggt ccggctctca gctgcgcctg ccgcggccac tgccagccgt gccgggggag 360

ctgaccgagg ccacgcccaa caggtaccgc tattaccaga atgtgtgcac gcaaagctac 420

tctttcgtgt ggtgggactg ggcccggtgg gagcgagaga tagactggat ggcgctgaat 480

ggcatcaacc tggcactggc atggagcggc caggaggcca tctggcagcg ggtgtacctg 540

gccttgggcc tgacccaggc agagatcaat gagttcttta ctggtcctgc cttcttggca 600

tgggggcgaa tgggcaacct gcacacctgg gatggccccc tgccccctc ctggcacatc 660

aagcagcttt atctgcagca ccgggtcctg gaccagatgc gctccttcgg catgacccca 720

gtgctgcctg cattcgcggg gcatgttccc gaggctgtca ccagggtgtt ccctcaggtc 780

aatgtcacga agatgggcag ttggggccac tttaactgtt cctactcctg ctccttcctt 840

ctggctccgg aagaccccat attccccatc atcgggagcc tcttcttgcg agagctgatc 900

aaagagtttg gcacagacca catctatggg gccgacactt tcaatgagat gcagccacct 960

tcctcagagc cctcctatct tgccgcagcc accactgccg tctatgaggc catgactgca 1020

gtggatactg aggctgtgtg gctgctccaa ggctggctct tccagcacca gccgcagttc 1080

tgggggcccg cccagatcag ggctgtgctg ggagctgtgc cccgtggccg cctcctggtt 1140

ctggacctgt ttgctgagag ccagcctgtg tatacccgca ctgcctcctt ccaaggccag 1200

cccttcatct ggtgcatgct gcacaacttt gggggaaatc atggtctttt tggagccttg 1260

gaggccgtga acggaggccc agaagctgcc cgcctcttcc ccaactccac aatggtaggc 1320

acgggcatgg ccccgaggg catcagccag aacgaagtgg tctattccct catggctgag 1380

ctgggctggc gaaaggaccc agtgccagat ttggcagcct gggtgaccag ctttgccgcc 1440

cggcggtatg gggtctccca cccggacgca ggggcagcgt ggaggctact gctccggagt 1500

gtgtacaact gctccgggga ggcatgcagg ggccacaatc gtagcccgct ggtcaggcgg 1560

[0007]

ccgtccctac agatgaatac cagcatctgg tacaaccgat ctgatgtgtt tgaggcctgg 1620

cggctgctgc tcacatctgc tccctccctg gccaccagcc ccgccttccg ctacgacctg 1680

ctggacctca ctcggcaggc agtgcaggag ctggtcagct tgtattatga ggaggcaaga 1740

agcgcctatc tgagcaagga gctggcctcc ttgttgaggg ctggaggcgt cctggcctat 1800

gagctgctgc cggcactgga cgaggtgctg gctagtgaca gccgcttctt gctgggcagc 1860

tggctagagc aggcccgagc agcggcagtc agtgaggccg aggccgattt ctacgagcag 1920

aacagccgct accagctgac cttgtggggg ccagaaggca acatcctgga ctatgccaac 1980

aagcagctgg cggggttggt ggccaactac tacaccctc gctggcggct ttcctggag 2040

gcgctggttg acagtgtggc ccagggcatc cctttccaac agcaccagtt tgacaaaaat 2100

gtcttccaac tggagcaggc cttcgttctc agcaagcaga ggtaccccag ccagccgcga 2160

ggagacactg tggacctggc caagaagatc ttcctcaaat attacccccg ctgggtggcc 2220

ggctcttggt gatt 2234

<210> 3
<211> 1132
<212> DNA
<213> 鸡

<400> 3
gttaagtcct cagacttggc aaggagaatg tagatttcca cagtatatat gttttcacaa 60

aaggaaggag agaaacaaaa gaaaatggca ctgactaaac ttcagctagt ggtataggaa 120

agtaattctg cttaacagag attgcagtga tctctatgta tgtcctgaag aattatgttg 180

tacttttttc ccccatttt aaatcaaaca gtgctttaca gaggtcagaa tggtttcttt 240

actgtttgtc aattctatta tttcaataca gaacaatagc ttctataact gaaatatatt 300

tgctattgta tattatgatt gtccctcgaa ccatgaacac tcctccagct gaatttcaca 360

attcctctgt catctgccag gccattaagt tattcatgga agatctttga ggaacactgc 420

[0008]

aagttcatat cataaacaca tttgaaattg agtattgttt tgcattgtat ggagctatgt 480

tttgctgtat cctcagaata aaagtttgtt ataaagcatt cacacccata aaaagataga 540

tttaaatatt ccaactatag gaaagaaagt gtgtctgctc ttcactctag tctcagttgg 600

ctccttcaca tgcacgcttc tttatttctc ctattttgtc aagaaaataa taggtcaagt 660

cttgttctca tttatgtcct gtctagcgtg gctcagatgc acattgtaca tacaagaagg 720

atcaaatgaa acagacttct ggtctgttac tacaaccata gtaataagca cactaactaa 780

taattgctaa ttatgttttc catctccaag gttcccacat ttttctgttt tcttaaagat 840

cccattatct ggttgtaact gaagctcaat ggaacatgag caatatttcc cagtcttctc 900

tcccatccaa cagtcctgat ggattagcag aacaggcaga aaacacattg ttacccagaa 960

ttaaaaacta atatttgctc tccattcaat ccaaaatgga cctattgaaa ctaaaatcta 1020

acccaatccc attaaaatgat ttctatggtg tcaaaggtca aacttctgaa gggaacctgt 1080

gggtgggtca caattcagac tatatattcc ccagggctca gccagtgtct gt 1132

<210> 4
<211> 10256
<212> DNA
<213> 人工序列

<220>
<223> pSIN-OV-1.1-I-rhNaGlu

<400> 4
ggccgcaaga agaaagctga aaaactctgt cccttccaac aagacccaga gcactgtagt 60

atcaggggta aaatgaaaag tatgttatct gctgcatcca gacttcataa aagctggagc 120

ttaattcaga aaaaaaatca gaaagaaatt acactgtgag aacaggtgca attcactttt 180

cctttacaca gagtaatact ggtaactcat ggatgaaggc ttaagggaat gaaattggac 240

tcacagtact gagtcatcac actgaaaaat gcaacctgat acatcagcag aaggtttatg 300

[0009]

ggggaaaaat gcagccttcc aattaagcca gatatctgta tgaccaagct gctccagaat 360

tagtcactca aaatctctca gattaaatta tcaactgtca ccaaccattc ctatgctgac 420

aaggcaattg cttgttctct gtgttcctga tactacaagg ctcttcctga cttcctaaag 480

atgcattata aaaatcttat aattcacatt tctccctaaa ctttgactca atcatggtat 540

gttggcaaat atggtatatt actattcaaa ttgttttcct tgtacccata tgtaatgggt 600

cttgtgaatg tgctcttttg ttcctttaat cataataaaa acatgtttaa gcaaacactt 660

ttcacttgta gtatttgaag gtaccggatc tcgagccgcc ttcaatgccc ccaaaaccaa 720

tccccaggtt tttaactctc ccgatttttcc aagtaccata gcccgctgag agagcgccgc 780

ggtaatggga tcccaggacc ccggggaata taagtctgag ggggacgtaa gcaacccttc 840

cttttgtaac agggacaaca tagcccctat ttccttctta gaaggagagg ttttcccgca 900

ataggtctta cacgcggacg aaatcacctt tatgacggct tccatgcttg atccaccggg 960

cgaccggaat cacgcagagc aaccggaatc acgcctgggg tggaccgctc agtcgtcggg 1020

cttccttccc gtcttccaac gactctctga gttctcggta gggtatgttg gccccctgca 1080

gtagggctcc ctccgacgcc actcagcttc tgccctccta agccgcagcc ccctctacta 1140

gggtcatcgt ccgctccccg aataagcgag acggatgagg acaggatcgc cacgccgcct 1200

gtggccgacc actattccct aacgatcacg tcggggtcac caaatgaagc cttctgcttc 1260

atgcatgtgc tcgtagtcgt cagggaatca acggtccggc catcaaccca ggtgcacacc 1320

aatgtggtga atggtcaaat ggcgtttatt gtatcgagct aggcacttaa atacaatatc 1380

tctgcaatgc ggaattcagt ggttcgtcca atccgtgtta gacccgtctg ttgccttcct 1440

aacaaggcac gatcatacca cgatcatacc accttactcc caccaatcgg catgcacggt 1500

gctttttctc tccttataag gcatgttgct aactcatcgt tacataagca tgttgcaaga 1560

ctacaagagt attgcataag actacatttc cccctcccta tgcaaaagcg aaactactat 1620

atcctgaggg gactcctaac cgcgtacaac cgaagccccg cttttcgcct aaacatgcta 1680

[0010]

ttgtccctc agtcaagcct tgcccgttac aacccgattc gcaagccttg ccctccccac 1740

attatccgta gcattatttc ctagcagtca tcagagctac agaagatact ctatgctgta 1800

gccaagtcta caagtttact attcagcgac ctcctatatt ccgcgtgcca gccgatcaat 1860

taccaatgcg cgcttggcgt aatcatggtc atagctgttt cctgtgtgaa attgttatcc 1920

gctcacaatt ccacacaaca tacgagccgg aagcataaag tgtaaagcct ggggtgccta 1980

atgagtgagc taactcacat taattgcgtt gcgctcactg cccgctttcc agtcgggaaa 2040

cctgtcgtgc cagctgcatt aatgaatcgg ccaacgcgcg gggagaggcg gtttgcgtat 2100

tgggcgctct tccgcttcct cgctcactga ctcgctgcgc tcggtcgttc ggctgcggcg 2160

agcggtatca gctcactcaa aggcggtaat acggttatcc acagaatcag gggataacgc 2220

aggaaagaac atgtgagcaa aaggccagca aaaggccagg aaccgtaaaa aggccgcgtt 2280

gctggcgttt ttccataggc tccgcccccc tgacgagcat cacaaaaatc gacgctcaag 2340

tcagaggtgg cgaaacccga caggactata aagataccag gcgtttcccc ctggaagctc 2400

cctcgtgcgc tctcctgttc cgaccctgcc gcttaccgga tacctgtccg cctttctccc 2460

ttcgggaagc gtggcgcttt ctcatagctc acgctgtagg tatctcagtt cggtgtaggt 2520

cgttcgctcc aagctgggct gtgtgcacga acccccccgtt cagcccgacc gctgcgcctt 2580

atccggtaac tatcgtcttg agtccaaccc ggtaagacac gacttatcgc cactggcagc 2640

agccactggt aacaggatta gcagagcgag gtatgtaggc ggtgctacag agttcttgaa 2700

gtggtggcct aactacggct acactagaag gacagtattt ggtatctgcg ctctgctgaa 2760

gccagttacc ttcggaaaaa gagttggtag ctcttgatcc ggcaaacaaa ccaccgctgg 2820

tagcggtggt ttttttgttt gcaagcagca gattacgcgc agaaaaaaag gatctcaaga 2880

agatcctttg atcttttcta cggggtctga cgctcagtgg aacgaaaact cacgttaagg 2940

gattttggtc atgagattat caaaaaggat cttcacctag atccttttaa attaaaaatg 3000

[0011]

aagttttaaa tcaatctaaa gtatatatga gtaaacttgg tctgacagtt accaatgctt 3060

aatcagtgag gcacctatct cagcgatctg tctatttcgt tcatccatag ttgcctgact 3120

ccccgtcgtg tagataacta cgatacggga gggcttacca tctggcccca gtgctgcaat 3180

gataccgcga gacccacgct caccggctcc agatttatca gcaataaacc agccagccgg 3240

aagggccgag cgcagaagtg gtcctgcaac tttatccgcc tccatccagt ctattaattg 3300

ttgccgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttgccat 3360

tgctacaggc atcgtggtgt cacgctcgtc gtttggtatg gcttcattca gctccggttc 3420

ccaacgatca aggcgagtta catgatcccc catgttgtgc aaaaaagcgg ttagctcctt 3480

cggtcctccg atcgttgtca gaagtaagtt ggccgcagtg ttatcactca tggttatggc 3540

agcactgcat aattctctta ctgtcatgcc atccgtaaga tgcttttctg tgactggtga 3600

gtactcaacc aagtcattct gagaatagtg tatgcggcga ccgagttgct cttgcccggc 3660

gtcaatacgg gataataccg cgccacatag cagaacttta aaagtgctca tcattggaaa 3720

acgttcttcg gggcgaaaac tctcaaggat cttaccgctg ttgagatcca gttcgatgta 3780

acccactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg 3840

agcaaaaaca ggaaggcaaa atgccgcaaa aaagggaata agggcgacac ggaaatgttg 3900

aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcagggtt attgtctcat 3960

gagcggatac atatttgaat gtatttagaa aaataaacaa atagggggtc cgcgcacatt 4020

tccccgaaaa gtgccacctg acgcgccctg tagcggcgca ttaagcgcgg cgggtgtggt 4080

ggttacgcgc agcgtgaccg ctacacttgc cagcgcccta gcgcccgctc ctttcgcttt 4140

cttcccttcc tttctcgcca cgttcgccgg ctttccccgt caagctctaa atcgggggct 4200

ccctttaggg ttccgattta gtgctttacg gcacctcgac cccaaaaaac ttgattaggg 4260

tgatggttca cgtagtgggc catcgccctg atagacggtt tttcgccctt tgacgttgga 4320

gtccacgttc tttaatagtg gactcttgtt ccaaactgga acaacactca accctatctc 4380

[0012]

ggtctattct tttgatttat aagggatttt gccgatttcg gcctattggt taaaaaatga 4440

gctgatttaa caaaaattta acgcgaattt taacaaaata ttaacgctta caatttccat 4500

tcgccattca ggctgcgcaa ctgttgggaa gggcgatcgg tgcgggcctc ttcgctatta 4560

cgccagctgg cgaaaggggg atgtgctgca aggcgattaa gttgggtaac gccagggttt 4620

tcccagtcac gacgttgtaa aacgacggcc agtgagcgcg tattccctaa cgatcacgtc 4680

ggggtcacca aatgaagcct tctgcttcat gcatgtgctc gtagtcgtca gggaatcaac 4740

ggtccggcca tcaacccagg tgcacaccaa tgtggtgaat ggtcaaatgg cgtttattgt 4800

atcgagctag gcacttaaat acaatatctc tgcaatgcgg aattcagtgg ttcgtccaat 4860

ccgtcccccc ccctatgcaa aagcgaaact actatatcct gaggggactc ctaaccgcgt 4920

acaaccgaag ccccgctttt cgcctaaaca tgctattgtc ccctcagtca agccttgccc 4980

gttacaaccc gattcgcaag ccttgccctc cccacattat ccgtagcatt atttcctagc 5040

agtcatcaga gctacagaag atactctatg ctgtagccaa gtctacaagt ttactattca 5100

gcgacctcct atattccgcg tgccagccga tcaattacca atccaaccag ctatcacacg 5160

gaatacaaga actcgcctac gctcttcttt cgggctgctt ataagcctcc tgtaattttt 5220

ttatattcct cgttaagtcc tcagacttgg caaggagaat gtagatttcc acagtatata 5280

tgttttcaca aaaggaagga gagaaacaaa agaaaatggc actgactaaa cttcagctag 5340

tggtatagga aagtaattct gcttaacaga gattgcagtg atctctatgt atgtcctgaa 5400

gaattatgtt gtacttttct cccccatttt taaatcaaac agtgctttac agaggtcaga 5460

atggtttctt tactgtttgt caattctatt atttcaatac agaacaatag cttctataac 5520

tgaaatatat ttgctattgt atattatgat tgtccctcga accatgaaca ctcctccagc 5580

tgaatttcac aattcctctg tcatctgcca ggccattaag ttattcatgg aagatctttg 5640

aggaacactg caagttcata tcataaacac atttgaaatt gagtattgtt ttgcattgta 5700

[0013]

tggagctatg ttttgctgta tcctcagaat aaaagtttgt tataaagcat tcacacccat 5760

aaaaagatag atttaaatat tccaactata ggaaagaaag tgtgtctgct cttcactcta 5820

gtctcagttg gctccttcac atgcacgctt ctttatttct cctattttgt caagaaaata 5880

ataggtcaag tcttgttctc atttatgtcc tgtctagcgt ggctcagatg cacattgtac 5940

atacaagaag gatcaaatga aacagacttc tggtctgtta ctacaaccat agtaataagc 6000

acactaacta ataattgcta attatgtttt ccatctccaa ggttcccaca tttttctgtt 6060

ttcttaaaga tcccattatc tggttgtaac tgaagctcaa tggaacatga gcaatatttc 6120

ccagtcttct ctcccatcca acagtcctga tggattagca gaacaggcag aaaacacatt 6180

gttacccaga attaaaaact aatatttgct ctccattcaa tccaaaatgg acctattgaa 6240

actaaaatct aacccaatcc cattaaatga tttctatggt gtcaaaggtc aaacttctga 6300

agggaacctg tgggtgggtc acaattcaga ctatatattc cccagggctc agccagtgtc 6360

tgtacataca gctagaaagc tgtattgcct ttagcagtca agctcgaaag gtaagcaact 6420

ctctggaatt accttctctc tatattagct cttacttgca cctaaacttt aaaaaattaa 6480

caattattgt gctatgtgtt gtatctttaa gggtgaagta cctgcgtgat acccccctata 6540

aaaacttctc acctgtgtat gcattctgca ctattttatt atgtgtaaaa gctttgtgtt 6600

tgttttcagg aggcttattc tttgtgctta aaatatgttt ttaatttcag aacatcttat 6660

cctgtcgttc actatctgat atgctttgca gtttgcttga ttaacttcta gccctacaga 6720

gtgcacagag agcaaaatca tggtgttcag tgaattctgg ggagttattt taatgtgaaa 6780

attctctaga agtttaattc ctgcaaagtg cagctgctga tcactacaca agataaaaat 6840

gtgggggtg cataaacgta tattcttaca ataatagata catgtgaact tatatacaga 6900

aaagaaaatg agaaaaatgt gtgtgtgtat actcacacac gtggtcagta aaaacttttg 6960

aggggtttaa tacagaaaat ccaatcctga ggccccagca ctcagtacgc atataaaggg 7020

ctgggctctg aaggacttct gactttcaca gattatataa atctcaggaa agcaactaga 7080

[0014]

ttcatgctgg ctccaaaagc tgtgctttat ataagcacac tggctataca atagttgtac 7140

agttcagctc tttataatag aaacagacag aacaagtata aatcttctat tggtctatgt 7200

catgaacaag aattcattca gtggctctgt tttatagtaa acattgctat tttatcatgt 7260

ctgcatttct cttctgtctg aatgtcacca ctaaaattta actccacaga aagtttatac 7320

tacagtacac atgcatatct ttgagcaaag caaaccatac ctgaaagtgc aatagagcag 7380

aatatgaatt acatgcgtgt ctttctccta gactacatga ccccatataa attacattcc 7440

ttatctattc tgccatcacc aaaacaaagg taaaaatact tttgaagatc tactcatagc 7500

aagtagtgtg caacaaacag atatttctct acatttattt ttagggaata aaaataagaa 7560

ataaaatagt cagcaagcct ctgctttctc atatatctgt ccaaacctaa agtttactga 7620

aatttgctct ttgaatttcc agttttgcaa gcctatcaga ttgtgttttt atcagaggta 7680

ctgaaaagta tcaatgaatt ctagctttca ctgaacaaaa atatgtagag gcaactggct 7740

tctgggacag tttgctaccc aaaagacaac tgaatgcaaa tacataaata gatttatgaa 7800

tatggttttg aacatgcaca tgagaggtgg atatagcaac agacacatta ccacagaatt 7860

actttaaaac tacttgttaa catttaattg cctaaaaact gctcgtaatt tactgttgta 7920

gcctaccata gagtaccctg catggtacta tgtacagcat tccatcctta cattttcact 7980

gttctgctgt ttgctctaga caactcagag ttcaccatgg aggcggtggc ggtggccgcg 8040

gcggtggggg tccttctcct ggccggggcc gggggcgcgg caggcgacga ggcccgggag 8100

gcggcggccg tgcgggcgct cgtggcccgg ctgctggggc caggccccgc ggccgacttc 8160

tccgtgtcgg tggagcgcgc tctggctgcc aagccgggct tggacaccta cagcctgggc 8220

ggcggcggcg cggcgcgcgt gcgggtgcgc ggctccacgg gcgtggcagc cgccgcgggg 8280

ctgcaccgct acctgcgcga cttctgtggc tgccacgtgg cctggtccgg ctctcagctg 8340

cgcctgccgc ggccactgcc agccgtgccg ggggagctga ccgaggccac gcccaacagg 8400

[0015]

taccgctatt accagaatgt gtgcacgcaa agctactctt tcgtgtggtg ggactgggcc 8460

cggtgggagc gagagataga ctggatggcg ctgaatggca tcaacctggc actggcatgg 8520

agcggccagg aggccatctg gcagcgggtg tacctggcct tgggcctgac ccaggcagag 8580

atcaatgagt tctttactgg tcctgccttc ttggcatggg ggcgaatggg caacctgcac 8640

acctgggatg gccccctgcc ccctcctgg cacatcaagc agctttatct gcagcaccgg 8700

gtcctggacc agatgcgctc cttcggcatg accccagtgc tgcctgcatt cgcggggcat 8760

gttcccgagg ctgtcaccag ggtgttccct caggtcaatg tcacgaagat gggcagttgg 8820

ggccactita actgttccta ctcctgctcc ttccttctgg ctccggaaga ccccatattc 8880

cccatcatcg ggagcctctt cttgcgagag ctgatcaaag agtttggcac agaccacatc 8940

tatggggccg acactttcaa tgagatgcag ccaccttcct cagagccctc ctatcttgcc 9000

gcagccacca ctgccgtcta tgaggccatg actgcagtgg atactgaggc tgtgtggctg 9060

ctccaaggct ggctcttcca gcaccagccg cagttctggg ggcccgccca gatcagggct 9120

gtgctgggag ctgtgccccg tggccgcctc ctggttctgg acctgtttgc tgagagccag 9180

cctgtgtata cccgcactgc ctccttccaa ggccagccct tcatctggtg catgctgcac 9240

aactttgggg gaaatcatgg tcttttgga gccttggagg ccgtgaacgg aggcccagaa 9300

gctgcccgcc tcttccccaa ctccacaatg gtaggcacgg gcatggcccc cgagggcatc 9360

agccagaacg aagtggtcta ttccctcatg gctgagctgg gctggcgaaa ggacccagtg 9420

ccagatttgg cagcctgggt gaccagcttt gccgcccggc ggtatggggt ctcccacccg 9480

gacgcagggg cagcgtggag gctactgctc cggagtgtgt acaactgctc cggggaggca 9540

tgcaggggcc acaatcgtag cccgctggtc aggcggccgt ccctacagat gaataccagc 9600

atctggtaca accgatctga tgtgtttgag gcctggcggc tgctgctcac atctgctccc 9660

tccctggcca ccagccccgc cttccgctac gacctgctgg acctcactcg gcaggcagtg 9720

caggagctgg tcagcttgta ttatgaggag gcaagaagcg cctatctgag caaggagctg 9780

[0016]

gcctccttgt tgagggctgg aggcgtcctg gcctatgagc tgctgccggc actggacgag 9840

gtgctggcta gtgacagccg cttcttgctg ggcagctggc tagagcaggc ccgagcagcg 9900

gcagtcagtg aggccgaggc cgatttctac gagcagaaca gccgctacca gctgaccttg 9960

tgggggccag aaggcaacat cctggactat gccaacaagc agctggcggg gttggtggcc 10020

aactactaca cccctcgctg gcggcttttc ctggaggcgc tggttgacag tgtggcccag 10080

ggcatccctt tccaacagca ccagtttgac aaaaatgtct tccaactgga gcaggccttc 10140

gttctcagca agcagaggta ccccagccag ccgcgaggag acactgtgga cctggccaag 10200

aagatcttcc tcaaatatta cccccgctgg gtggccggct cttggtgatt cgaagc 10256

<210> 5
<211> 12
<212> PRT
<213> 人工序列

<220>
<223> 转铁蛋白受体配体(TfRL)

<400> 5

Thr His Arg Pro Pro Met Trp Ser Pro Val Trp Pro
1 5 10

人NaGlu氨基酸序列(信号肽:1-23，加下划线)

```
MEAVAVAAAV GVLLLAGAGG AAGDEAREAA AVRALVARLL GPGPAADFSV SVERALAAKP      60
GLDTYSLGGG GAARVRVRGS TGVAAAAGLH RYLRDFCGCH VAWSGSQLRL PRPLPAVPGE     120
LTEATPNRYR YYQNVCTQSY SFVWWDWARW EREIDWMALN GINLALAWSG QEAIWQRVYL     180
ALGLTQAEIN EFFTGPAFLA WGRMGNLHTW DGPLPPSWHI KQLYLQHRVL DQMRSFGMTP     240
VLPAFAGHVP EAVTRVFPQV NVTKMGSWGH FNCSYSCSFL LAPEDPIFPI IGSLFLRELI     300
KEFGTDHIYG ADTFNEMQPP SSEPSYLAAA TTAVYEAMTA VDTEAVWLLQ GWLFQHQPQF     360
WGPAQIRAVL GAVPRGRLLV LDLFAESQPV YTRTASFQGQ PFIWCMLHNF GGNHGLFGAL     420
EAVNGGPEAA RLFPNSTMVG TGMAPEGISQ NEVVYSLMAE LGWRKDPVPD LAAWVTSFAA     480
RRYGVSHPDA GAAWRLLLRS VYNCSGEACR GHNRSPLVRR PSLQMNTSIW YNRSDVFEAW     540
RLLLTSAPSL ATSPAFRYDL LDLTRQAVQE LVSLYYEEAR SAYLSKELAS LLRAGGVLAY     600
ELLPALDEVL ASDSRFLLGS WLEQARAAAV SEAEADFYEQ NSRYQLTLWG PEGNILDYAN     660
KQLAGLVANY YTPRWRLFLE ALVDSVAQGI PFQQHQFDKN VFQLEQAFVL SKQRYPSQPR     720
GDTVDLAKKI FLKYYPRWVA GSW                                             743
```

(SEQ ID NO:1)

图 1

人 NaGlu 编码序列 (cDNA)

```
atggaggcgg tggcggtggc cgcggcggtg ggggtccttc tcctggccgg ggccgggggc      60
gcggcaggcg acgaggcccg ggaggcggcg gccgtgcggg cgctcgtggc ccggctgctg     120
gggccaggcc ccgcggccga cttctccgtg tcggtggagc gcgctctggc tgccaagccg     180
ggcttggaca cctacagcct gggcggcggc ggcgcggcgc gcgtgcgggt gcgcggctcc     240
acgggcgtgg cagccgccgc ggggctgcac cgctacctgc gcgacttctg tggctgccac     300
gtggcctggt ccggctctca gctgcgcctg ccgcggccac tgccagccgt gccgggggag     360
ctgaccgagg ccacgcccaa caggtaccgc tattaccaga atgtgtgcac gcaaagctac     420
tctttcgtgt ggtgggactg ggcccggtgg gagcgagaga tagactggat ggcgctgaat     480
ggcatcaacc tggcactggc atggagcggc caggaggcca tctggcagcg ggtgtacctg     540
gccttgggcc tgacccaggc agagatcaat gagttcttta ctggtcctgc cttcttggca     600
tgggggcgaa tgggcaacct gcacacctgg gatggccccc tgccccctc ctggcacatc      660
aagcagcttt atctgcagca ccgggtcctg gaccagatgc gctccttcgg catgacccca     720
gtgctgcctg cattcgcggg gcatgttccc gaggctgtca ccagggtgtt ccctcaggtc     780
aatgtcacga agatgggcag ttggggccac tttaactgtt cctactcctg ctccttcctt     840
ctggctccgg aagaccccat attccccatc atcgggagcc tcttcttgcg agagctgatc     900
aaagagtttg gcacagacca catctatggg gccgacactt tcaatgagat gcagccacct     960
tcctcagagc cctcctatct tgccgcagcc accactgccg tctatgaggc catgactgca    1020
gtggatactg aggctgtgtg gctgctccaa ggctggctct tccagcacca gccgcagttc    1080
tgggggcccg cccagatcag ggctgtgctg ggagctgtgc cccgtggccg cctcctggtt    1140
ctggacctgt ttgctgagag ccagcctgtg tatacccgca ctgcctcctt ccaaggccag    1200
cccttcatct ggtgcatgct gcacaacttt gggggaaatc atggtctttt tggagccttg    1260
gaggccgtga acggaggccc agaagctgcc cgcctcttcc ccaactccac aatggtaggc    1320
acgggcatgg cccccgaggg catcagccag aacgaagtgg tctattccct catggctgag    1380
ctgggctggc gaaaggaccc agtgccagat ttggcagcct gggtgaccag ctttgccgcc    1440
cggcggtatg gggtctccca cccggacgca ggggcagcgt ggaggctact gctccggagt    1500
gtgtacaact gctccgggga ggcatgcagg ggccacaatc gtagcccgct ggtcaggcgg    1560
ccgtccctac agatgaatac cagcatctgg tacaaccgat ctgatgtgtt tgaggcctgg    1620
cggctgctgc tcacatctgc tccctccctg gccaccagcc ccgccttccg ctacgacctg    1680
ctggacctca ctcggcaggc agtgcaggag ctggtcagct tgtattatga ggaggcaaga    1740
agcgcctatc tgagcaagga gctggcctcc ttgttgaggg ctggaggcgt cctggcctat    1800
gagctgctgc cggcactgga cgaggtgctg gctagtgaca gccgcttctt gctgggcagc    1860
tggctagagc aggcccgagc agcggcagtc agtgaggccg aggccgattt ctacgagcag    1920
aacagccgct accagctgac cttgtggggg ccagaaggca acatcctgga ctatgccaac    1980
aagcagctgg cggggttggt ggccaactac tacacccctc gctggcggct ttcctggag     2040
gcgctggttg acagtgtggc ccagggcatc cctttccaac agcaccagtt tgacaaaaat    2100
gtcttccaac tggagcaggc cttcgttctc agcaagcaga ggtaccccag ccagccgcga    2160
ggagacactg tggacctggc caagaagatc ttcctcaaat attacccccg ctgggtggcc    2220
ggctcttggt gatt                                                      2234
```

(SEQ ID NO:2)

图 2

1.1kb OV 启动子

```
gttaagtcct cagacttggc aaggagaatg tagatttcca cagtatatat gttttcacaa      60
aaggaaggag agaaacaaaa gaaaatggca ctgactaaac ttcagctagt ggtataggaa     120
agtaattctg cttaacagag attgcagtga tctctatgta tgtcctgaag aattatgttg     180
tacttttttc ccccattttt aaatcaaaca gtgctttaca gaggtcagaa tggtttcttt     240
actgtttgtc aattctatta tttcaataca gaacaatagc ttctataact gaaatatatt     300
tgctattgta tattatgatt gtccctcgaa ccatgaacac tcctccagct gaatttcaca     360
attcctctgt catctgccag gccattaagt tattcatgga agatctttga ggaacactgc     420
aagttcatat cataaacaca tttgaaattg agtattgttt tgcattgtat ggagctatgt     480
tttgctgtat cctcagaata aaagtttgtt ataaagcatt cacacccata aaaagataga     540
tttaaatatt ccaactatag gaaagaaagt gtgtctgctc ttcactctag tctcagttgg     600
ctccttcaca tgcacgcttc tttatttctc ctattttgtc aagaaaataa taggtcaagt     660
cttgttctca tttatgtcct gtctagcgtg gctcagatgc acattgtaca tacaagaagg     720
atcaaatgaa acagacttct ggtctgttac tacaaccata gtaataagca cactaactaa     780
taattgctaa ttatgttttc catctccaag gttcccacat ttttctgttt tcttaaagat     840
cccattatct ggttgtaact gaagctcaat ggaacatgag caatatttcc cagtcttctc     900
tcccatccaa cagtcctgat ggattagcag aacaggcaga aaacacattg ttacccagaa     960
ttaaaaacta atatttgctc tccattcaat ccaaaatgga cctattgaaa ctaaaatcta    1020
acccaatccc attaaatgat ttctatggtg tcaaaggtca aacttctgaa gggaacctgt    1080
gggtgggtca caattcagac tatatattcc ccagggctca gccagtgtct gt             1132
```

{SEQ ID NO:3 }

图 3

pSIN-OV-1.1-I-rhNaGlu

```
ggccgcaaga agaaagctga aaaactctgt cccttccaac aagacccaga gcactgtagt      60
atcaggggta aaatgaaaag tatgttatct gctgcatcca gacttcataa aagctggagc     120
ttaattcaga aaaaaaatca gaaagaaatt acactgtgag aacaggtgca attcactttt     180
cctttacaca gagtaatact ggtaactcat ggatgaaggc ttaagggaat gaaattggac     240
tcacagtact gagtcatcac actgaaaaat gcaacctgat acatcagcag aaggtttatg     300
ggggaaaaat gcagccttcc aattaagcca gatatctgta tgaccaagct gctccagaat     360
tagtcactca aaatctctca gattaaatta tcaactgtca ccaaccattc ctatgctgac     420
aaggcaattg cttgttctct gtgttcctga tactacaagg ctcttcctga cttcctaaag     480
atgcattata aaaatcttat aattcacatt tctccctaaa ctttgactca atcatggtat     540
gttggcaaat atggtatatt actattcaaa ttgttttcct tgtacccata tgtaatgggt     600
cttgtgaatg tgctcttttg ttcctttaat cataataaaa acatgtttaa gcaaacactt     660
ttcacttgta gtatttgaag gtaccggatc tcgagccgcc ttcaatgccc ccaaaaccaa     720
tccccaggtt tttaactctc ccgattttcc aagtaccata gcccgctgag agagcgccgc     780
ggtaatggga tcccaggacc ccggggaata taagtctgag ggggacgtaa gcaacccttc     840
cttttgtaac agggacaaca tagccccctat ttccttctta gaaggagagg ttttcccgca     900
ataggtctta cacgcggacg aaatcacctt tatgacggct tccatgcttg atccaccggg     960
cgaccggaat cacgcagagc aaccggaatc acgcctgggg tggaccgctc agtcgtcggg    1020
cttccttccc gtcttccaac gactctctga gttctcggta gggtatgttg gccccctgca    1080
gtagggctcc ctccgacgcc actcagcttc tgccctccta agccgcagcc ccctctacta    1140
gggtcatcgt ccgctccccg aataagcgag acggatgagg acaggatcgc cacgccgcct    1200
gtggccgacc actattccct aacgatcacg tcggggtcac caaatgaagc cttctgcttc    1260
atgcatgtgc tcgtagtcgt cagggaatca acggtccggc catcaaccca ggtgcacacc    1320
aatgtggtga atggtcaaat ggcgtttatt gtatcgagct aggcacttaa atacaatatc    1380
tctgcaatgc ggaattcagt ggttcgtcca atccgtgtta gacccgtctg ttgccttcct    1440
aacaaggcac gatcatacca cgatcatacc accttactcc caccaatcgg catgcacggt    1500
gcttttttctc tccttataag gcatgttgct aactcatcgt tacataagca tgttgcaaga    1560
ctacaagagt attgcataag actacatttc cccctcccta tgcaaaagcg aaactactat    1620
atcctgaggg gactcctaac cgcgtacaac cgaagccccg cttttcgcct aaacatgcta    1680
ttgtcccctc agtcaagcct tgcccgttac aacccgattc gcaagccttg ccctccccac    1740
attatccgta gcattatttc ctagcagtca tcagagctac agaagatact ctatgctgta    1800
gccaagtcta caagtttact attcagcgac ctcctatatt ccgcgtgcca gccgatcaat    1860
taccaatgcg cgcttggcgt aatcatggtc atagctgttt cctgtgtgaa attgttatcc    1920
gctcacaatt ccacacaaca tacgagccgg aagcataaag tgtaaagcct ggggtgccta    1980
atgagtgagc taactcacat taattgcgtt gcgctcactg cccgctttcc agtcgggaaa    2040
cctgtcgtgc cagctgcatt aatgaatcgg ccaacgcgcg gggagaggcg gtttgcgtat    2100
tgggcgctct tccgcttcct cgctcactga ctcgctgcgc tcggtcgttc ggctgcggcg    2160
```

图 4A

```
agcggtatca gctcactcaa aggcggtaat acggttatcc acagaatcag gggataacgc  2220
aggaaagaac atgtgagcaa aaggccagca aaaggccagg aaccgtaaaa aggccgcgtt  2280
gctggcgttt ttccataggc tccgcccccc tgacgagcat cacaaaaatc gacgctcaag  2340
tcagaggtgg cgaaacccga caggactata aagataccag gcgtttcccc ctggaagctc  2400
cctcgtgcgc tctcctgttc cgaccctgcc gcttaccgga tacctgtccg cctttctccc  2460
ttcgggaagc gtggcgcttt ctcatagctc acgctgtagg tatctcagtt cggtgtaggt  2520
cgttcgctcc aagctgggct gtgtgcacga accccccgtt cagcccgacc gctgcgcctt  2580
atccggtaac tatcgtcttg agtccaaccc ggtaagacac gacttatcgc cactggcagc  2640
agccactggt aacaggatta gcagagcgag gtatgtaggc ggtgctacag agttcttgaa  2700
gtggtggcct aactacggct acactagaag gacagtattt ggtatctgcg ctctgctgaa  2760
gccagttacc ttcggaaaaa gagttggtag ctcttgatcc ggcaaacaaa ccaccgctgg  2820
tagcggtggt tttttgtttg gcaagcagca gattacgcgc agaaaaaaag gatctcaaga  2880
agatcctttg atcttttcta cggggtctga cgctcagtgg aacgaaaact cacgttaagg  2940
gattttggtc atgagattat caaaaaggat cttcacctag atccttttaa attaaaaatg  3000
aagttttaaa tcaatctaaa gtatatatga gtaaacttgg tctgacagtt accaatgctt  3060
aatcagtgag gcacctatct cagcgatctg tctatttcgt tcatccatag ttgcctgact  3120
ccccgtcgtg tagataacta cgatacggga gggcttacca tctggccccа gtgctgcaat  3180
gataccgcga gacccacgct caccggctcc agatttatca gcaataaacc agccagccgg  3240
aagggccgag cgcagaagtg gtcctgcaac tttatccgcc tccatccagt ctattaattg  3300
ttgccgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttgccat  3360
tgctacaggc atcgtggtgt cacgctcgtc gtttggtatg gcttcattca gctccggttc  3420
ccaacgatca aggcgagtta catgatcccc catgttgtgc aaaaaagcgg ttagctcctt  3480
cggtcctccg atcgttgtca gaagtaagtt ggccgcagtg ttatcactca tggttatggc  3540
agcactgcat aattctctta ctgtcatgcc atccgtaaga tgcttttctg tgactggtga  3600
gtactcaacc aagtcattct gagaatagtg tatgcggcga ccgagttgct cttgcccggc  3660
gtcaatacgg gataataccg cgccacatag cagaacttta aaagtgctca tcattggaaa  3720
acgttcttcg gggcgaaaac tctcaaggat cttaccgctg ttgagatcca gttcgatgta  3780
acccactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg  3840
agcaaaaaca ggaaggcaaa atgccgcaaa aaagggaata agggcgacac ggaaatgttg  3900
aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcagggtt attgtctcat  3960
gagcggatac atatttgaat gtatttagaa aaataaacaa ataggggttc cgcgcacatt  4020
tccccgaaaa gtgccacctg acgcgccctg tagcggcgca ttaagcgcgg cgggtgtggt  4080
ggttacgcgc agcgtgaccg ctacacttgc cagcgcccta gcgcccgctc ctttcgcttt  4140
cttcccttcc tttctcgcca cgttcgccgg ctttccccgt caagctctaa atcgggggct  4200
ccctttaggg ttccgattta gtgctttacg gcacctcgac cccaaaaaac ttgattaggg  4260
tgatggttca cgtagtgggc catcgccctg atagacggtt tttcgccctt tgacgttgga  4320
gtccacgttc tttaatagtg gactcttgtt ccaaactgga acaacactca accctatctc  4380
ggtctattct tttgatttat aagggatttt gccgatttcg gcctattggt taaaaaatga  4440
gctgatttaa caaaaattta acgcgaattt taacaaaata ttaacgctta caatttccat  4500
tcgccattca ggctgcgcaa ctgttgggaa gggcgatcgg tgcgggcctc ttcgctatta  4560
cgccagctgg cgaaaggggg atgtgctgca aggcgattaa gttgggtaac gccagggttt  4620
tcccagtcac gacgttgtaa aacgacggcc agtgagcgcg tattcctaa cgatcacgtc  4680
ggggtcacca aatgaagcct tctgcttcat gcatgtgctc gtagtcgtca gggaatcaac  4740
ggtccggcca tcaacccagg tgcacaccaa tgtggtgaat ggtcaaatgg cgtttattgt  4800
atcgagctag gcacttaaat acaatatctc tgcaatgcgg aattcagtgg ttcgtccaat  4860
ccgtcccccc ccctatgcaa aagcgaaact actatatcct gaggggactc ctaaccgcgt  4920
acaaccgaag ccccgctttt cgcctaaaca tgctattgtc ccctcagtca agccttgccc  4980
gttacaaccc gattcgcaag ccttgccctc cccacattat ccgtagcatt atttcctagc  5040
agtcatcaga gctacagaag atactctatg ctgtagccaa gtctacaagt ttactattca  5100
```

图 4B

```
gcgacctcct atattccgcg tgccagccga tcaattacca atccaaccag ctatcacacg 5160
gaatacaaga actcgcctac gctcttcttt cgggctgctt ataagcctcc tgtaattttt 5220
ttatattcct cgttaagtcc tcagacttgg caaggagaat gtagatttcc acagtatata 5280
tgttttcaca aaaggaagga gagaaacaaa agaaaatggc actgactaaa cttcagctag 5340
tggtatagga aagtaattct gcttaacaga gattgcagtg atctctatgt atgtcctgaa 5400
gaattatgtt gtacttttct cccccatttt taaatcaaac agtgctttac agaggtcaga 5460
atggtttctt tactgtttgt caattctatt atttcaatac agaacaatag cttctataac 5520
tgaaatatat ttgctattgt atattatgat tgtccctcga accatgaaca ctcctccagc 5580
tgaatttcac aattcctctg tcatctgcca ggccattaag ttattcatgg aagatctttg 5640
aggaacactg caagttcata tcataaacac atttgaaatt gagtattgtt ttgcattgta 5700
tggagctatg ttttgctgta tcctcagaat aaaagtttgt tataaagcat tcacacccat 5760
aaaaagatag atttaaatat tccaactata ggaaagaaag tgtgtctgct cttcactcta 5820
gtctcagttg gctccttcac atgcacgctt ctttatttct cctattttgt caagaaaata 5880
ataggtcaag tcttgttctc atttatgtcc tgtctagcgt ggctcagatg cacattgtac 5940
atacaagaag gatcaaatga aacagacttc tggtctgtta ctacaaccat agtaataagc 6000
acactaacta ataattgcta attatgtttt ccatctccaa ggttcccaca tttttctgtt 6060
ttcttaaaga tcccattatc tggttgtaac tgaagctcaa tggaacatga gcaatatttc 6120
ccagtcttct ctcccatcca acagtcctga tggattagca gaacaggcag aaaacacatt 6180
gttacccaga attaaaaact aatatttgct ctccattcaa tccaaaatgg acctattgaa 6240
actaaaatct aacccaatcc cattaaatga tttctatggt gtcaaaggtc aaacttctga 6300
agggaacctg tgggtgggtc acaattcaga ctatatattc cccagggctc agccagtgtc 6360
tgtacataca gctagaaagc tgtattgcct ttagcagtca agctcgaaag gtaagcaact 6420
ctctggaatt accttctctc tatattagct cttacttgca cctaaacttt aaaaaattaa 6480
caattattgt gctatgtgtt gtatctttaa gggtgaagta cctgcgtgat acccccctata 6540
aaaacttctc acctgtgtat gcattctgca ctattttatt atgtgtaaaa gctttgtgtt 6600
tgttttcagg aggcttattc tttgtgctta aaatatgttt ttaatttcag aacatcttat 6660
cctgtcgttc actatctgat atgctttgca gtttgcttga ttaacttcta gccctacaga 6720
gtgcacagag agcaaaatca tggtgttcag tgaattctgg ggagttattt taatgtgaaa 6780
attctctaga agtttaattc ctgcaaagtg cagctgctga tcactacaca agataaaaat 6840
gtgggggtg cataaacgta tattcttaca ataatagata catgtgaact tatatacaga 6900
aaagaaaatg agaaaaatgt gtgtgtgtat actcacacac gtggtcagta aaaacttttg 6960
aggggtttaa tacagaaaat ccaatcctga ggccccagca ctcagtacgc atataaaggg 7020
ctgggctctg aaggacttct gactttcaca gattatataa atctcaggaa agcaactaga 7080
ttcatgctgg ctccaaaagc tgtgctttat ataagcacac tggctataca atagttgtac 7140
agttcagctc tttataatag aaacagacag aacaagtata aatcttctat tggtctatgt 7200
catgaacaag aattcattca gtggctctgt tttatagtaa acattgctat tttatcatgt 7260
ctgcatttct cttctgtctg aatgtcacca ctaaaattta actccacaga aagtttatac 7320
tacagtacac atgcatatct ttgagcaaag caaaccatac ctgaaagtgc aatagagcag 7380
aatatgaatt acatgcgtgt ctttctccta gactacatga ccccatataa attacattcc 7440
ttatctattc tgccatcacc aaaacaaagg taaaaatact tttgaagatc tactcatagc 7500
aagtagtgtg caacaaacag atatttctct acatttattt ttagggaata aaaataagaa 7560
ataaaatagt cagcaagcct ctgctttctc atatatctgt ccaaacctaa agtttactga 7620
aatttgctct ttgaatttcc agttttgcaa gcctatcaga ttgtgtttta atcagaggta 7680
ctgaaaagta tcaatgaatt ctagctttca ctgaacaaaa atatgtagag gcaactggct 7740
tctgggacag tttgctaccc aaaagacaac tgaatgcaaa tacataaata gatttatgaa 7800
tatggttttg aacatgcaca tgagaggtgg atatagcaac agacacatta ccacagaatt 7860
actttaaaac tacttgttaa catttaattg cctaaaaact gctcgtaatt tactgttgta 7920
gcctaccata gagtaccctg catggtacta tgtacagcat tccatcctta cattttcact 7980
```

图 4C

```
gttctgctgt ttgctctaga caactcagag ttcaccatgg aggcggtggc ggtggccgcg      8040
gcggtggggg tccttctcct ggccggggcc gggggcgcgg caggcgacga ggcccgggag      8100
gcggcggccg tgcgggcgct cgtggcccgg ctgctggggc caggccccgc ggccgacttc      8160
tccgtgtcgg tggagcgcgc tctggctgcc aagccgggct tggacaccta cagcctgggc      8220
ggcggcggcg cggcgcgcgt gcgggtgcgc ggctccacgg gcgtggcagc cgccgcgggg      8280
ctgcaccgct acctgcgcga cttctgtggc tgccacgtgg cctggtccgg ctctcagctg      8340
cgcctgccgc ggccactgcc agccgtgccg ggggagctga ccgaggccac gcccaacagg      8400
taccgctatt accagaatgt gtgcacgcaa agctactctt tcgtgtggtg ggactgggcc      8460
cggtgggagc gagagataga ctggatggcg ctgaatggca tcaacctggc actggcatgg      8520
agcggccagg aggccatctg gcagcgggtg tacctggcct tgggcctgac ccaggcagag      8580
atcaatgagt tctttactgg tcctgccttc ttggcatggg ggcgaatggg caacctgcac      8640
acctgggatg gcccccctgcc ccctcctgg cacatcaagc agctttatct gcagcaccgg      8700
gtcctggacc agatgcgctc cttcggcatg accccagtgc tgcctgcatt cgcggggcat      8760
gttcccgagg ctgtcaccag ggtgttccct caggtcaatg tcacgaagat gggcagttgg      8820
ggccacttta actgttccta ctcctgctcc ttccttctgg ctccggaaga ccccatattc      8880
cccatcatcg ggagcctctt cttgcgagag ctgatcaaag agtttggcac agaccacatc      8940
tatggggccg acactttcaa tgagatgcag ccaccttcct cagagccctc ctatcttgcc      9000
gcagccacca ctgccgtcta tgaggccatg actgcagtgg atactgaggc tgtgtggctg      9060
ctccaaggct ggctcttcca gcaccagccg cagttctggg ggcccgccca gatcagggct      9120
gtgctgggag ctgtgccccg tggccgcctc ctggttctgg acctgtttgc tgagagccag      9180
cctgtgtata cccgcactgc ctccttccaa ggccagccct tcatctggtg catgctgcac      9240
aactttgggg gaaatcatgg tcttttgga gccttggagg ccgtgaacgg aggcccagaa      9300
gctgcccgcc tcttccccaa ctccacaatg gtaggcacgg gcatggcccc cgagggcatc      9360
agccagaacg aagtggtcta ttccctcatg gctgagctgg gctggcgaaa ggacccagtg      9420
ccagatttgg cagcctgggt gaccagcttt gccgcccggc ggtatggggt ctcccacccg      9480
gacgcagggg cagcgtggag gctactgctc cggagtgtgt acaactgctc cggggaggca      9540
tgcaggggcc acaatcgtag cccgctggtc aggcggccgt ccctacagat gaataccagc      9600
atctggtaca accgatctga tgtgtttgag gcctggcggc tgctgctcac atctgctccc      9660
tccctggcca ccagccccgc cttccgctac gacctgctgg acctcactcg gcaggcagtg      9720
caggagctgg tcagcttgta ttatgaggag gcaagaagcg cctatctgag caaggagctg      9780
gcctccttgt tgagggctgg aggcgtcctg gcctatgagc tgctgccggc actggacgag      9840
gtgctggcta gtgacagccg cttcttgctg ggcagctggc tagagcaggc ccgagcagcg      9900
gcagtcagtg aggccgaggc cgatttctac gagcagaaca gccgctacca gctgaccttg      9960
tggggccag aaggcaacat cctggactat gccaacaagc agctggcggg gttggtggcc     10020
aactactaca cccctcgctg gcggcttttc ctggaggcgc tggttgacag tgtggcccag     10080
ggcatccctt tccaacagca ccagtttgac aaaaatgtct tccaactgga gcaggccttc     10140
gttctcagca agcagaggta ccccagccag ccgcgaggag acactgtgga cctggccaag     10200
aagatcttcc tcaaatatta cccccgctgg gtggccggct cttggtgatt cgaagc          10256
```

(SEQ ID NO:4)

图 4D

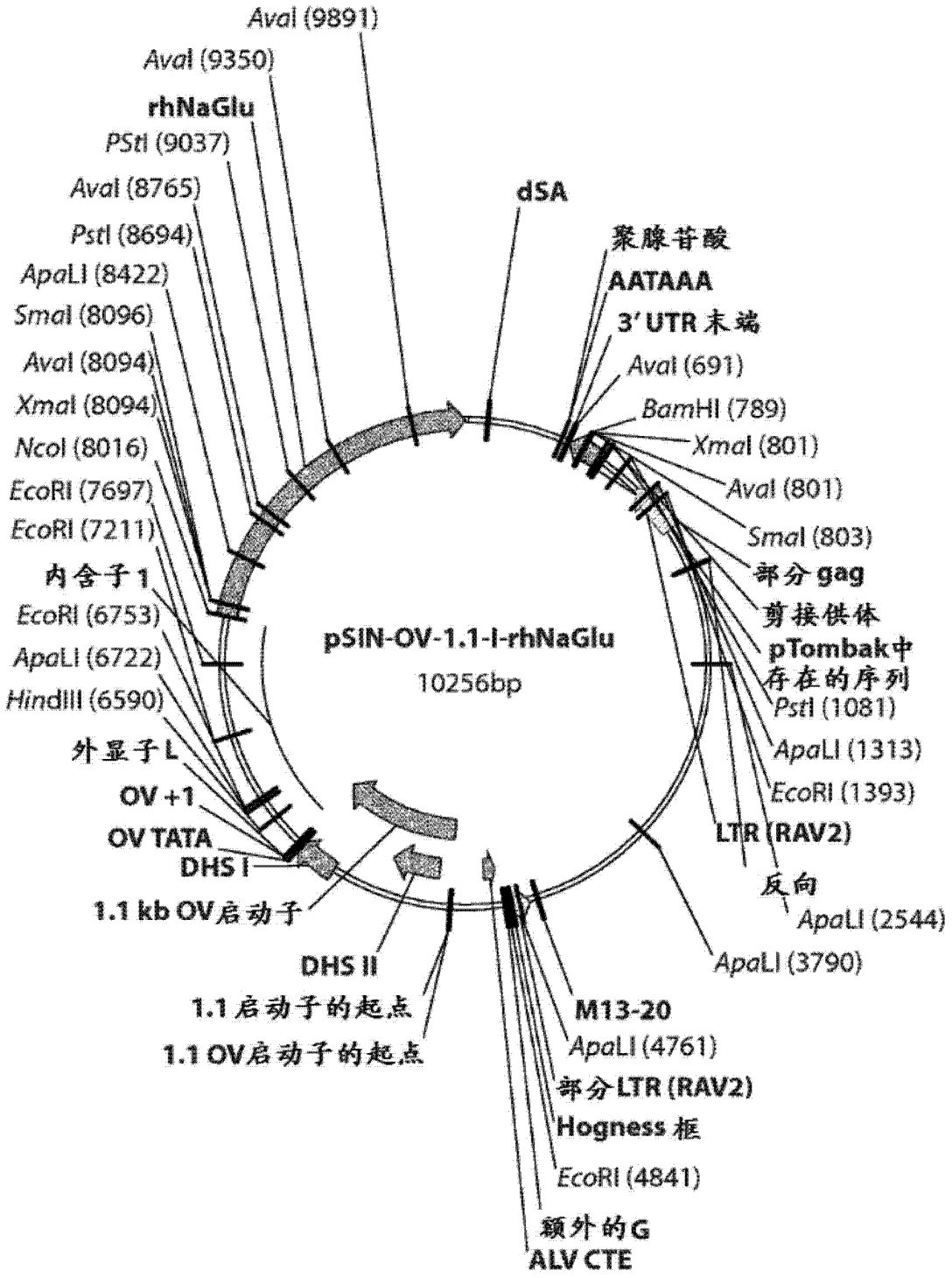
AvaI (9891)
AvaI (9350)
rhNaGlu
PstI (9037)
AvaI (8765)
PstI (8694)
ApaLI (8422)
SmaI (8096)
AvaI (8094)
XmaI (8094)
NcoI (8016)
EcoRI (7697)
EcoRI (7211)
内含子1
EcoRI (6753)
ApaLI (6722)
HindIII (6590)
外显子L
OV +1
OV TATA
DHS I
1.1 kb OV启动子
DHS II
1.1 启动子的起点
1.1 OV启动子的起点
pSIN-OV-1.1-I-rhNaGlu
10256bp
dSA
聚腺苷酸
AATAAA
3′UTR 末端
AvaI (691)
BamHI (789)
XmaI (801)
AvaI (801)
SmaI (803)
部分 gag
剪接供体
pTombak中存在的序列
PstI (1081)
ApaLI (1313)
EcoRI (1393)
LTR (RAV2)
反向
ApaLI (2544)
ApaLI (3790)
M13-20
ApaLI (4761)
部分LTR (RAV2)
Hogness 框
EcoRI (4841)
额外的G
ALV CTE

图 5

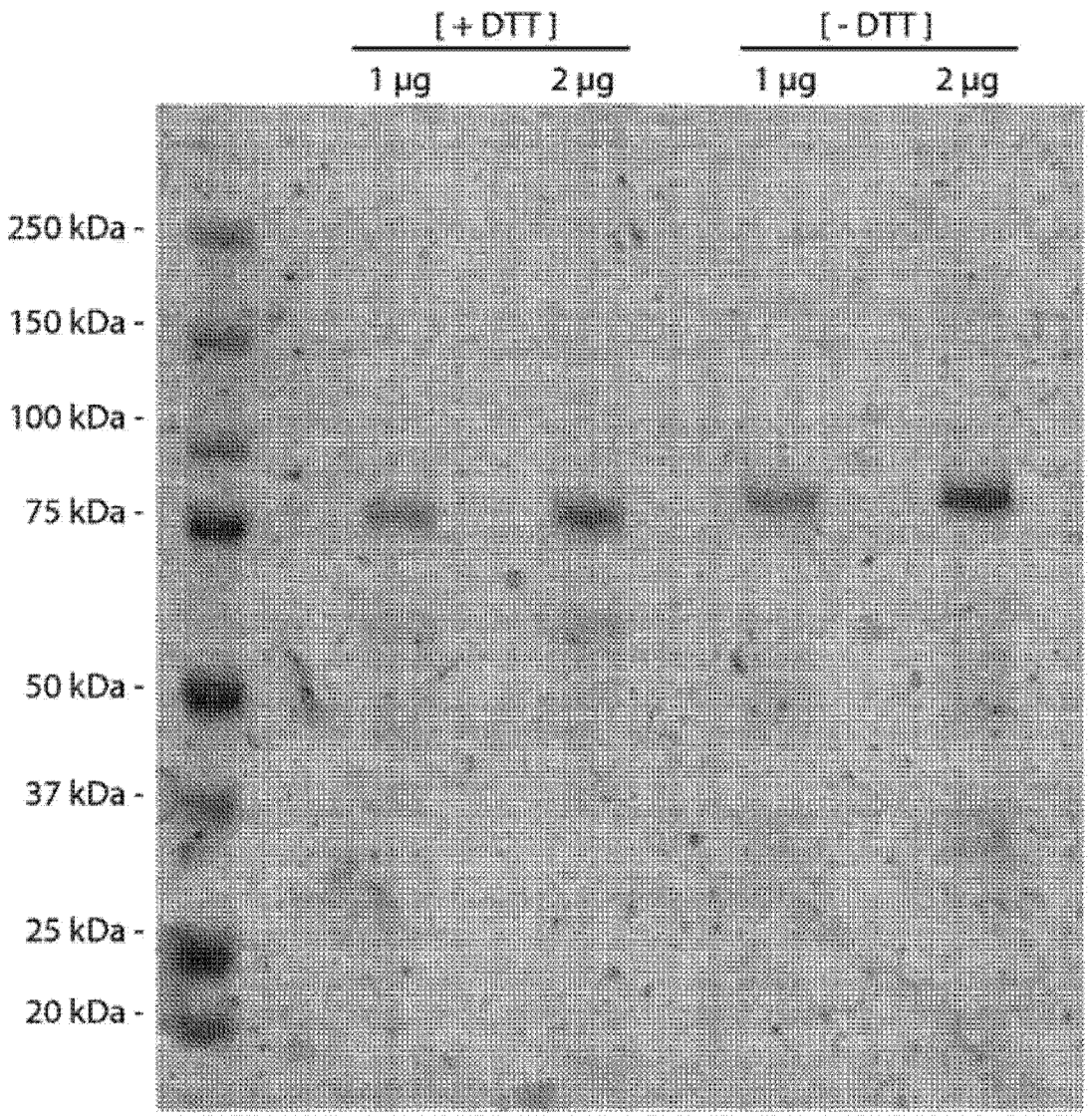
[+ DTT]
[- DTT]
1 µg
2 µg
1 µg
2 µg
250 kDa -
150 kDa -
100 kDa -
75 kDa -
50 kDa -
37 kDa -
25 kDa -
20 kDa -
考马斯蛋白质染色

图 6

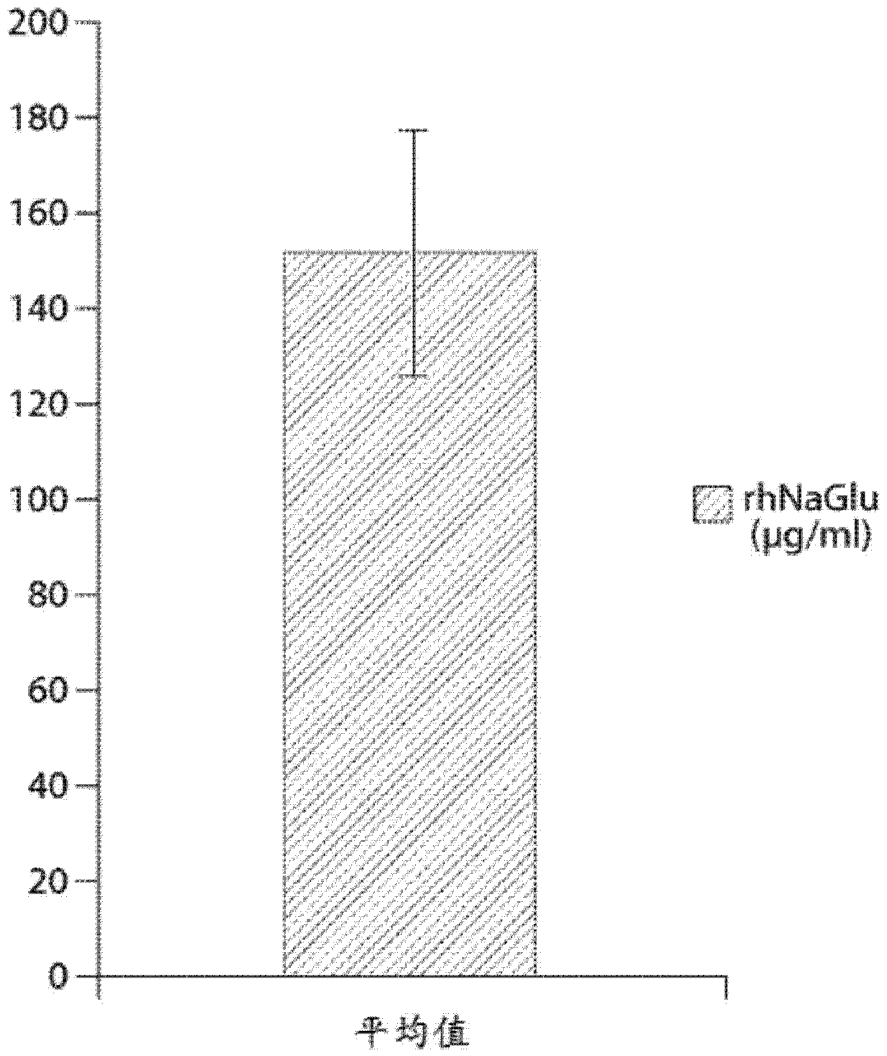

图 7

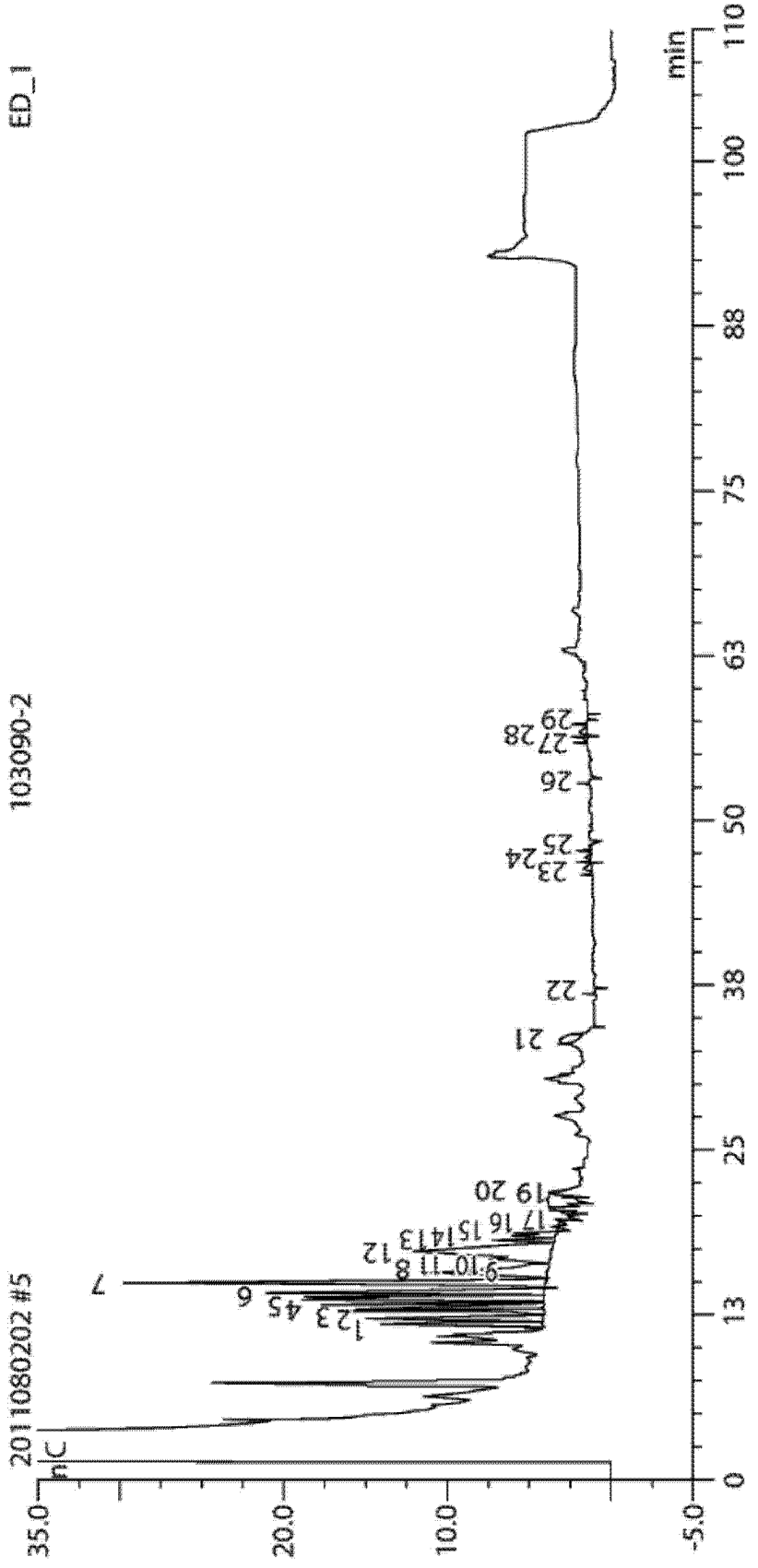
ED_1
103090-2
20110802202 #5
nC
35.0
20.0
10.0
-5.0
0
13
25
38
50
63
75
88
100
110
min
1 2 3 4 5
6
7
8 9 10 11
12
13 14 15 16 17
19 20
21
22
23 24
25
26
27 28 29

图 8

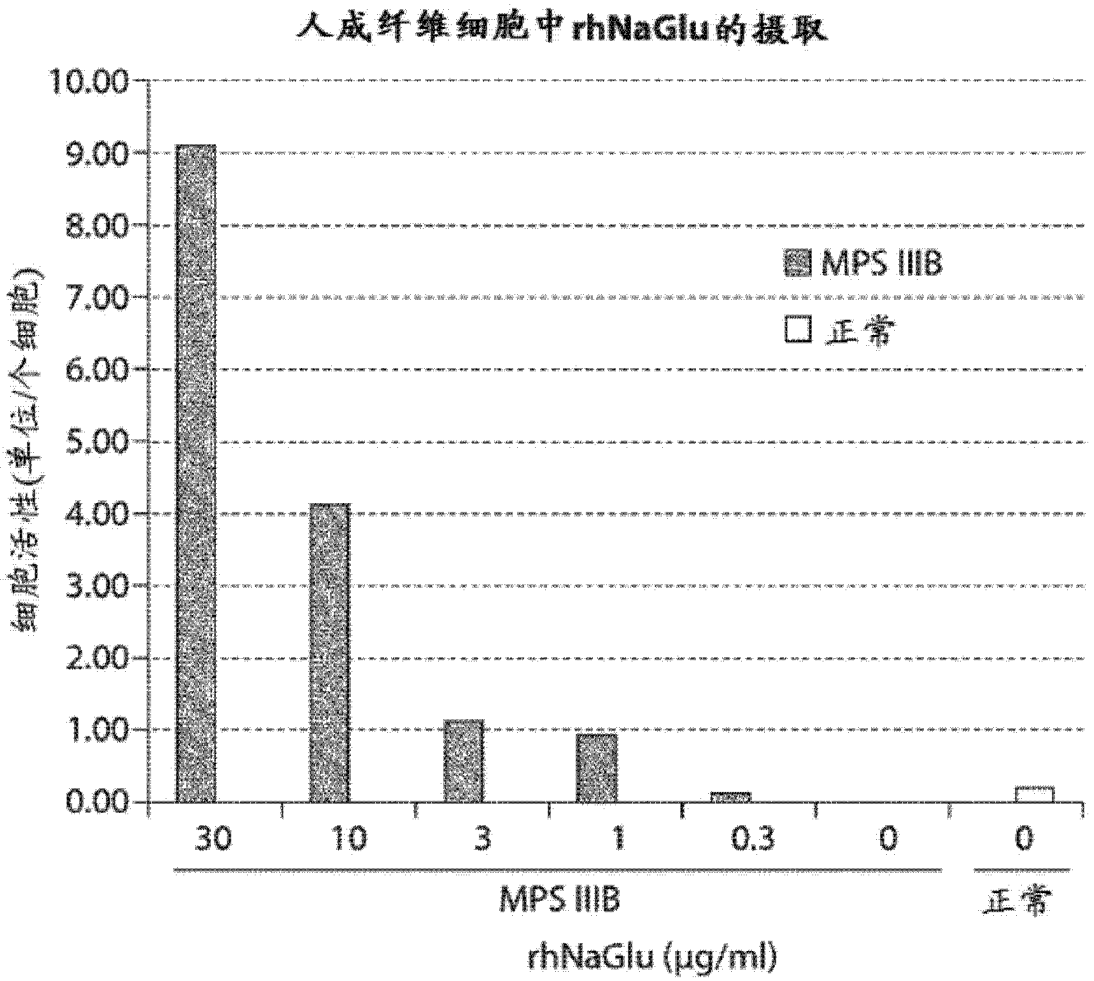
人成纤维细胞中rhNaGlu的摄取
细胞活性(单位/个细胞)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
MPS IIIB
正常
30
10
3
1
0.3
0
0
MPS IIIB
正常
rhNaGlu (μg/ml)

图 9

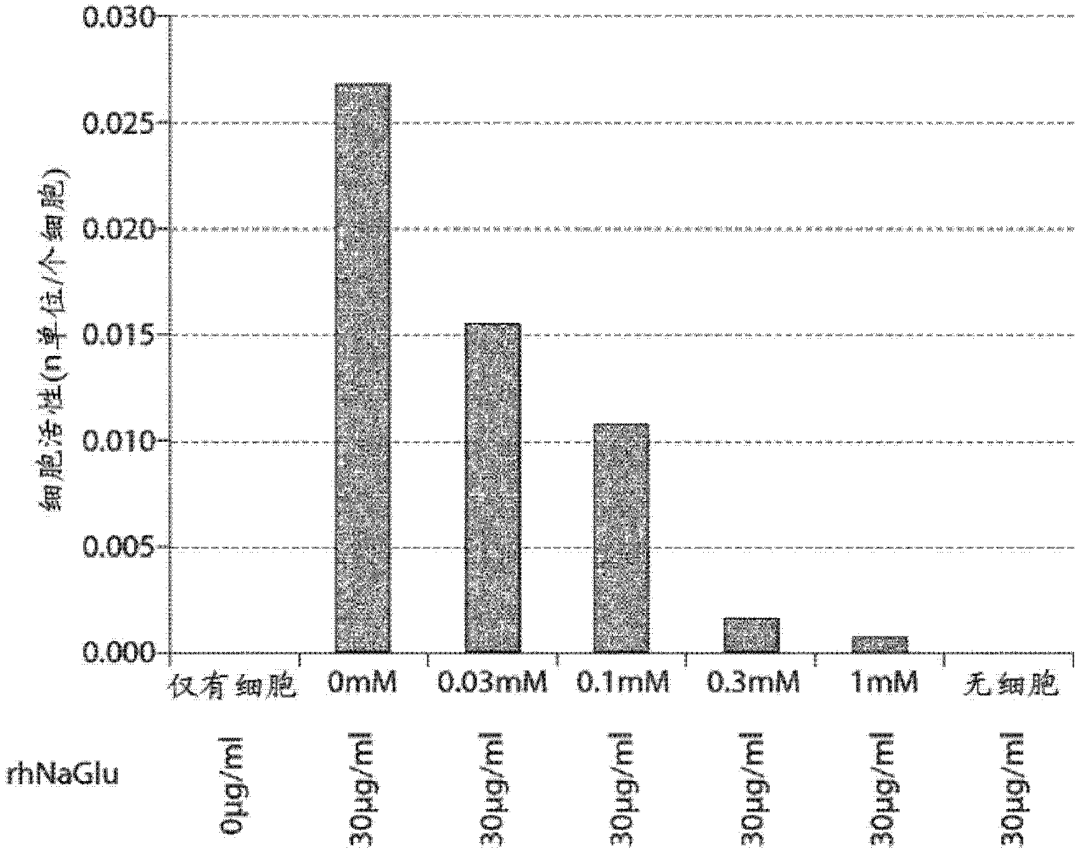
0.030
0.025
0.020
0.015
0.010
0.005
0.000
细胞活性(n单位/个细胞)
仅有细胞
0mM
0.03mM
0.1mM
0.3mM
1mM
无细胞
rhNaGlu
0μg/ml
30μg/ml
30μg/ml
30μg/ml
30μg/ml
30μg/ml
30μg/ml

图 10

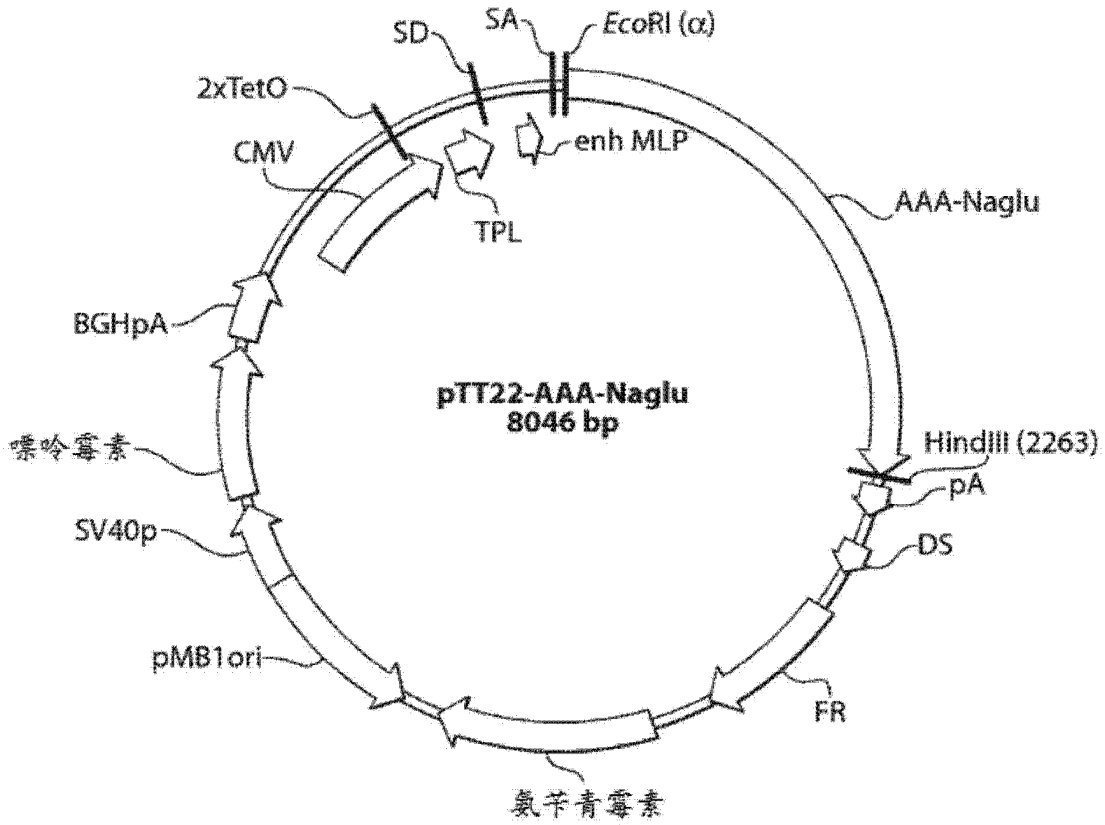
SD
SA
EcoRI (α)
2xTetO
CMV
enh MLP
TPL
AAA-Naglu
BGHpA
pTT22-AAA-Naglu
8046 bp
HindIII (2263)
pA
噪呤霉素
SV40p
DS
pMB1ori
FR
氨苄青霉素

图 11

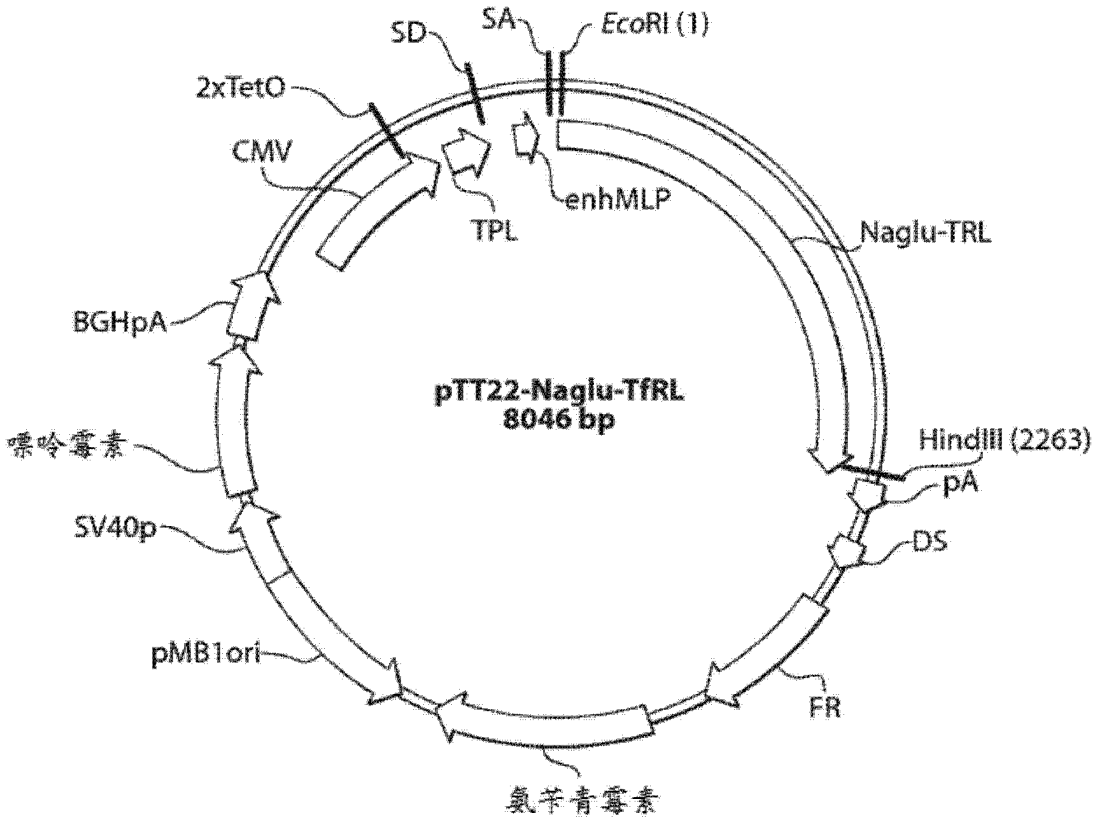
SD
SA
EcoRI (1)
2xTetO
CMV
TPL
enhMLP
Naglu-TRL
BGHpA
pTT22-Naglu-TfRL
8046 bp
HindIII (2263)
pA
DS
SV40p
pMB1ori
FR
氨苄青霉素

图 12

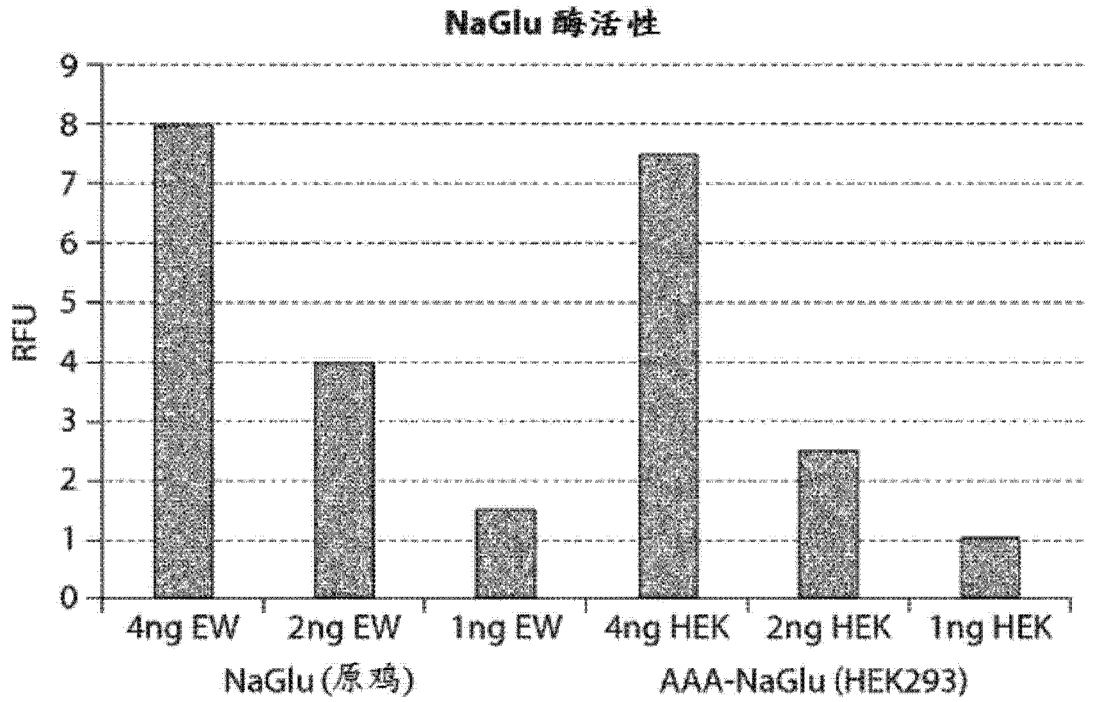
NaGlu 酶活性
RFU
9
8
7
6
5
4
3
2
1
0
4ng EW
2ng EW
1ng EW
4ng HEK
2ng HEK
1ng HEK
NaGlu (原鸡)
AAA-NaGlu (HEK293)

图 13

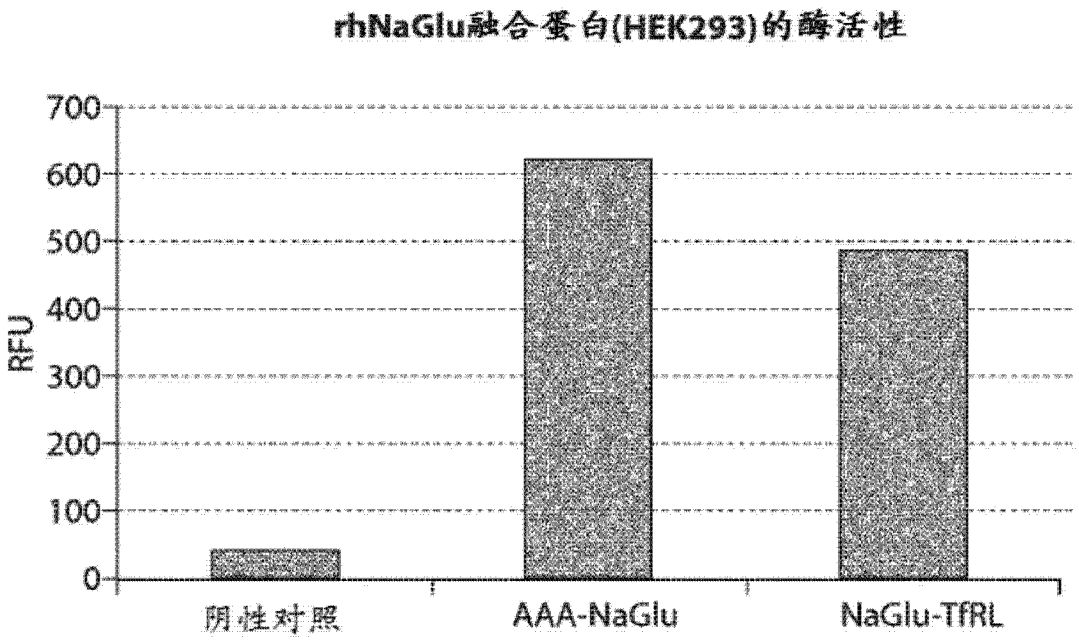
rhNaGlu融合蛋白(HEK293)的酶活性
RFU
700
600
500
400
300
200
100
0
阴性对照
AAA-NaGlu
NaGlu-TfRL

图 14

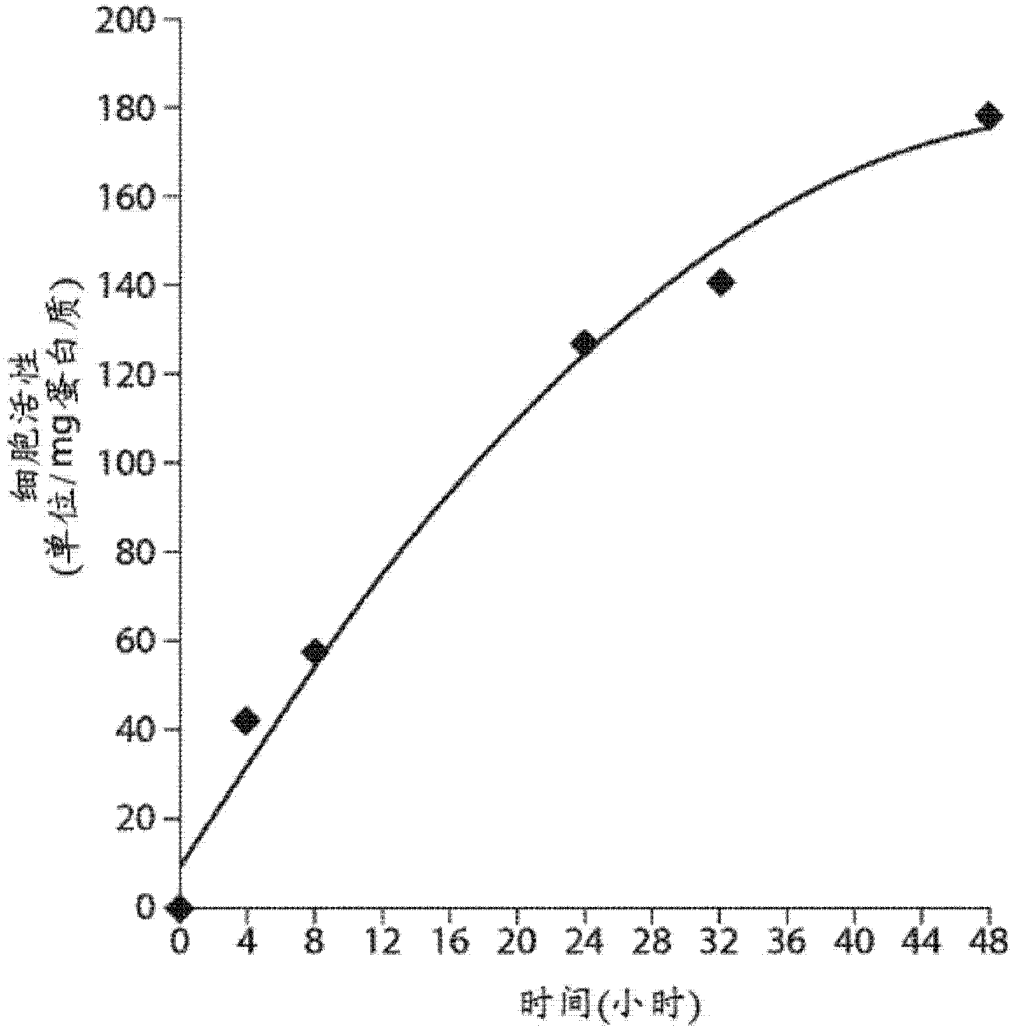
200
180
160
140
120
100
80
60
40
20
0
细胞活性
(单位/mg蛋白质)
0
4
8
12
16
20
24
28
32
36
40
44
48
时间(小时)

图 15

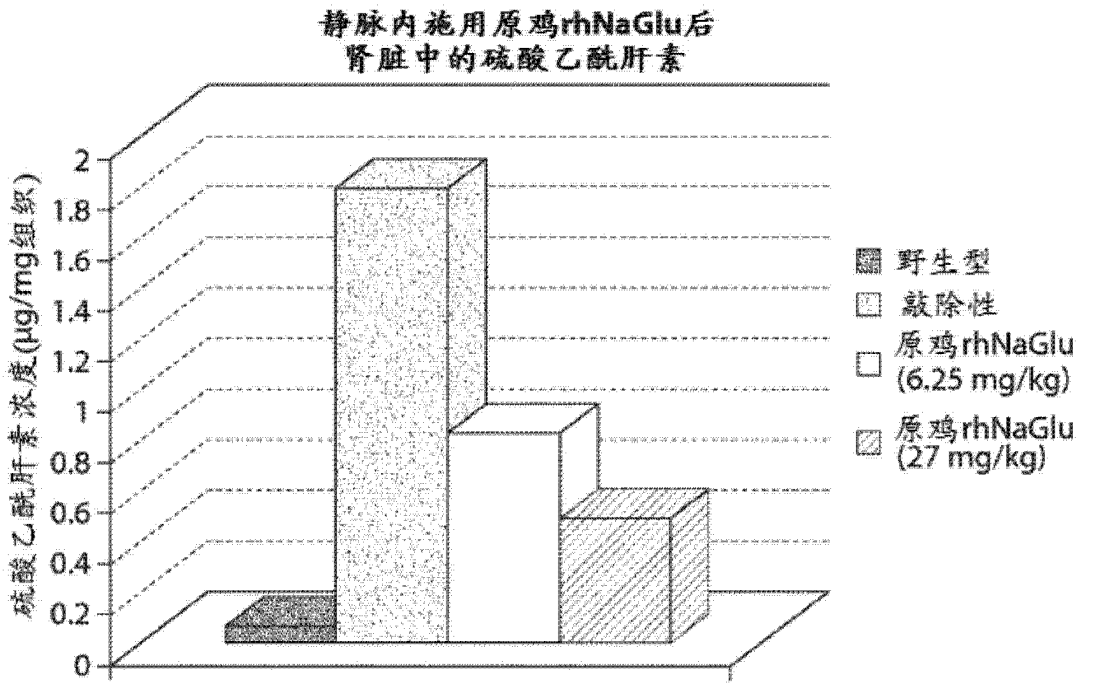
静脉内施用原鸡rhNaGlu后
肾脏中的硫酸乙酰肝素
硫酸乙酰肝素浓度(μg/mg组织)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
野生型
敲除性
原鸡rhNaGlu (6.25 mg/kg)
原鸡rhNaGlu (27 mg/kg)

图 16

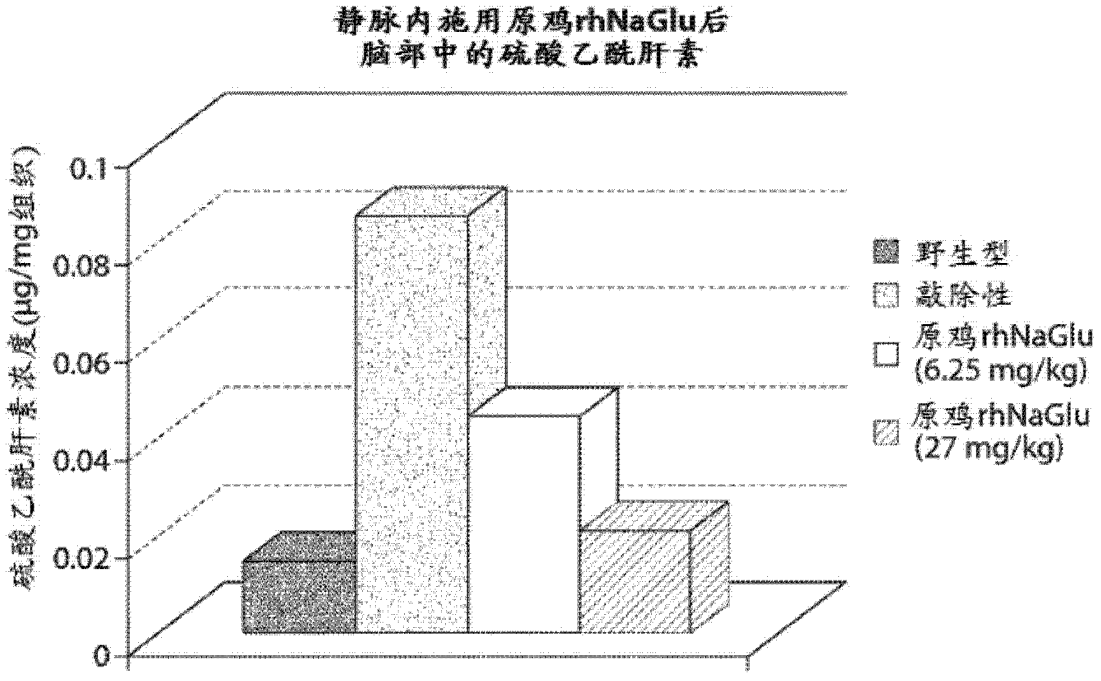
静脉内施用原鸡rhNaGlu后
脑部中的硫酸乙酰肝素
硫酸乙酰肝素浓度(μg/mg组织)
0.1
0.08
0.06
0.04
0.02
0
野生型
敲除性
原鸡rhNaGlu (6.25 mg/kg)
原鸡rhNaGlu (27 mg/kg)

图 17

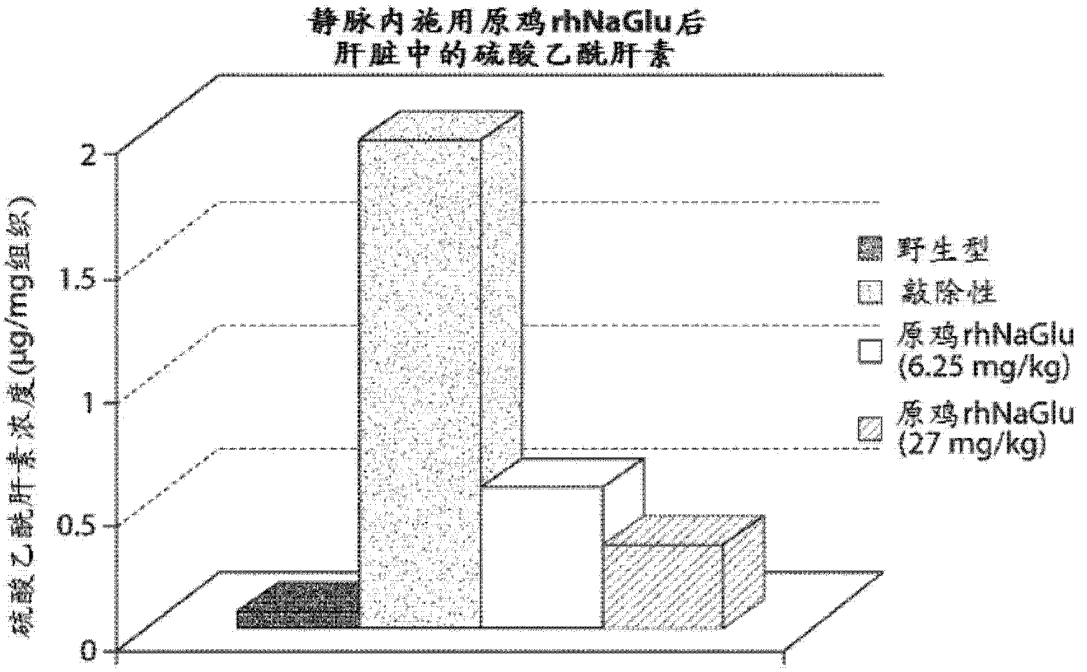
静脉内施用原鸡rhNaGlu后
肝脏中的硫酸乙酰肝素
硫酸乙酰肝素浓度(μg/mg组织)
2
1.5
1
0.5
0
野生型
敲除性
原鸡rhNaGlu
(6.25 mg/kg)
原鸡rhNaGlu
(27 mg/kg)

图 18

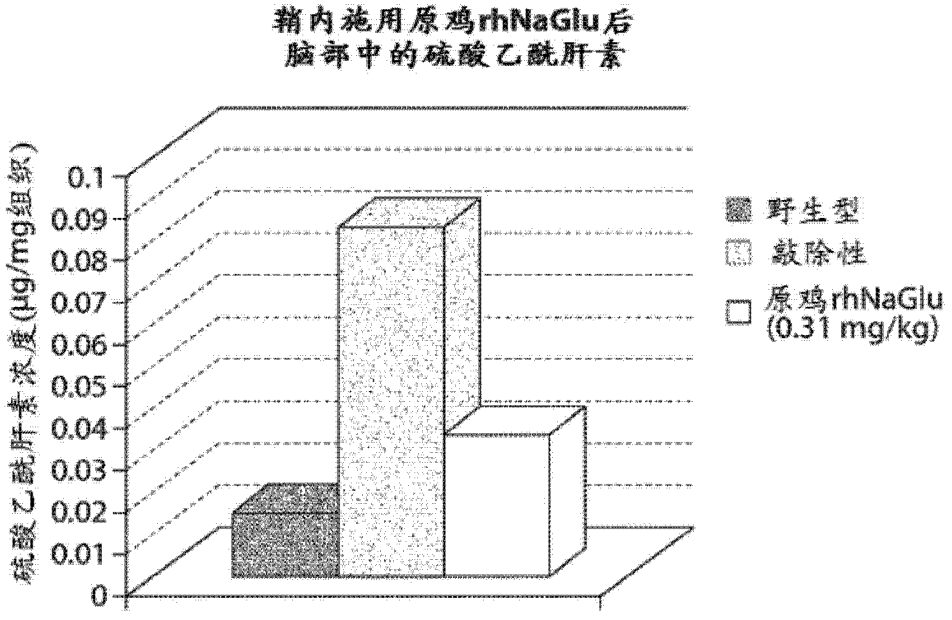
鞘内施用原鸡rhNaGlu后
脑部中的硫酸乙酰肝素
硫酸乙酰肝素浓度(μg/mg组织)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
野生型
敲除性
原鸡rhNaGlu
(0.31 mg/kg)

图 19

Abstract

The present invention provides compositions comprising an isolated mixture of recombinant human NaGlu proteins in which a substantial amount of the NaGlu proteins in the mixture has increased levels of phosphorylated mannose that confer the proteins to be efficiently internalized into human cells. The present invention also provides methods of producing such mixture of NaGlu proteins, vectors used in transgenesis and expression, host cells harboring such vectors, and methods of isolating and purifying the mixture of NaGlu proteins. The invention further provides methods of treating NaGlu associated diseases.