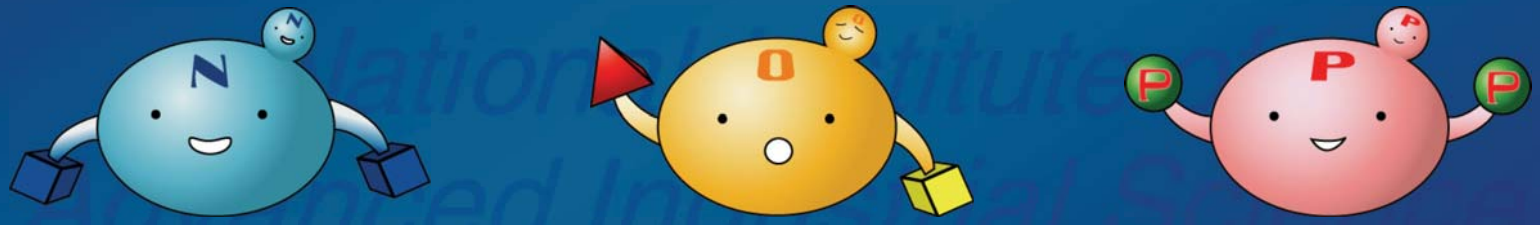
02/14/2014@BITEC, Bangkok, Thailand

Glycan engineering and production of ‘humanized‘ glycoprotein by yeast cells



Yasunori Chiba

Bioproduction Research Institute

National Institute of Advanced Industrial Science and Technology

Japan

RESEARCH LETTER

Heterologous protein expression in *Pichia thermomethanolica* BCC16875, a thermotolerant methylotrophic yeast and characterization of *N*-linked glycosylation in secreted protein

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Glycan engineering and production of 'humanized' glycoprotein by yeast cells

- Introduction

- Suppression of *O*-mannosylation for expression of antibody in yeast cells

- Mannose-6-phosphate-type glycoprotein production in yeast and application for enzyme replacement therapy of lysosomal disease

Hosts for glycoprotein production

Biological products

Cytokines, Enzymes
Vaccines, hormones
Antibody drugs

Mammalian Cells (CHO cells)

Advantage
- High secretion
- Post translational modification

Disadvantage
- Difficulty in breeding
- Costly production
- Viral infection

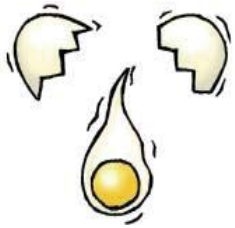
Yeast

Advantage
- Rapid growth
- Low cost
- Easy breeding
- High secretion ability
- Post translational modification

E. coli

Fungi
Moss

Silkworm

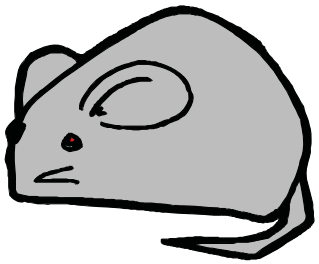


Egg

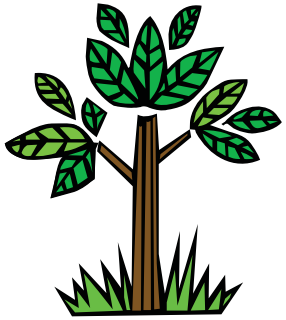
Plant

Transgenic animal

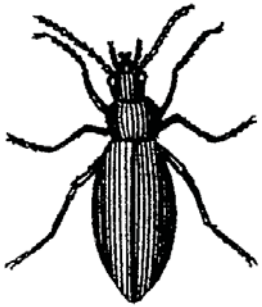
Dependency of the glycan structure on the host cells



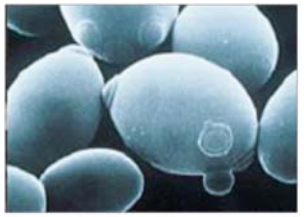
Mammal



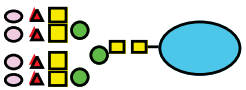
Plant



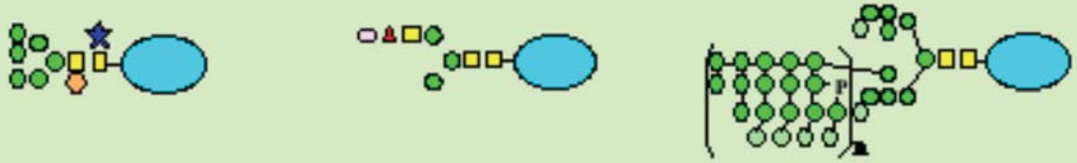
Insect



Yeast



N-glycan structure



Difference of glycan structure

↓

Immune response

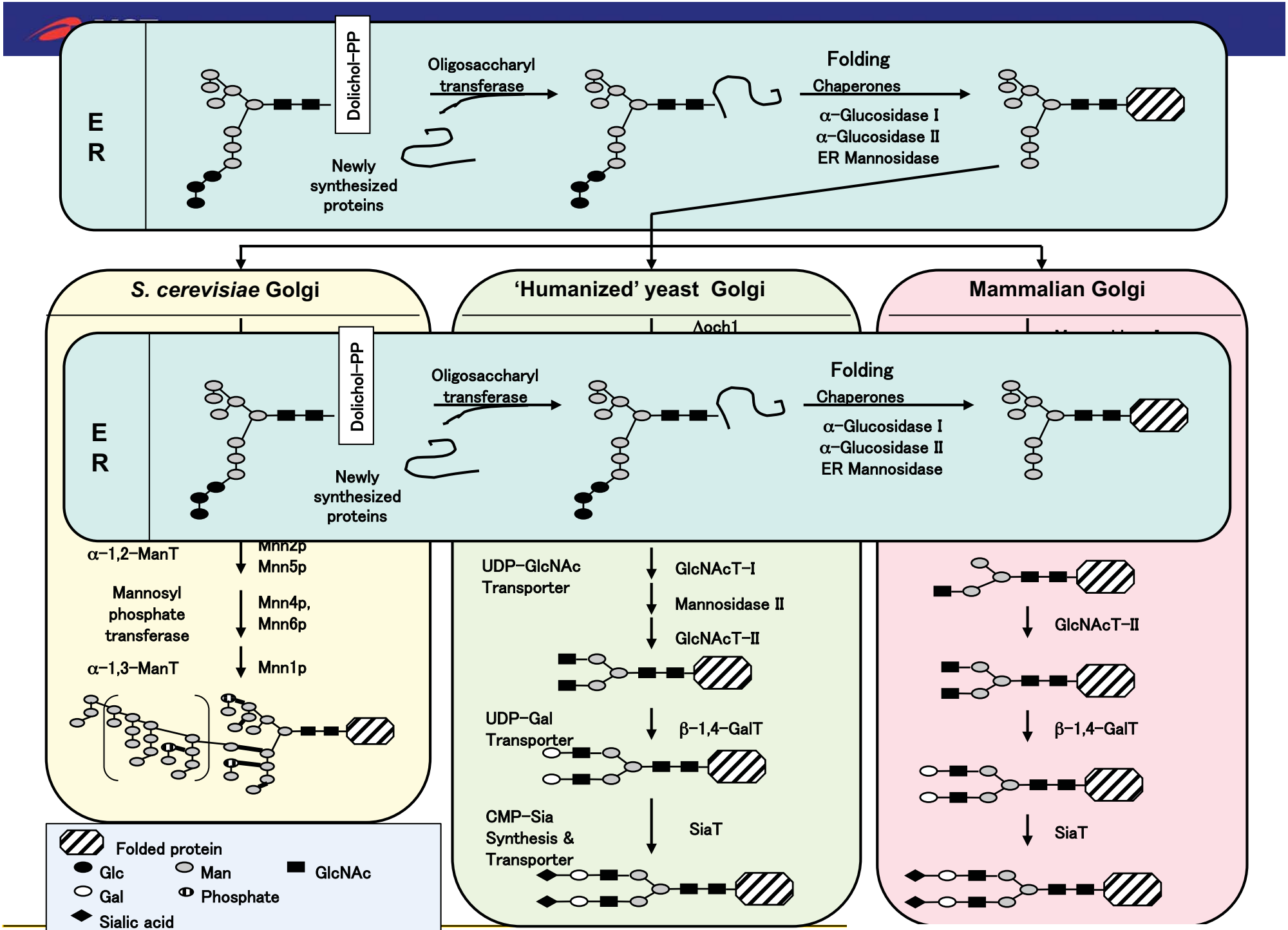
Glycan remodeling of yeast cells must be required!

The goals of our study

- Establishment of yeast cells capable of producing mammalian-type glycans (“Humanization” “human-compatible”)

- Effective production of human glycoprotein in “human-compatible” yeast cells

How do we create “humanized” yeast?



Chiba et al. JBC 273:26298-304 (1998)

How to create “humanized” glycans in yeast cells

Wild-type yeast

OCH1
MNN1
MNN4

KRE2
KTR1, 3

Mannan-less yeast

~~OCH1~~
~~MNN1~~
~~KRE2~~
~~MNN4~~
~~KTR1~~

Humanized glycoprotein production in yeast

***N*-glycan**

- **High-mannose type**
- **Complex type**
- **Mannose-6-phosphate type**

***O*-glycan**

- **Mucin-type**
- **Dystroglycan (O-man) type**
- **O-Fuc type**
- **O-Xyl type**

Disruption of yeast specific glycogenes

Introduction of glycogenes for humanization

Production of full-length antibody by *P. pastoris* (GlycoFi)

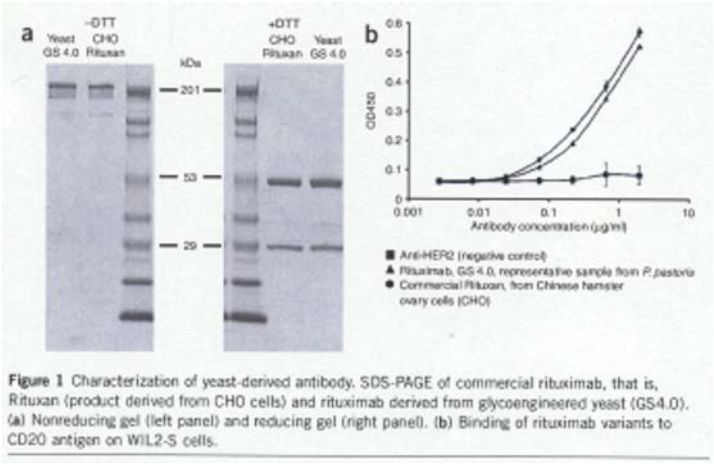
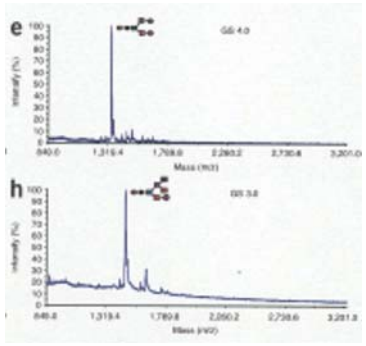


Figure 1 Characterization of yeast-derived antibody. SDS-PAGE of commercial rituximab, that is, Rituxan (product derived from CHO cells) and rituximab derived from glycoengineered yeast (GS4.0). (a) Nonreducing gel (left panel) and reducing gel (right panel). (b) Binding of rituximab variants to CD20 antigen on WIL2-S cells.

N-glycan analysis of antibodies by MALDI-TOF mass spectrometry



GS4.0=M3GN2

GS3.0=M5GN1

* *Pichia pastoris*
* *N*-glycan：WT=M9-12
* Anti-CD20 Ab (rituximab)
* Productivity: 1 g/L
* Degradation products and deglycosylated antibody were observed.

Nat Biotechnol. 24:210-215 (2006) .

GS4.0

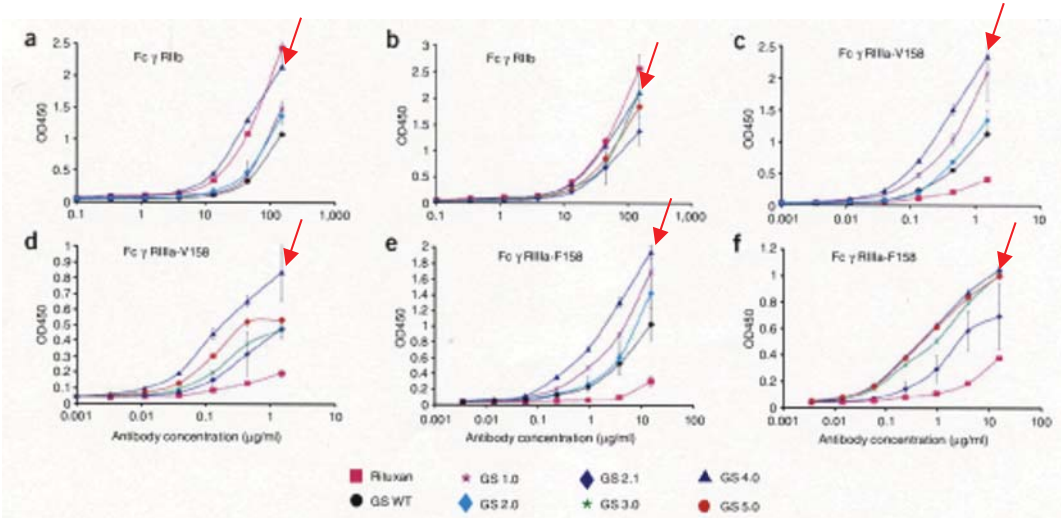
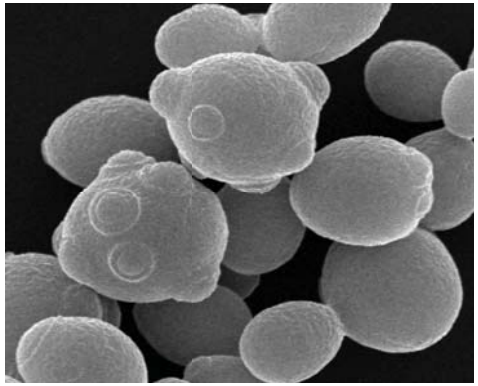


Figure 3 Receptor binding assays of rituximab glycovariants. (a–f) Binding to human FcγRIIb (a,b), FcγRIIIa 158V (c,d), FcγRIIIa 158F (e,f).

Full-length antibody production in *O. minuta*

Kuroda *et al.*, *FEMS Yeast Res.*, **7**, 1307-1316 (2007)

Collaborative Research between AIST, Kyowa-Kirin Co. and Daiichi-Sankyo Pharma Co.



Host cells:

Ogataea minuta:

YK6 cell *(Δoch1 Δyps1 Δade1 Δura3)*

Vector:

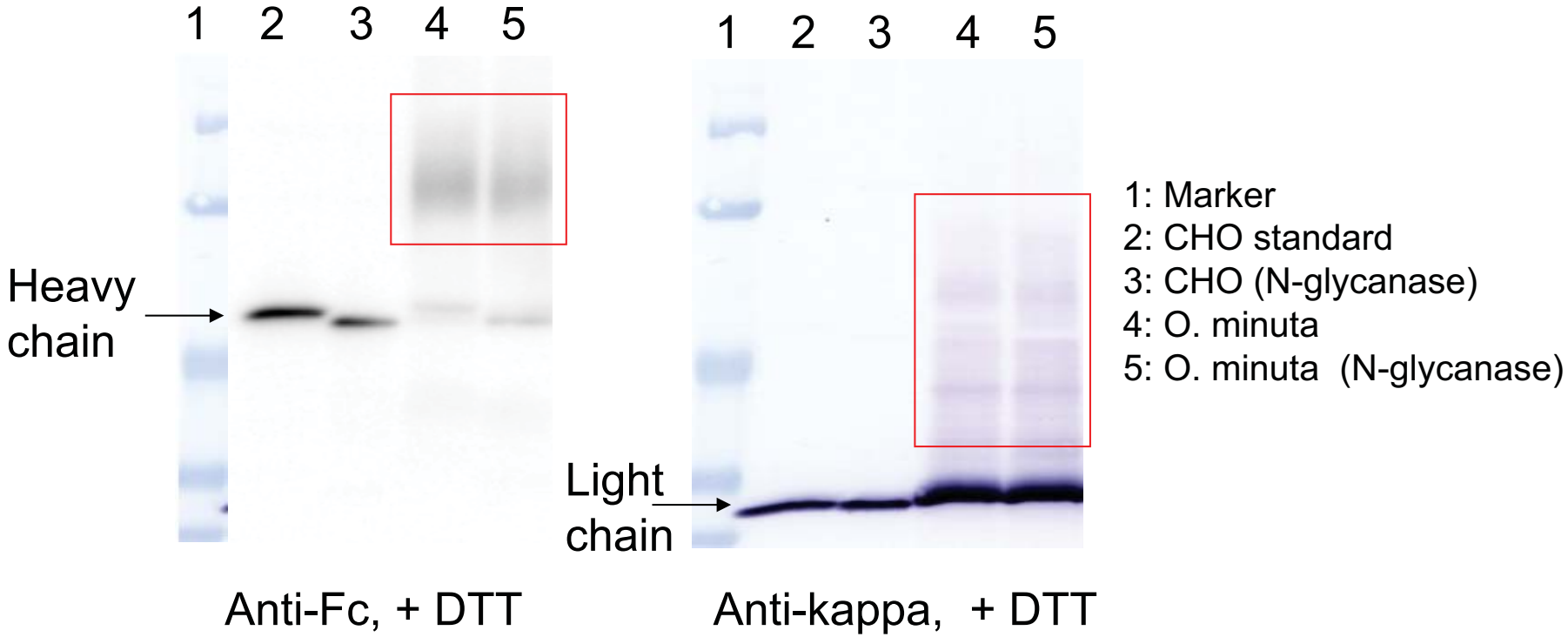
***OmTDH1 (GAP)* promoter,**

***ADE1 or URA3* marker**

Expressed antibody:

human anti-TRAIL-R antibody light and heavy chain fused with Sc invertase secretion signal (sL/pOMGPA1 & sH/pOMGU1ΔSp)

Detection of unusual *O*-glycosylation of antibodies in yeast



Western blot analysis of secreted antibodies

Smear signals with higher molecular mass of heavy and light chains were observed even after *N*-glycanase treatment, indicating the presence of *O*-glycosylation for antibodies.

Inhibition of yeast protein:*O*-mannosylation by a chemical reagent

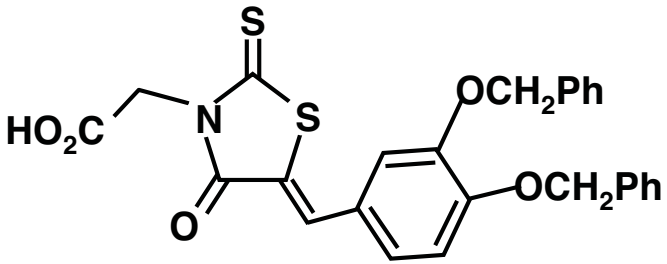
O*-glycan structure in *S. cerevisiae

Ser/Thr –αMan2αMan2αMan3

αMan3

αMan

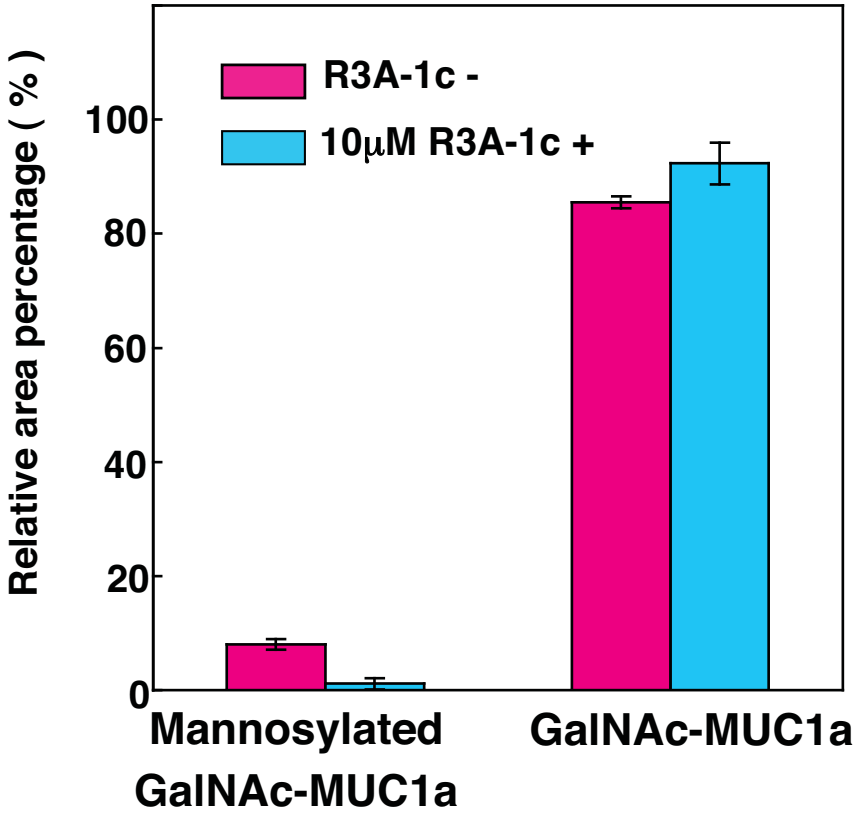
Pmt proteins



Rhodanine-3-acetic acid 1c (R3A-1c)

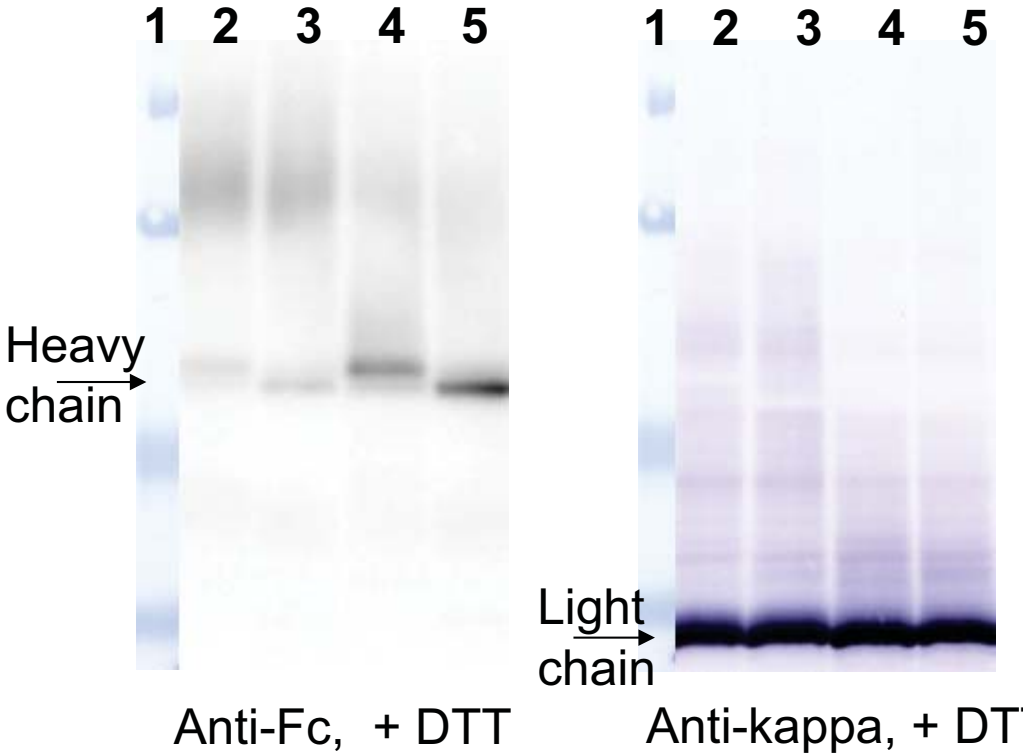
(Inhibitor of *C. albicans* Pmt1p)

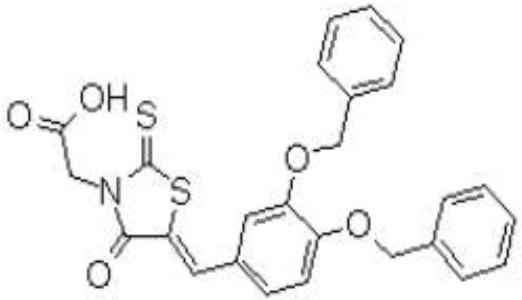
Bioorganic & Medicinal Chemistry Letters, **14:**3975-3978 (2004)



Amano *et al., PNAS*, **105**:3232-7 (2008).

Effect of R3A-1c on *O*-mannosylation of secreted antibodies (1)



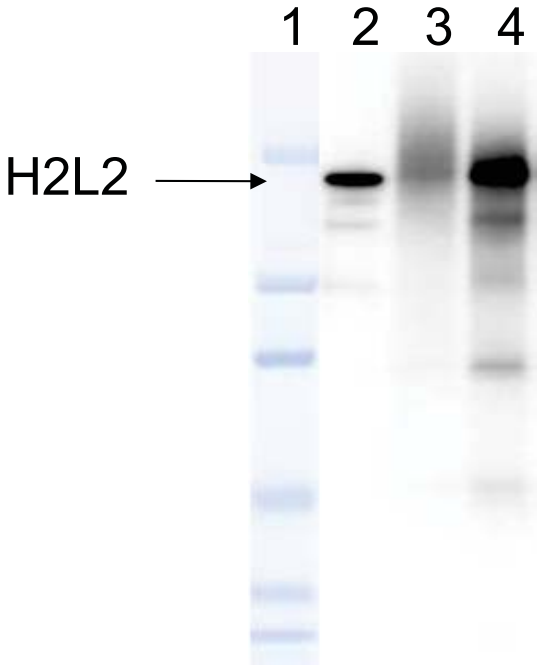


1: Marker
2: - R3A-1c
3: - R3A-1c
(N-Glycanase treatment)
4: + R3A-1c (10μM)
5: + R3A-1c (10μM)
(N-Glycanase treatment)

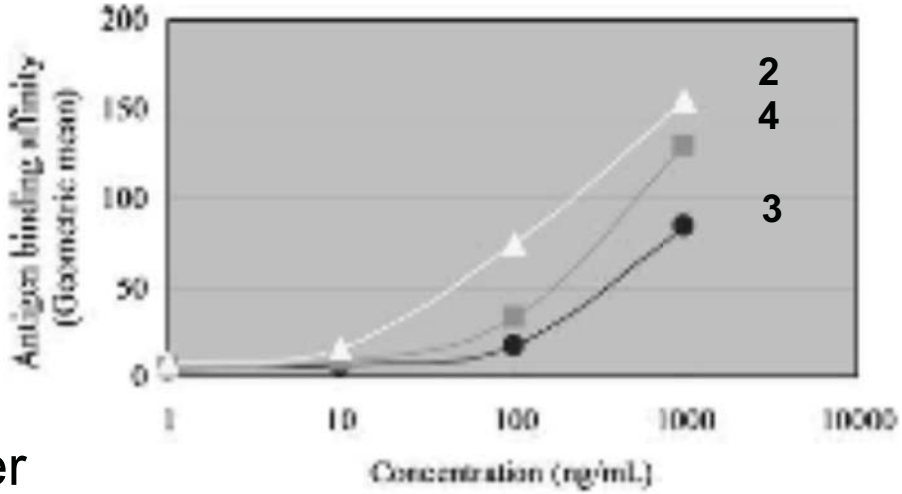
Western blot analysis of secreted antibodies with or w/o R3A-1c

Smear signals decreased by the addition of R3A-1c.

Effect of R3A-1c on O-mannosylation of secreted antibodies (2)



Anti-Fc, - DTT



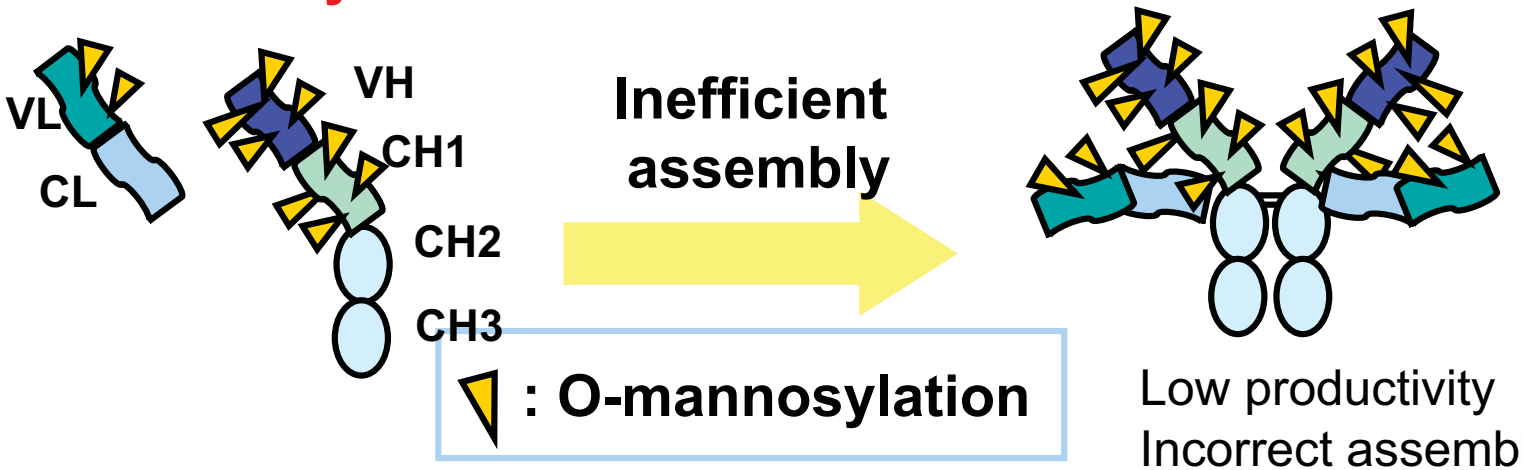
1: Marker
2: CHO standard
3: - R3A-1c
4: + R3A-1c

Western blot analysis of secreted antibodies with and w/o R3A-1c

Smear signals decreased and assembled antibody signals (H2L2) increased, indicating the inhibition of O-mannosylation leads to correct assembly of heavy chains and light chains

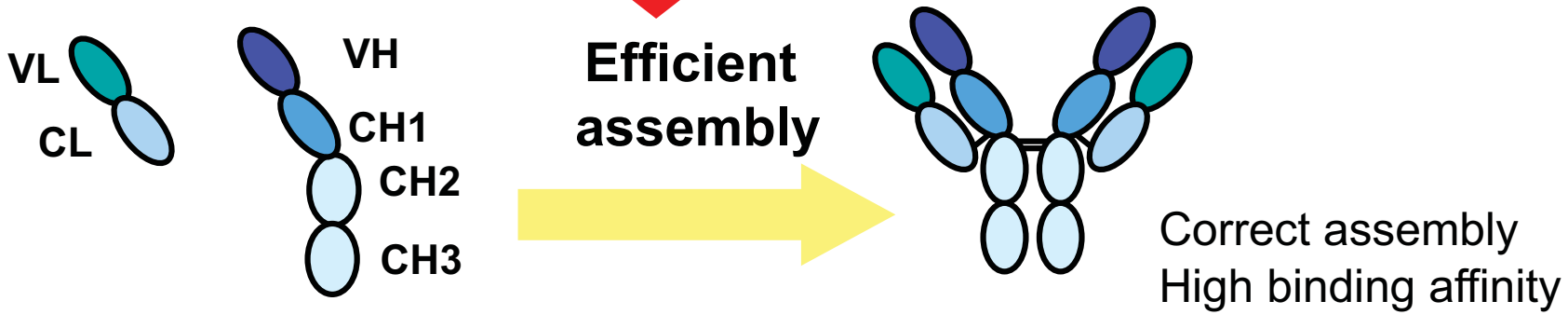
Suppression of O-mannosylation improved full-length antibody productivity in *O. minuta*

With O-mannosylation



Suppression of O-mannosylation by PMT inhibitor (R3A-1c)

Without O-mannosylation



Lysosomal diseases

Gaucher disease

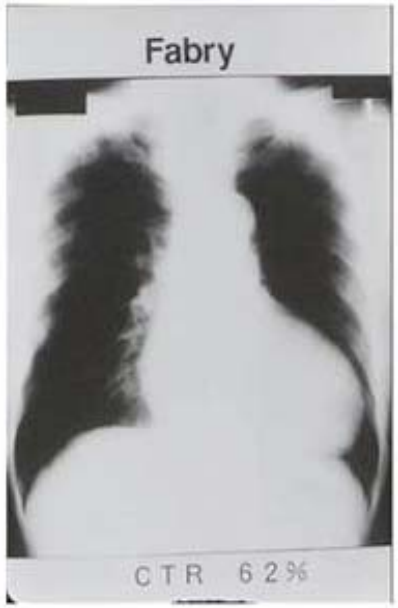
Incidence:
one in 3,000 (Jewish)
one in 100,000 (others)

Pompe disease

Incidence:
one in 60,000
people

Mucolipidosis
(-I、-II、-VI)

Incidence:
one in 60,000 (type-I)
one in 80,000 (type-II)



Fabry disease

Incidence:
one in 3,000- 6,000

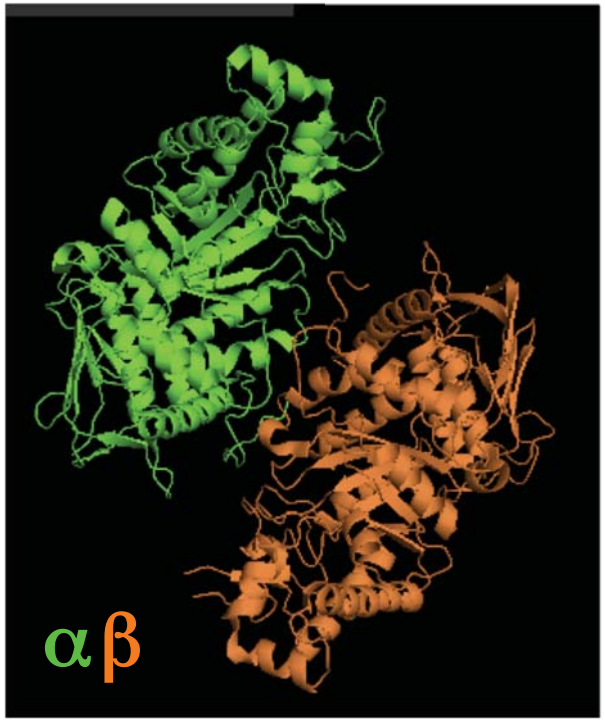
Problems

− High-cost production and unstable supply of the enzyme for treatment

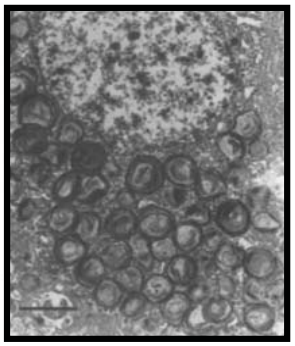
Answer

- Development of expression system for lysosomal enzymes with low-cost and safety.

GM2-gangliosidosis



HexA deficiency

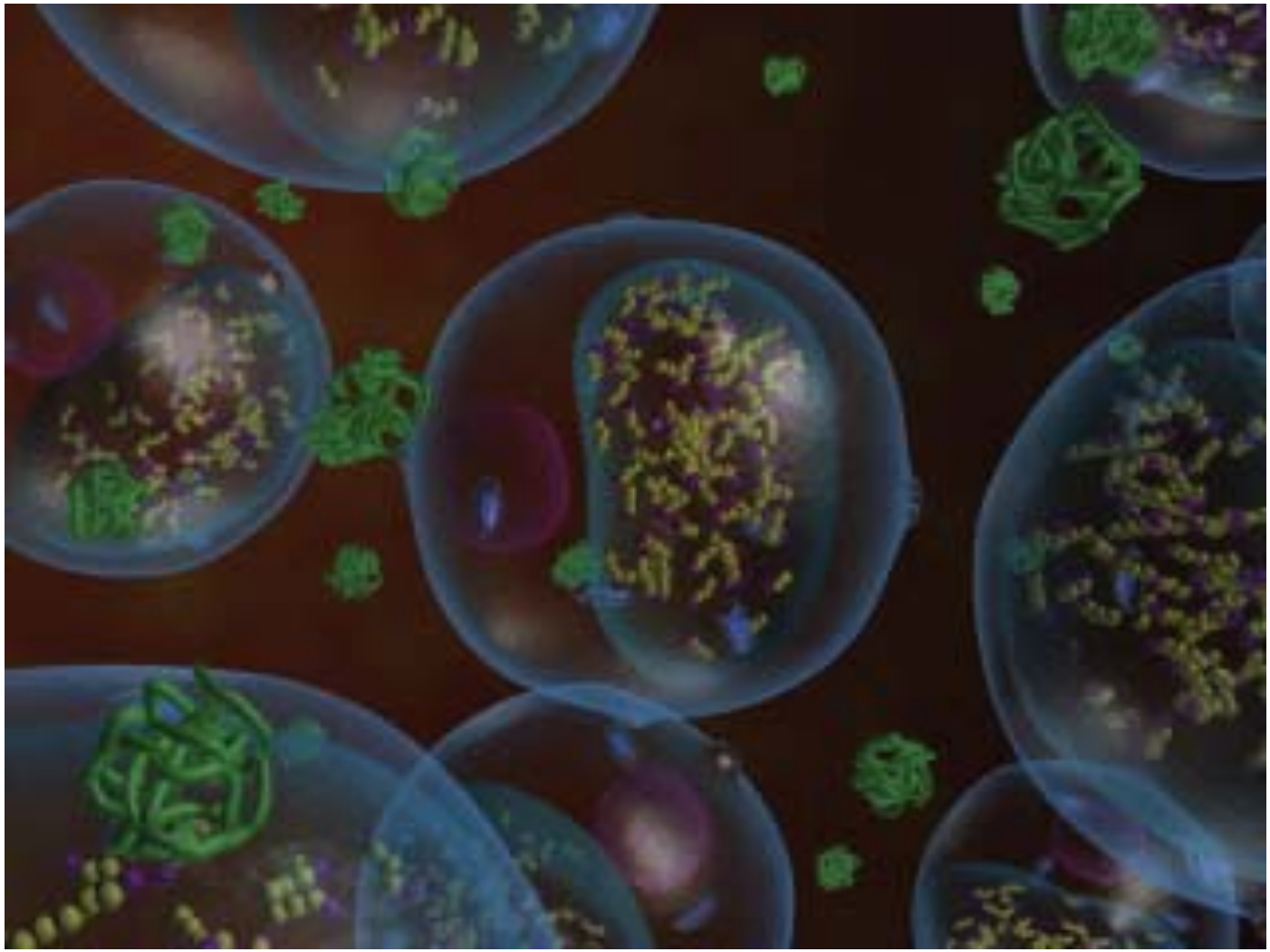


GM2 accumulates in neural cells

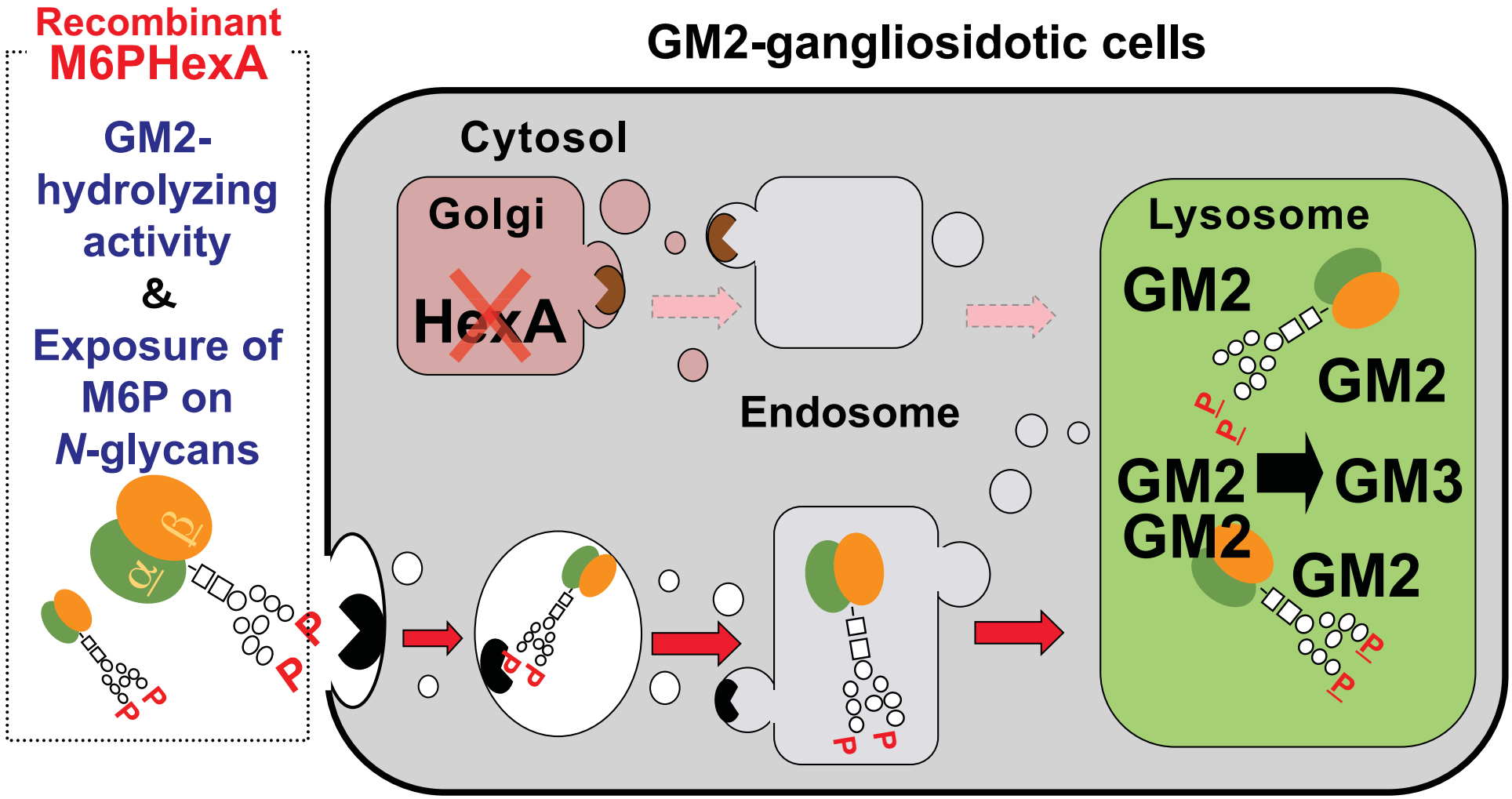
Onset of GM2-gangliosidosis

- Pathology of GM2-gangliosidosis is exhibited as neural disorders.
- Rapid progression leads to a vegetative state within a few years.
- In the case of slower progression of the disease, muscle weakness are also observed.

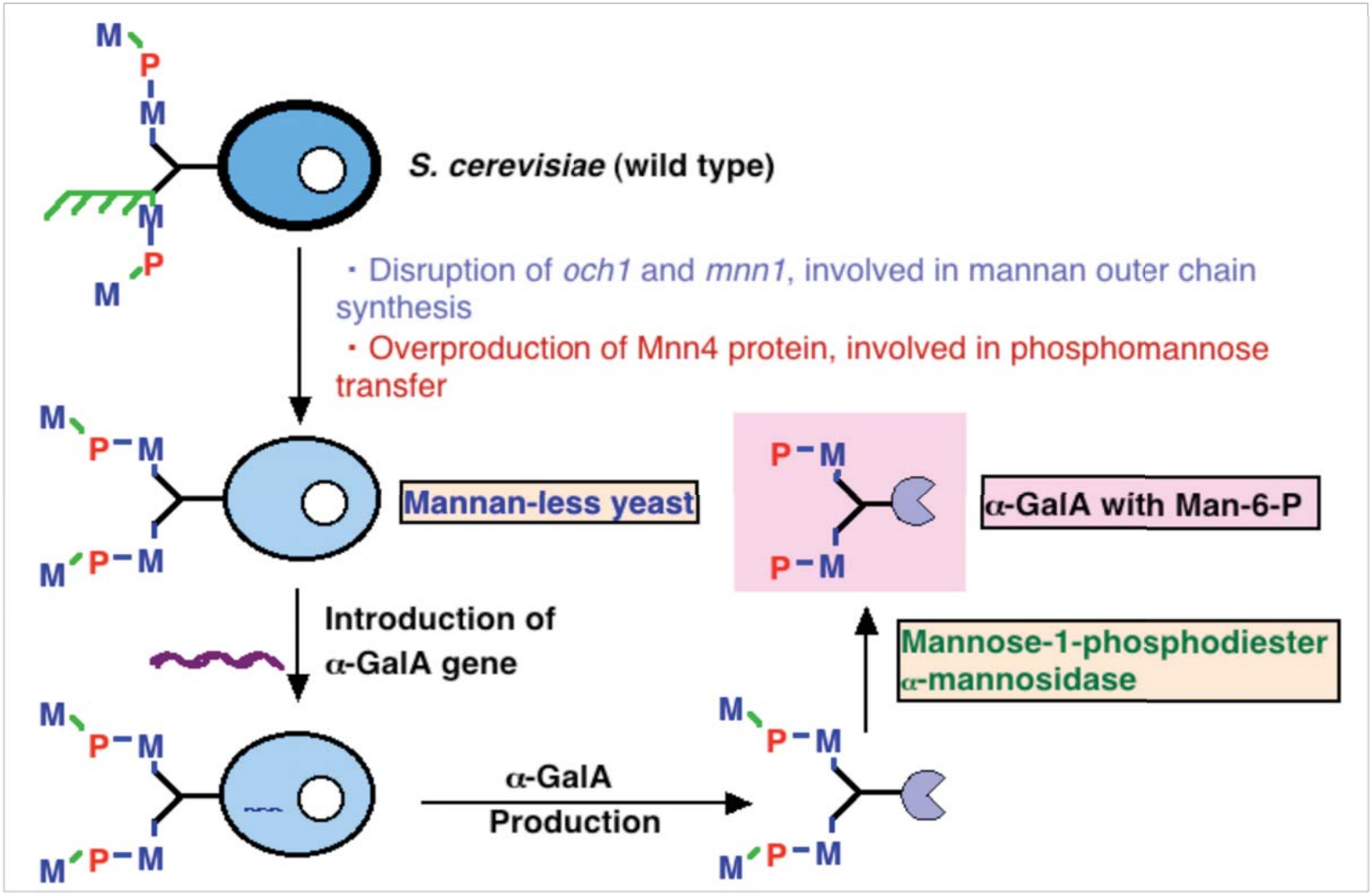
Enzyme replacement therapy for lysosomal diseases



Enzyme replacement therapy (ERT) of GM2-gangliosidosis



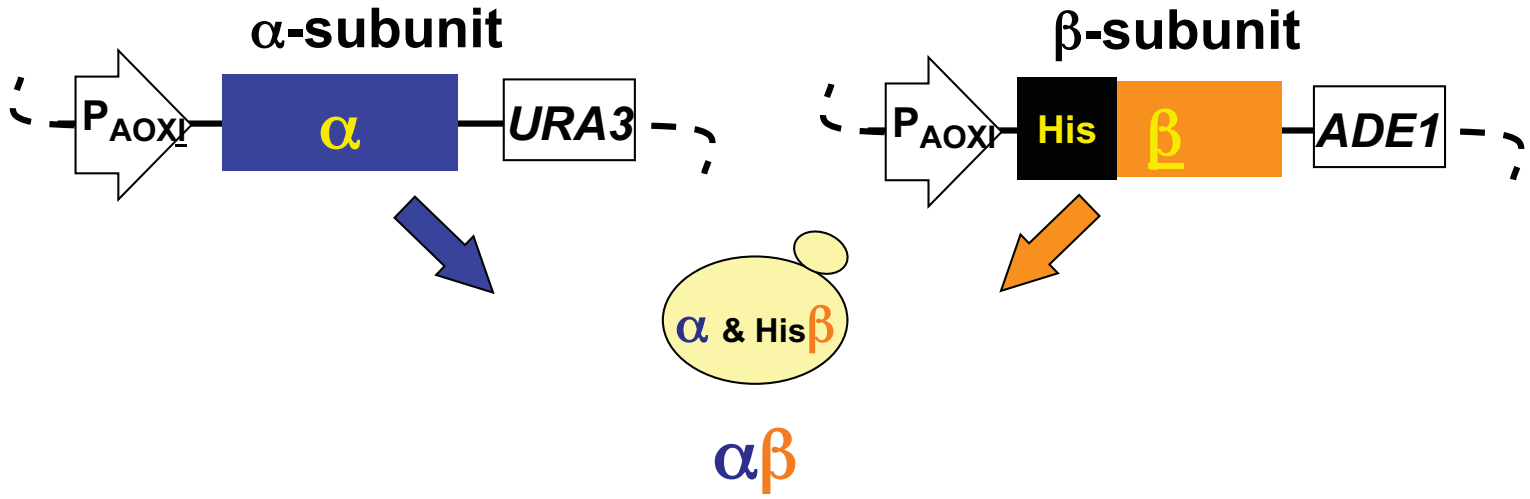
Production of glycoprotein with mannose-6-phosphate-type glycan by yeast cells



Chiba *et al*., *Glycobiology*, **12**, 821-828 (2002)

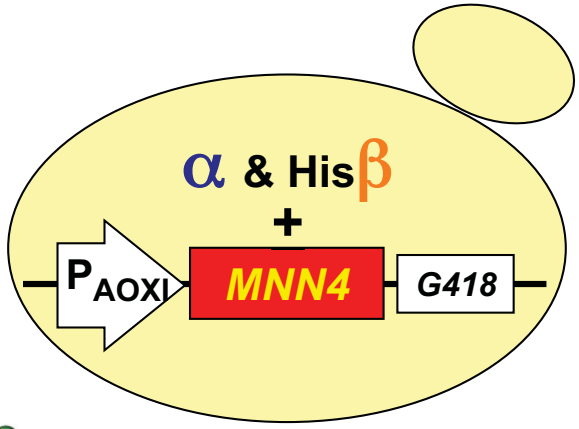
Expression of recombinant HexA

Host : *O. minuta* TK5-3 (Δ*ade1*, Δ*ura3*, Δ*och1*)



Expression of recombinant HexA

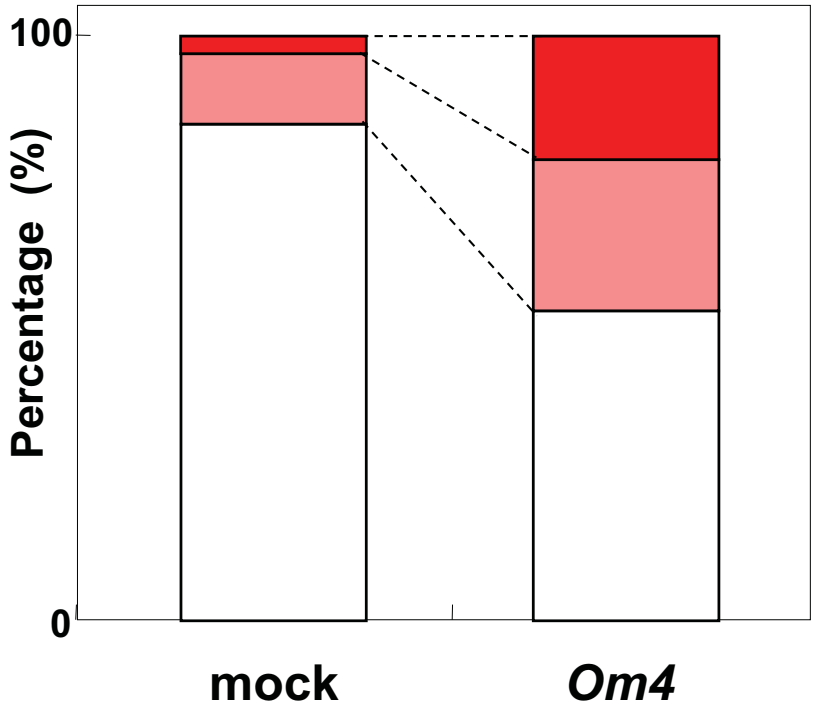
Expression of highly mannosylphosphorylated recombinant HexA



***MNN4* expression vectors**

OmMNN4-1, -2, -3, -4* & *ScMNN4

Glycan contents in the recombinant HexA produced by *O. minuta*



	mock	*Om4*
	(%)	(%)
Neutral glycans (GN2M5 – GN2M33)	86.0	55.0
Acidic glycans	**14.0**	**45.1**
1*M6P (GN2M7 – GN2M33)	9.6	26.1
2*M6P (GN2M7 – GN2M16)	4.4	19.0

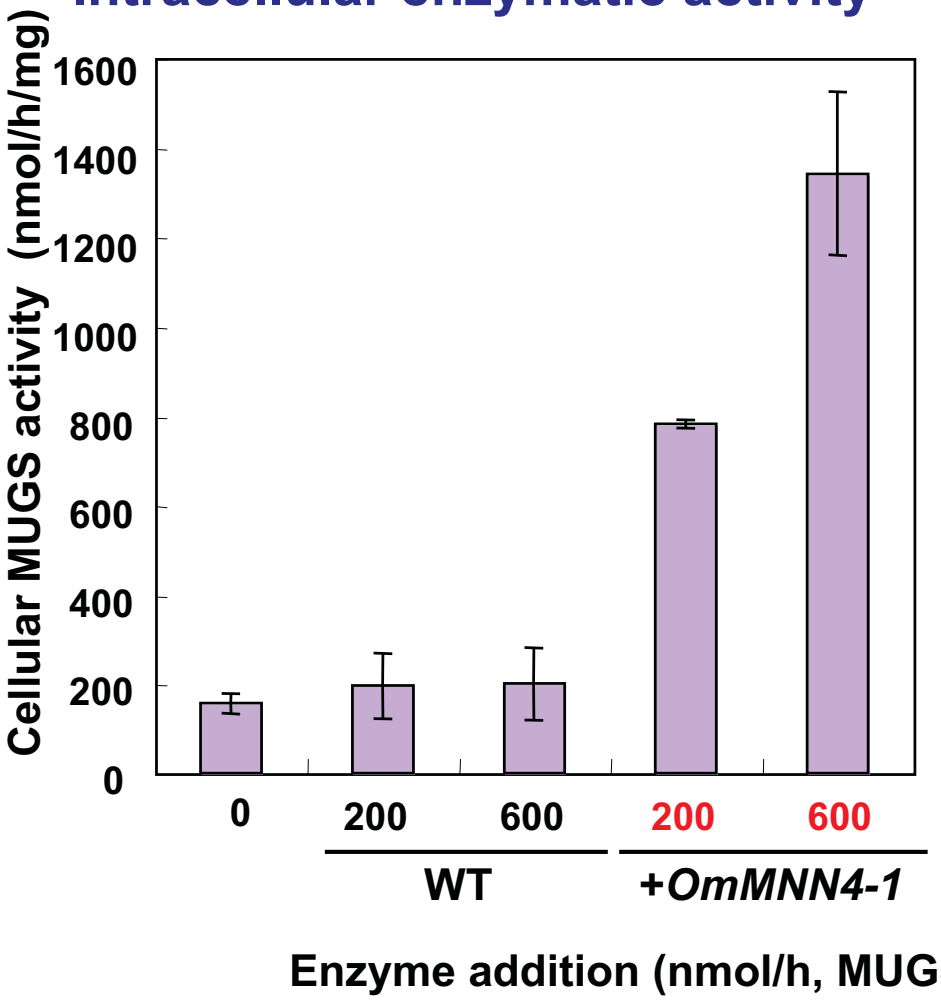
(n=3)

Overexpression of OmMNN4 gene caused increase in the percentage of phosphorylated glycans.

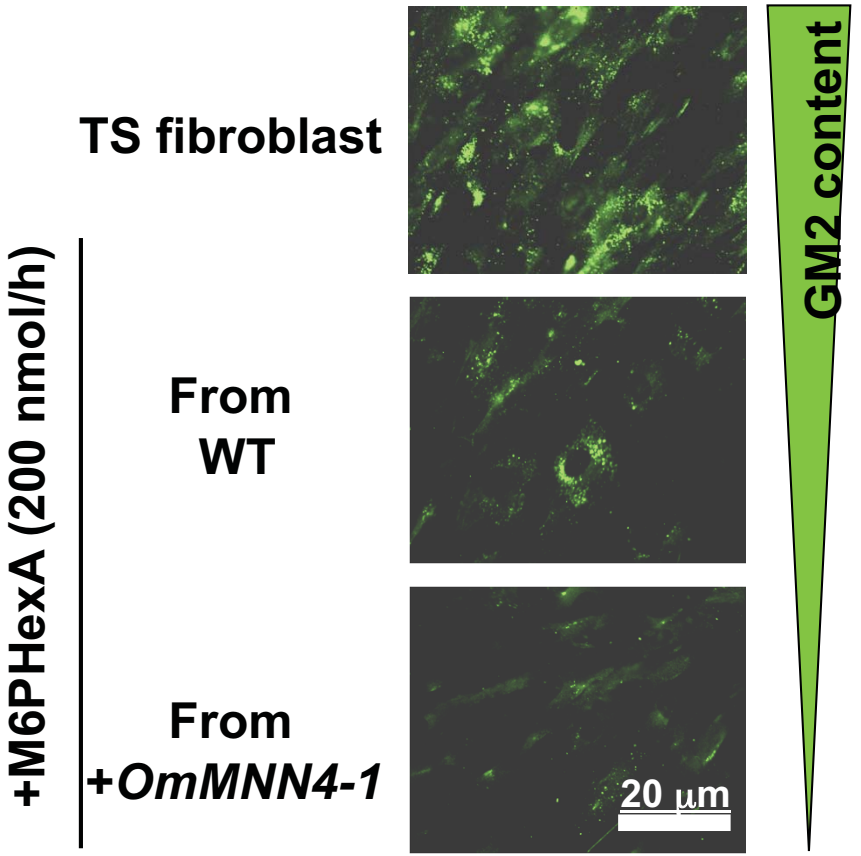
Akeboshi et al., Glycobiology. 2009 Sep;19(9):1002-9.

Incorporation of recombinant HexA into cells

Intracellular enzymatic activity



Degradation of intracellular GM2



Enzyme uptake & GM2 hydrolyzing activity
WT (Low M6P) < *+OmMNN4-1* (High M6P)

Akeboshi *et al.*, *Glycobiology* **19**:1002-9 (2009).

Intracerebroventricularly administration to Sandhoff disease model mice

PBS

hHexA *MNN4* (-)

hHexA *MNN4* (+)

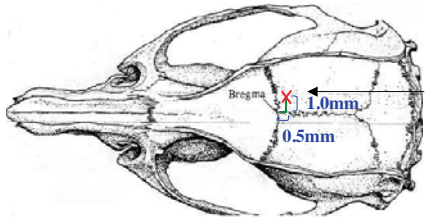
SD mice (*Hexb* -/-)
10 weeks ages

ICV administration

Rota-rod test

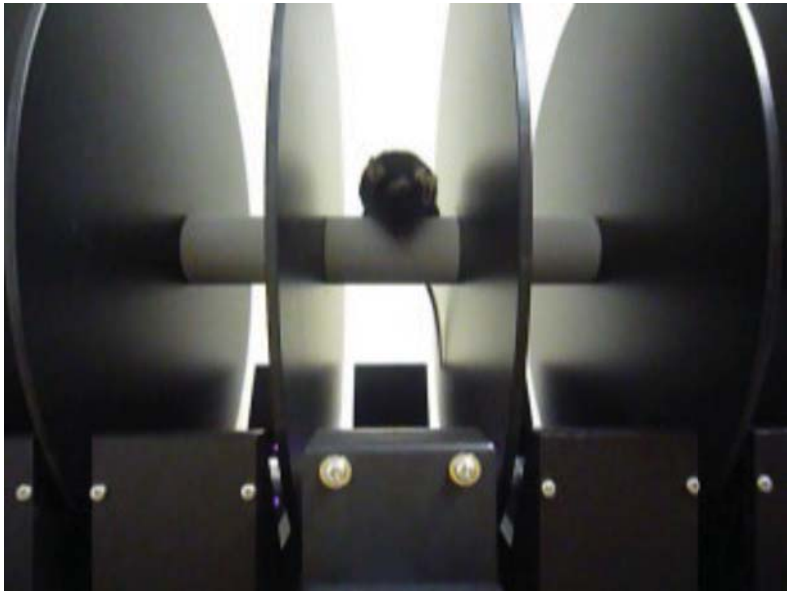
Foot print test

0.6mg/kg
MUG : 1.0×10^4nmol/h
MUGS: 0.4×10^4nmol/h



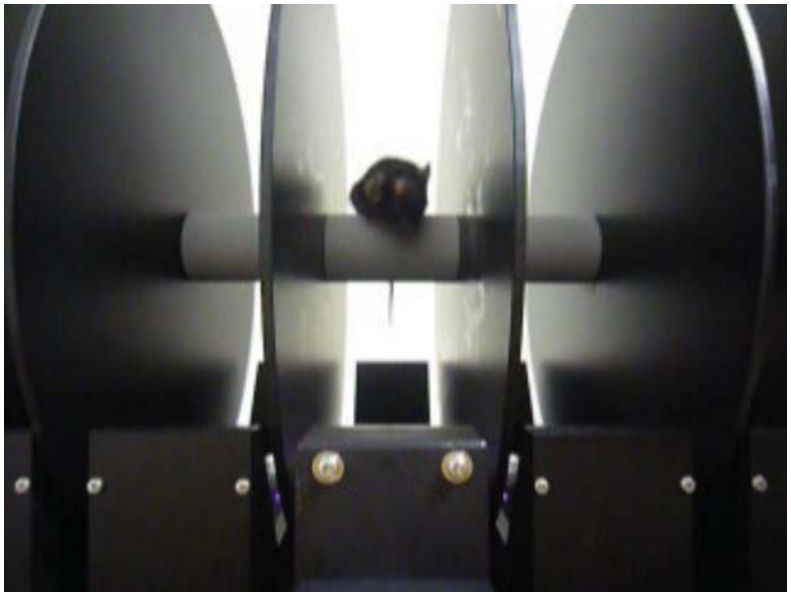
injection site

Tsuji *et al.*, *Ann Neurol.* **69**:691-701 (2011).



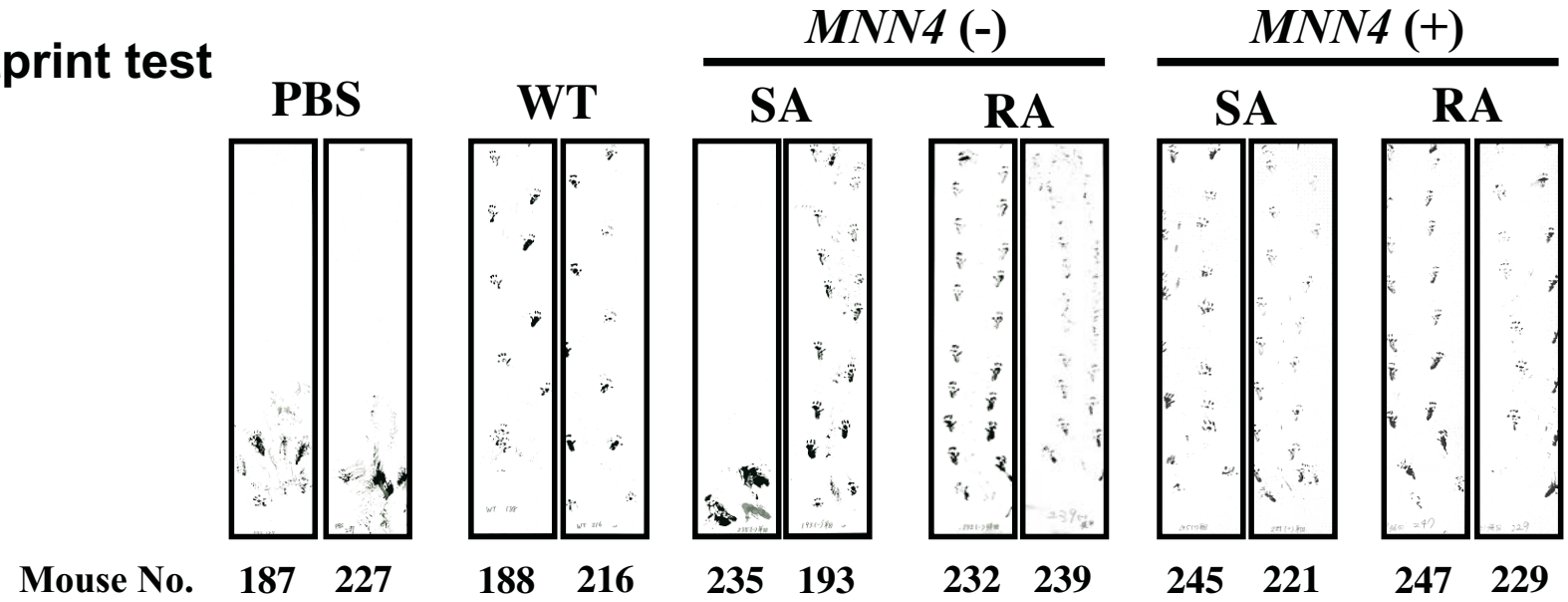
PBS administration

HexA administration

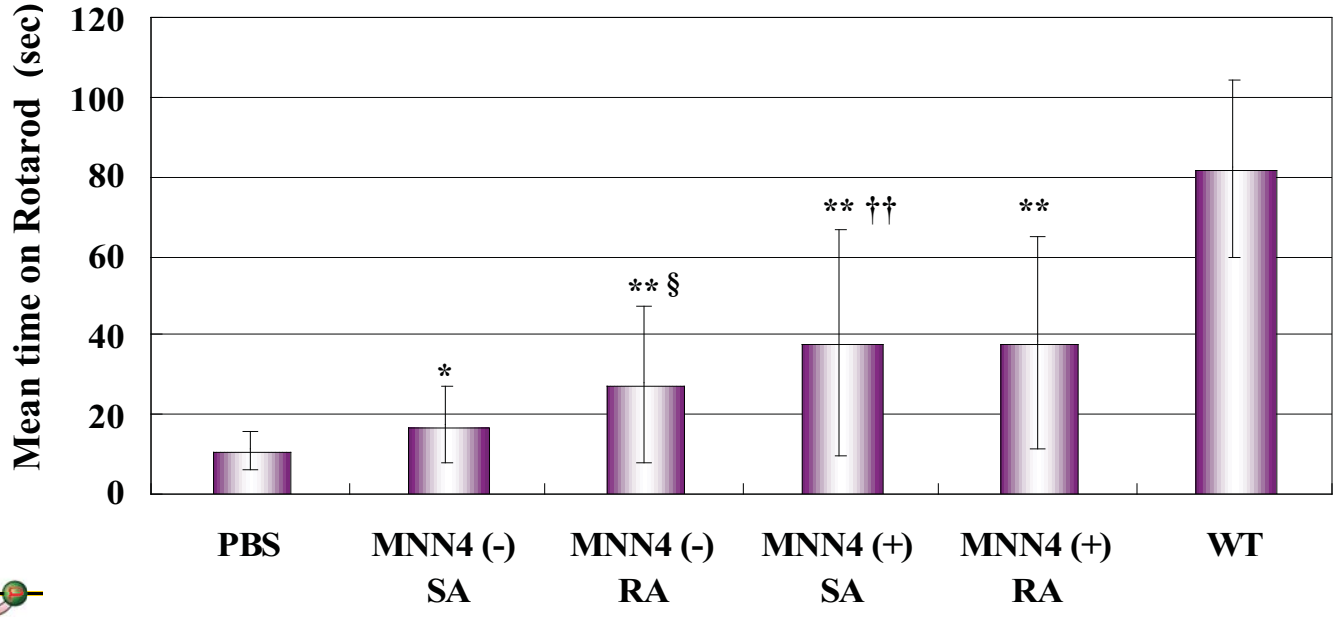


M6PHexA administration

Footprint test



Rota-rod test



* : $P < 0.05$ (versus SD Untreated)
** : $P < 0.01$ (versus SD Untreated)
†† : $P < 0.01$ (versus *MNN4* (-))
§ : $P < 0.05$ (SA versus RA)

Acknowledgement

AIST

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Tokushima Univ.

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Kazuo Kobayashi
Kosuke Kuroda

Dai-ichi Sankyo Co.

Koichi Nonaka
Yoshihisa Ichikawa

Otsuka Chemical Co.

Hiroaki Asai
Akio Kanatani
Hiroshi Shibutani
Kazuhiro Fukae

NEDO
JST・CREST
NIBIO

Thank you for your attention.