Review

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Recent progress in development of transgenic silkworms overexpressing recombinant human proteins with therapeutic potential in silk glands

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Summary **Since 2000, transgenic silkworms have been developed to produce recombinant proteins with therapeutic potential for future clinical use, including antibody preparations. Lysosomal storage diseases (LSDs) are inherited metabolic disorders caused by mutations of lysosomal enzymes associated with excessive accumulation of natural substrates and neurovisceral symptoms. Over the past few years, enzyme replacement therapy (ERT) with human lysosomal enzymes produced by genetically engineered mammalian cell lines has been used clinically to treat several patients with an LSD involving multi-organ symptoms. ERT is based on the incorporation of recombinant glycoenzymes by their binding to glycan receptors on the surface of target cells and their subsequent delivery to lysosomes. However, ERT has several disadvantages, including difficulty mass producing human enzymes, dangers of pathogen contamination, and high costs. Recently, the current authors have succeeded in producing transgenic silkworms overexpressing human lysosomal enzymes in the silk glands and the authors have purified catalytically active enzymes from the middle silk glands. Silk gland-derived human enzymes carrying high-mannose and pauci-mannose N-glycans were endocytosed by monocytes via the mannose receptor pathway and were then delivered to lysosomes. Conjugates with cell-penetrating peptides were also taken up by cultured fibroblasts derived from patients with enzyme deficiencies to restore intracellular catalytic activity and reduce the excessive accumulation of substrates in patient fibroblasts. Transgenic silkworms overexpressing human lysosomal enzymes in the silk glands could serve as future bioresources that provide safe therapeutic enzymes for the treatment of LSDs. Combining recent developments in transglycosylation technology with microbial endoglycosidases will promote the development of therapeutic glycoproteins as bio-medicines.**

Keywords: Transgenic silkworm, bio-medicine, lysosomal storage diseases, lysosomal enzyme replacement therapy, glycotechnology

1. Introduction

A transgenic (Tg) silkworm *Bombyx mori* was first produced by gene transfer utilizing a *piggyBac* transposon-derived vector in 2000 by Tamura *et al*. (*1*). Since then, Tg silkworms have been developed to produce recombinant human proteins, including procollagen (*2*), cytokines (*3,4*), and monoclonal antibodies (MoAb) (*5-7*), in their cocoons and silk glands. The advantages of Tg silkworms producing recombinant human proteins are: *i*) somewhat larger amounts of recombinant proteins (> 1 mg per larva and/or cocoon) are expressed than those derived from other hosts, *ii*) physical containment of Tg silkworms is easy because the larvae move slowly and seldom

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escape and because the moths cannot fly, *iii*) human pathogens have not been found in *Bombyx mori*, and *iv*) year-round breeding is possible with an artificial diet and expanded facilities, reducing the cost of producing recombinant proteins.

Lysosomal storage diseases (LSDs) are metabolic inborn errors caused by defects in lysosomal enzymes and their related co-factors (*8,9*). LSDs are associated with excessive accumulation of intact substrates in lysosomes due to an enzyme deficiency and exhibit manifestations that are quite heterogeneous. Intravenous (*iv*) enzyme replacement therapy (ERT) has been developed with recombinant enzyme preparations produced by mammalian cell lines, including the Chinese hamster ovary (CHO) and human fibrosarcoma (HT1080) cell lines, that stably overexpress genes that encode human lysosomal enzymes. Since 1991, ERT has been clinically used to treat LSDs, including Gaucher's disease (*10*), Fabry's disease (*11*), Pompe's disease (*12*), mucopolysaccharidoses (MPS) I (*13*), II (*14*), VI (*15*), and Wolman's disease (*16*) involving multi-order disorders such as hepatosplenomegaly, vascular disorders, cardiomegaly, and dysostosis multiplex. *iv* ERT is based on incorporation of a recombinant enzyme *via* cell surface glycan receptors, including the mannose receptor (MR) (*10*) and cation-independent mannose 6-phosphate receptor (CI-M6PR)/insulin-like growth factor type II receptor (*17*), into target organs and their subsequent delivery to intracellular lysosomes to degrade accumulated substrates. However, using *iv* ERT with recombinant human enzyme preparations produced by mammalian cell lines to treat LSDs has several disadvantages, *i.e. i*) large-scale production of recombinant human lysosomal enzymes by mammalian cells is limited and costly in comparison to the large-scale production of therapeutic antibodies (*5-7*) and *ii*) the risk of pathogen infection and the risk of an immune response and adverse effects including production of neutralizing antibodies against enzyme preparations in patients with an LSD and an enzyme deficiency as a result of continuous administration (*18*). Alternative human glycoprotein expression systems need to be developed as more effective, safer, and cheaper lysosomal enzyme sources to overcome these problems. Recently, the current authors succeeded in producing Tg silkworms overexpressing human lysosomal enzymes, including cathepsin A (CTSA) (*19,20*), specifically in the middle silk glands (MSGs) and purifying a catalytically active mature enzyme, allowing elucidation of their crystal structures. The purified enzyme was found to have human-like high-mannose and pauci-mannose N-glycans without insect-specific saccharides. The recombinant human glycoproteins produced by the silk glands of Tg silkworms could be clinically used to treat human diseases as glycobiologics that are slightly antigenic because of their glycan structures.

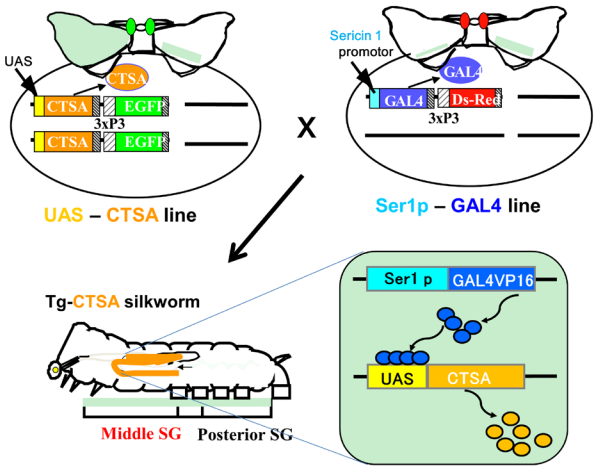

Figure 1. Establishment of transgenic silkworm line overexpressing human lysosomal cathepsin A (CTSA) in the middle silk glands. A Tg silkworm line (Tg-CTSA) overexpressing the human CTSA specifically in the middle silk glands (MSG) was established by crossbreeding between the UAS-CTSA [UAS-CTSA, 3xP3-EGFP] and the Ser1p-GAL4 [Ser1p-GAL4VP16, 3xP3-DsRed2] lines, both are established by utilizing *piggyBac* vectors and fluorescence (due to EGFP or DsRed2) screening of the G1 embryos. In the MSGs of the obtained Tg-CTSA silkworm line harboring both transgenes of the UAS-CTSA and the Ser1p-GAL4VP16, the human CTSA is overproduced under transcriptional control of the MSG-specific sericin 1 gene promotor (Ser1p)–driven GAL4VP16 and the upstream activation sequence recognized by GAL4VP16.

2. Utilizing *piggyBac* vectors to establish transgenic silkworms expressing human lysosomal enzymes

A Tg silkworm line expressing human CTSA in its MSGs (Tg-CTSA) was established as shown in Figure 1. A cloned open reading frame (ORF) fragment of CTSA was inserted downstream of the upstream activation sequence (UAS) of the *piggyBac* vector pBac[UAS-3xP3-EGFP] plasmid (*21*). The MSG-specific sericin I gene promoter (Ser1p) and its 3'-UTR region, including the BlnI site, was amplified from the genomic DNA of *B. mori* with PCR to construct the plasmid pBac[Ser1p-BlnI-UTR, 3xP3-DsRed2] (*22*), and this plasmid was inserted into regions including the *Nhe* I and *Bgl* II sites of pBacMCS (*23,24*). To construct the plasmid pBac[Ser1p-Gal4VP16, 3xP3-DsRed2], a Gal4VP16 *Nhe*I fragment (*22*) was inserted into the *Bln*I site of the plasmid pBac[Ser1-BlnI-UTR, 3xP3-DsRed2]. To generate a transgenic line with UAS-CTSA or Ser1p-Gal4VP16, the *piggyBac* vector pBac[UAS-CTSA, 3xP3-egfp] or pBac[Ser1p-Gal4VP16, 3xP3-DsRed2] was co-injected with the helper plasmid pHA3PIG into pre-blastoderm embryos of the *w-1 pnd* strain (*1*). The hatched larvae were reared on an artificial mulberry diet at 25ºC. G1 embryos were screened under a fluorescence stereomicroscope equipped with an EGFP or DsRed2 filer and a UAS-CTSA or Ser1-Gal4VP16 line was obtained. The UAS-CTSA line was crossed with the Ser1p-Gal4VP16 line, and silkworms (Tg-CTSA) harboring two transgenes, UAS-CTSA and Ser1p-Gal4VP16, were produced.

3. Molecular properties of recombinant human lysosomal enzymes derived from silk glands of the transgenic silkworm

Studies by the current authors have succeeded in functional expression of genes encoding human lysosomal enzymes and posttranslational modification of the MSGs of Tg silkworms. CTSA is a multifunctional lysosomal glycoprotein that exhibits serine carboxypeptidase (cathepsin A) activity as well as protective action to activate lysosomal neuraminidase 1 (NEU1) and stabilize acid β-galactosidase (GLB1) by formation of a multienzyme complex in lysosomes (*19*). In mammalian cell lines, CTSA is biosynthesized as a 54-kDa precursor (catalytically inactive zymogen) and is processed into a catalytically active mature 32/20-kDa form while en route to lysosomes (Figure 2A) (*20*). As shown in Figure 2B, the cathepsin A activity of human mature CTSA at a pH 5.6 towards the artificial substrate Z-L-phenylalanyl-L-leucine was specifically observed in the MSGs but not in the posterior silk glands (PSGs) of the 5th instar larvae of Tg-CTSA harboring two transgenes, UAS-CTSA and Ser1p-Gal4VP16, indicating that expression of CTSA is controlled by the MSG-specific Ser1 promotor. In light of the specific activity (2.7 mmol/h/mg protein) of the human cathepsin A purified from a CHO cell line stably expressing *CSTA* cDNA (*20*), the estimated amount of CTSA expressed in the 5th instar larvae of Tg-CTSA was about 0.1 mg/larva. Approximately one gram of enzyme preparations is reported to be necessary to continuously treat a patient with an LSD per year. An estimated 50,000 larvae are necessary to obtain and purify the same amount of functional human enzymes from the MSGs of Tg-CTSA silkworms.

Immunoblotting with each antibody against the 32-kDa and 20-kDa subunits of mature CTSA (*25*) revealed that three specific immunoreactive bands migrated at 52, 31, and 20 kDa, corresponding to the precursor form and mature subunits, which migrated slightly faster than those (54, 32, and 20 kDa) derived from the CHO cell line stably expressing *CTSA* (20) (Figure 2C). Treatment of the recombinant CTSA proteins with peptide-N-glycosidase F (PNGaseF) revealed the presence of *N*-glycans attached to each precursor and mature subunit, which migrated faster at 50, 30, and 18 kDa, respectively (Figure 2C). These bands were equivalent in size to those of digested DNA from the CHO cell line (Figure 2C). These results indicate that all of the precursor (52-kDa) and mature subunits (31-kDa/20-kDa) expressed in the MSGs carry *N*-glycans.

Recombinant human mature CTSA (cathepsin A) was purified with three-step chromatography utilizing concanavalin A (ConA)-, butyl-, and SP-Sepharose beads. The total cathepsin A activity in the extracts derived from 1,000 larvae was 132 mmol/h. The

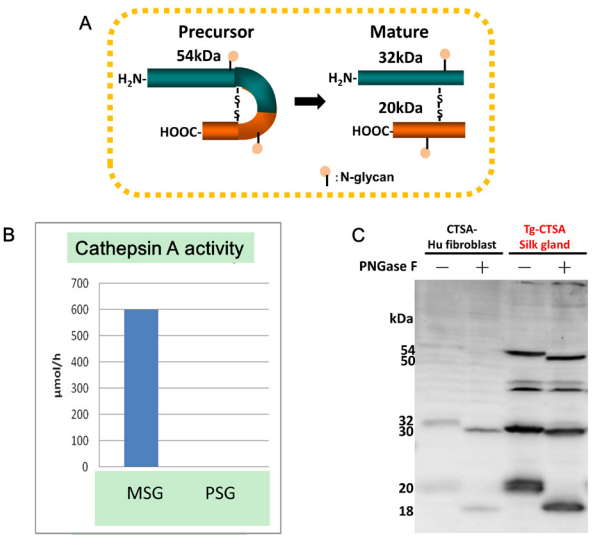

Figure 2. CTSA expression in the middle silk glands of Tg CTSA silkworm. (A) Posttranslational modification of CTSA in mammalian cells. CTSA is biosynthesized in endoplasmic reticulum (ER) as a catalytically inactive 54-kDa precursor (zymogen) and processed to the active 32/20-kDa mature enzyme in the course of transport to lysosomes. **(B)** Cathepsin A activity due to tIne human CTSA was specifically expressed in the middle silk glands (MSGs) but not in the posterior silk glands (PSGs) of Tg-CTSA silkworm under the transcriptional control of the MSG-specific sericin-1 promotor. **(C)** In the MSGs of Tg-CTSA both 52-kDa precursor and 31/20-kDa mature enzyme were expressed as soluble N-glycosylated proteins. Because of their sensitivity to PNGase F. The 52-kDa precursor was considered to be processed to the active 31/20-kDa mature form in the MSGs similarly in mammmalian cells.

total recovery of cathepsin A activity was 18%, and the degree of purification was above 98% (67-fold), according to high-performance liquid chromatography (HPLC)–MS/MS under native conditions.

The N-glycan structures attached to the recombinant mature CTSA purified from the MSGs are summarized in Figure 3 (center). Each mature subunit carries one N-glycan attached to the N-glycan sequon (*19,20*). According to MALDI-TOF-MS, the major *N*-glycan structures were Man3GlcNAc2- (M3b), GlcNAcMan3GlcNAc2- (GNb), Man2Man3GlcNAc2- (M5), GlcNAc2Man3GlcNAc2- (GN2), Man3Man3GlcNAc2- (M6), Man4Man3GlcNAc2- (M7), Man3Man3GlcNAc2- (M8), and Man5Man4GlcNAc2-PA (M9). Most of the major *N*-glycans were high-mannose forms such as M8 and pauci-mannose forms such as M3. The latter are typically found in insects and are also present in mammals as metabolic intermediates. However, fucose residues linked to the core insect-specific α-1,3-linked to GlcNAc residues were seldom detected. The N-glycosylated MoAbs purified from the cocoons of the Tg silkworm have been found to co-express heavy and light chains containing human-like high-mannose N-glycans with no or less insect-specific core-fucose residues (*7*), and these N-glycosylated MoAbs have markedly higher antibody-dependent cellular cytotoxicity as antibody preparations (*5,6*). These

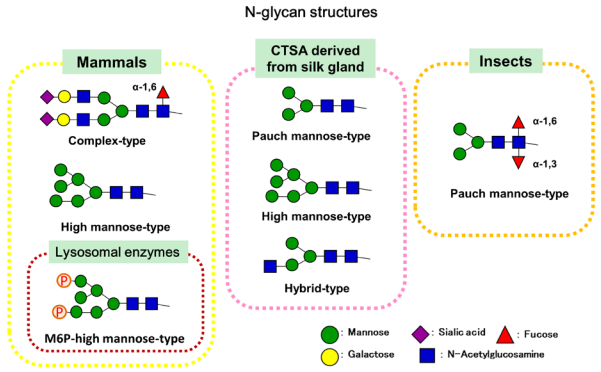

Figure 3. Comparison of N-glycan structures among human, MSG of Tg-CTSA silkworm and insects. In mammals the complex-, hybrid- and high mannose-type N-glycans are present. Lysosomal matrix enzymes have the terminal mannose 6-phosphate (M6P)-carrying high mannose-type (Left bottom). In contrast, insects have pauch mannose-type N-glycans containing insect-specific α-1,3 core fucose as well as α-1,6 fucose residues (Right). The N-glycans attached to the human CTSA in the MSGs of Tg-CTSA silkworms were human-like high mannose- and hybrid as well as pauchi-mannose type (Center), although the terminal M6Ps were not contained in the high mannose-type.

findings suggest the recombinant human glycoproteins produced by the silk glands of Tg silkworms could be clinically used to treat human diseases as glycobiologics that are slightly antigenic because of their glycan structures.

4. Intracellular delivery of mature CTSA purified from silk glands to lysosomes

As described above, mature CTSA derived from the MSGs was found to carry human-like N-glycans with terminal Man and GlcNAc residues. Therefore, purified mature CTSA should be endocytosed by macrophages and monocytes by binding to the cell surface mannose receptor. However, CTSA cannot be readily incorporated *via* CI-M6PR and delivered to lysosomes because CTSA derived from the MSGs does not carry terminal mannose 6-phosphate residues (M6P) in its N-glycans. A study has shown that fluorescein-conjugated mature CTSA at 1 μM can be incorporated into a cultured murine monocyte cell line after incubation at 37ºC for 24 h. Intracellular granular fluorescence due to the fluorescein-CTSA was found to co-localize with that of LysoTracker, which is a late endosome/lysosomal marker (Figure 4). These findings indicate that CTSA can be incorporated *via* the mannose receptor and that the fluorescein-CTSA derived from the MSGs of Tg-CTSA can be delivered to lysosomes.

5. Therapeutic effects of modified CTSA derived from Tg-CTSA on fibroblasts from a patient with galactosialidosis

Over the past few years, cell-penetrating peptides

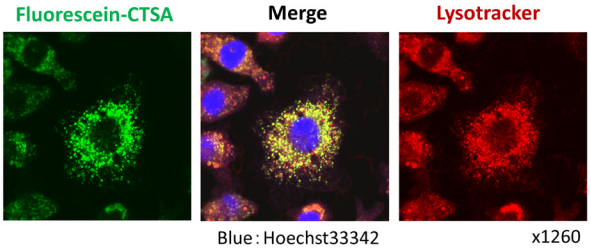

Figure 4. Lysosomal distribution of fluorescein-conjugated CTSA derived from MSGs of Tg-CTSA silkworm in murine monocytic cell line. Fluorescein-conjugated CTSA purified from the MSGs of Tg-CTSA carring terminal mannose-type N-glycans were incorporated into the murine monocytic cell line and transported to lysosomes (Left, green fluorescence), colocalized (Center) with Lysotracker (Right, red fluorescence). Nuclei were stained with Hoechst33342 (Blue). Magnificaion (×1260).

(CPPs) such as oligoarginine vectors have been a powerful tool for intracellular delivery of cargo molecules, including recombinant proteins and nucleic acids (*26,27*). Mature CTSA derived from the MSGs of Tg-CTSA does not carry the terminal M6P residues in its N-glycans, so intracellular uptake of CTSA derived from Tg-CTSA *via* CI-M6PR is not expected. CTSA, NEU1, and GLB activity are deficient in skin fibroblasts derived from a patient with galactosialidosis (GS). Previous studies demonstrated that the recombinant human CTSA produced by CHO cell lines expressing *CSTA* cDNA is taken up *via* cell surface CI-M6PR for delivery and that this recombinant human CTSA partially restores deficient enzyme activity. Oligoarginine (R8)-conjugated CTSA (R8-CTSA) was prepared by mixing BS^3-modified R8 peptides (*26*) with mature CTSA purified from MSGs at 37ºC, and the replacement (therapeutic) effects on a fibroblast cell line from a patient with GS were examined. R8-CTSA but not CTSA itself was found to restore deficient enzyme activity, including deficiencies in CTSA, NEU1, and GLB1, in a dose- and time-dependent manner. Cathepsin A activity recovered to a level equal to that of fibroblasts from a normal subject 24 h after treatment with R8-CTSA (2 μmol/h). The protection of NEU1 and GLB was also found to be partly restored. The current authors previously reported that sialylglycoconjugates, including sialyloligosaccharides, accumulate in fibroblasts from patients with GS (*28*). The addition of R8-CTSA to culture medium caused a marked reduction in the sialylglycoconjugates that accumulated in fibroblasts from a patient with GS according to *Maackia amurensis* (MAM) lectin staining, which recognizes and binds to terminal sialic acid residues of sialylglycoconjugates (*28*). Thus, R8-CTSA can be incorporated into fibroblasts *via* macropinocytosis with an R8 tag as a CPP, it can be delivered to lysosomes, and it can activate NEU1 to degrade accumulated sialyloligosaccharides, suggesting that R8-CTSA would therapeutically benefit patients with GS.

6. Perspectives for the future

Transgenic silkworms producing recombinant human proteins and enzymes in their silk glands are a promising bioresource to provide and promote the development of slightly antigenic therapeutic glycoproteins as safe bio-medicines for the treatment of human diseases, including LSDs. Recently, the current authors established another transgenic silkworm line overexpressing a functional human lysosomal enzyme. That enzyme was produced in the MSGs at an amount of 1 mg of glycoprotein per larva and/or cocoon, but the N-glycosylated enzyme does not carry terminal M6P residues. Genetic engineering will need to add terminal M6P residues to the N-glycans attached to human lysosomal enzymes; glycosyltransferase and/or glycosidase genes could be co-expressed in the MSGs of transgenic silkworm lines. Alternatively, combining recent developments in transglycosylation technology with microbial endoglycosidases is a possibility (*29*). A glycotechnology approach to the chemo-enzymatic synthesis of artificial glycopeptides/glycoproteins (neoglycoproteins) has been developed using N-glycan oxazoline derivatives and the transglycosylation activity of microbial endoglycosidases and their mutants, including endo β-N-acetylglucosaminidases such as Endo-M from *Mucor hiemalis* (*30*), Endo-A from *Arthrobacter protophormiae* (*31*), and Endo-D from *Streptococcus pneumoniae* (*32*). If high-mannose N-glycans carrying terminal M6P residues can be obtained as the N-glycan donor in large quantities, neoglycoenzymes could be produced for ERT to treat patients with an LSD. This could be accomplished through transglycosylation by utilizing the N-glycosylated human lysosomal enzymes derived from the silk glands of transgenic silkworms as acceptors for M6P-carrying N-glycans.

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References

1. Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P. Germline transformation of the silkworm *Bombyx mori* L. using a *piggyBac* transposon-derived vector. Nat Biotechnol. 2000;18:81-84.
2. Tomita M, Munetsuna H, Sato T, Adachi T, Hino R, Hayashi M, Shimizu K, Nakamura N, Tamura T, Yoshizato K. Transgenic silkworms produce recombinant human type III procollagen in cocoons. Nat Biotechnol. 2003; 21:52-56.
3. Shin S, Kim BY, Jeon HY, Lee A, Lee S, Sung SH, Park CS, Lee CK, Kong H, Song Y, Kim K. Expression system for production of bioactive compounds, recombinant human adiponectin, in the silk glands of transgenic silkworms. Arch Pharm Res. 2014; 37:645-651.
4. Xue R, Chen H, Cui L, Cao G, Zhou W, Zheng X, Gong C. Expression of hGM-CSF in silk glands of transgenic silkworms using gene targeting vector. Transgenic Res. 2012; 21:101-111.
5. Kurogochi M, Mori M, Osumi K, Tojino M, Sugawara S, Takashima S, Hirose Y, Tsukimura W, Mizuno M, Amano J, Matsuda A, Tomita M, Takayanagi A, Shoda S, Shirai T. Glycoengineered monoclonal antibodies with homogeneous glycan (M3, G0, G2, and A2) using a chemoenzymatic approach have different affinities for FcγRIIIa and variable antibody-dependent cellular cytotoxicity activities. PLoS One. 2015; 10:e0132848.
6. Tada M, Tatematsu K, Ishii-Watabe A, Harazono A, Takakura D, Hashii N, Sezutsu H, Kawasaki N. Characterization of anti-CD20 monoclonal antibody produced by transgenic silkworms (*Bombyx mori*). MAbs. 2015; 7:1138-1150.
7. Iizuka M, Ogawa S, Takeuchi A, Nakakita S, Kubo Y, Miyawaki Y, Hirabayashi J, Tomita M. Production of a recombinant mouse monoclonal antibody in transgenic silkworm cocoons. FEBS J. 2009; 276:5806-5820.
8. Meikle PJ, Hopwood JJ, Clague AE, Carey WF. Prevalence of lysosomal storage disorders. JAMA. 1999; 281:249-254.
9. Parenti G, Andria G, Ballabio A. Lysosomal storage diseases: From pathophysiology to therapy. Annu Rev Med. 2015; 66:471-486
10. Barton NW, Brady RO, Dambrosia JM, *et al*. Replacement therapy for inherited enzyme deficiency − Macrophage-targeted glucocerebrosidase for Gaucher's disease. N Engl J Med. 1991; 324:1464-1470.
11. Eng CM, Banikazemi M, Gordon RE, Goldman M, Phelps R, Kim L, Gass A, Winston J, Dikman S, Fallon JT, Brodie S, Stacy CB, Mehta D, Parsons R, Norton K, O'Callaghan M, Desnick RJ. A phase 1/2 clinical trial of enzyme replacement in Fabry disease: Pharmacokinetic, substrate clearance, and safety studies. Am J Hum Genet. 2001; 68:711-722.
12. Kishnani PS, Corzo D, Nicolino M, *et al*. Recombinant human acid α-glucosidase: Major clinical benefits in infantile-onset Pompe disease. Neurology. 2007; 68:99-109.
13. Kakkis ED, Muenzer J, Tiller GE, Waber L, Belmont J,

Passage M, Izykowski B, Phillips J, Doroshow R, Walot I, Hoft R, Neufeld EF. Enzyme-replacement therapy in mucopolysaccharidosis I. N Engl J Med. 2001; 344:182-188.
14. Wraith JE, Scarpa M, Beck M, Bodamer OA, De Meirleir L, Guffon N, Meldgaard Lund A, Malm G, Van der Ploeg AT, Zeman J. Mucopolysaccharidosis type II (Hunter syndrome): A clinical review and recommendations for treatment in the era of enzyme replacement therapy. Eur J Pediatr. 2008; 167:267-277.
15. Harmatz P, Whitley CB, Waber L, Pais R, Steiner R, Plecko B, Kaplan P, Simon J, Butensky E, Hopwood JJ. Enzyme replacement therapy in mucopolysaccharidosis VI (Maroteaux-Lamy syndrome). J Pediatr. 2004; 144:574-580.
16. Burton BK, Balwani M, Feillet F, *et al*. A phase 3 trial of sebelipase alfa in lysosomal acid lipase deficiency. N Engl J Med. 2015; 373:1010-1020.
17. Hawkes C, Kar S. Insulin-like growth factor-II/mannose-6-phosphate receptor: Widespread distribution in neurons of the central nervous system including those expressing cholinergic phenotype. J Comp Neurol. 2003; 458:113-127.
18. Wang J, Lozier J, Johnson G, Kirshner S, Verthelyi D, Pariser A, Shores E, Rosenberg A. Neutralizing antibodies to therapeutic enzymes: Considerations for testing, prevention and treatment. Nat Biotechnol. 2008; 26:901-908.
19. Galjart NJ, Gillemans N, Harris A, van der Horst GT, Verheijen FW, Galjaard H, d'Azzo A. Expression of cDNA encoding the human "protective protein" associated with lysosomal beta-galactosidase and neuraminidase: Homology to yeast proteases. Cell. 1988; 54:755-764.
20. Itoh K, Takiyama N, Kase R, Kondoh K, Sano A, Oshima A, Sakuraba H, Suzuki Y. Purification and characterization of human lysosomal protectiveprotein expressed in stably transformed Chinese hamster ovary cells. J Biol Chem. 1993; 268:1180-1186.
21. Sakudoh T, Sezutsu H, Nakashima T, Kobayashi I, Fujimoto H, Uchino K, Banno Y, Iwano H, Maekawa H, Tamura T, Kataoka H, Tsuchida K. Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the Yellow blood gene. Proc Natl Acad Sci U S A. 2007; 104:8941-8946.
22. Kobayashi I, Kojima K, Uchino K, Sezutsu H, Iizuka T, Tatematsu K, Yonemura N, Tanaka H, Yamakawa M, Ogura E, Kamachi Y, Tamura T. An efficient binary system for gene expression in the silkworm, *Bombyx mori*, using GAL4 variants. Arch Insect Biochem Physiol. 2001; 76:195-210.
23. Uchino K, Sezutsu H, Imamura M, Kobayashi I, Tatematsu K, Iizuka T, Yonemura N, Mita K, Tamura T. Construction of a piggyBac-based enhancer trap system for the analysis of gene function in silkworm *Bombyx mori*. Insect Biochem Mol Biol. 2008; 38:1165-1173.
24. Tatematsu K, Kobayashi I, Uchino K, Sezutsu H, Iizuka T, Yonemura N, Tamura T. Construction of a binary transgenic gene expression system for recombinant protein production in the middle silk gland of the silkworm *Bombyx mori*. Transgenic Res. 2010; 19:473-487.
25. Satake A, Itoh K, Shimmoto M, Saido TC, Sakuraba H, Suzuki Y. Distribution of lysosomal protective protein in human tissues. Biochem Biophys Res Commun. 1994; 205:38-43.
26. Kosuge M, Takeuchi T, Nakase I, Jones AT, Futaki S. Cellular internalization and distribution of arginine-rich peptides as a function of extracellularpeptide concentration, serum, and plasma membrane associated proteoglycans. Bioconjug Chem. 2008; 19:656-664.
27. Nakase I, Osaki K, Tanaka G, Utani A, Futaki S. Molecular interplays involved in the cellular uptake of octaarginine on cell surfaces and the importance of syndecan-4 cytoplasmic V domain for the activation of protein kinase Cα. Biochem Biophys Res Commun. 2014; 446:857-862.
28. Oheda Y, Kotani M, Murata M, Sakuraba H, Kadota Y, Tatano Y, Kuwahara J, Itoh K. Elimination of abnormal sialylglycoproteins in fibroblasts with sialidosis and galactosialidosis by normal gene transfer and enzyme replacement. Glycobiology. 2006; 16:271-280.
29. Yamamoto K. Recent advances in glycotechnology for glycoconjugate synthesis using microbial endoglycosidases. Biotechnol Lett. 2013; 35:1733-1743.
30. Yamamoto K, Kadowaki S, Watanabe J, Kumagai H. Transglycosylation activity of Mucor hiemalis endo-β-N-acetylglucosaminidase which transfers complex oligosaccharides to the N-acetylglucosamine moieties of peptides. Biochem Biophys Res Commun. 1994; 203:244-252.
31. Takegawa K, Yamaguchi S, Kondo A, Iwamoto H, Nakoshi M, Kato I, Iwahara S. Transglycosylation activity of endo-β-N-acetylglucosaminidase from Arthrobacter protophormiae. Biochem Int. 1991; 24:849-855.
32. Muramatsu H, Tachikui H, Ushida H, Song X, Qiu Y, Yamamoto S, Muramatsu T. Molecular cloning and expression of endo-β-N-acetylglucosaminidase D, which acts on the core structure of complex type asparagine-linked oligosaccharides. J Biochem. 2001; 129:923-928.

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