Glycoconj J (2008) 25:121–136
DOI 10.1007/s10719-007-9064-x

Temporal changes in the carbohydrates expressed on BG01 human embryonic stem cells during differentiation as embryoid bodies

Kimberly A. Wearne · Harry C. Winter · Irwin J. Goldstein

Received: 25 April 2007 / Revised: 27 June 2007 / Accepted: 2 July 2007 / Published online: 3 August 2007

Abstract Cell surface carbohydrates present on BG01 human embryonic stem cells after 28 days of differentiation were examined using two classes of carbohydrate binding proteins: lectins and antibodies specific for carbohydrate epitopes. Specificity of lectin staining was verified using carbohydrate ligands to block lectin interaction, glycohydrolases to cleave specific sugar residues that are receptors for these proteins, and periodate oxidation to destroy susceptible sugar residues. Specific antibodies were used to identify various tissue types and germ layers present in the 12- and 28-day differentiating embryoid bodies. Results from 12 and 28-day differentiated embryoid bodies were compared to determine changes over time. A slight increase in the sialylation of α-GalNAc was seen between 12 and 28 days of differentiation due to the presence of sialyl Tn and/or other sialylated α-GalNAc residues. Increases were also observed in GalNAc, the T antigen (Gal β1,3 GalNAc), and difucosylated LacNAc residues during this time interval. Additionally, some distinct differences in the *pattern* of lectin staining between 12 and 28 days were observed. Not unexpectedly, the presence of most differentiated cell-types increased during this time period with the exception of neural progenitors, which decreased. Undifferentiated cells, which were prevalent in the 12-day EBs, were undetectable after 28 days. We conclude that several changes in glycosylation occurred during the differentiation of embryonic stem cells, and that these changes may play a role in embryonic development.

Lectin abbreviations can be found in Table 1.

K. A. Wearne · H. C. Winter · I. J. Goldstein (✉)
Department of Biological Chemistry, University of Michigan,
1150 West Medical Center Drive, 3220E MSRBIII,
Ann Arbor, MI 48109, USA
e-mail: igoldste@umich.edu

Keywords Lectin · Human embryonic stem cell · Differentiation · Embryoid body · Antibody

Abbreviations

ABS	acetate buffered saline
BSA	bovine serum albumin
EB	embryoid body
HESC	human embryonic stem cell
PBS	phosphate buffered saline
PFA	paraformaldehyde
T antigen	Thomsen–Friedenreich antigen, Gal β1,3 GalNAc α1—Ser/Thr
Tn antigen	GalNAc α1—Ser/Thr

Introduction

The important roles that cell-surface carbohydrates play in the early development of many organisms is well established. Phenomena such as cell–cell interaction and communication, cellular adhesion and subcellular targeting have been shown to involve glycoconjugates [1]. We have examined the carbohydrate residues present on the surfaces of BG01 human embryonic stem cells (hESCs) and the changes in expression of these glycoconjugates during the course of differentiation as embryoid bodies (EBs). In an earlier study, we probed the carbohydrate residues present on hESCs differentiated for 12 days as EBs using carbohydrate-binding proteins (lectins and anti-carbohydrate antibodies, [2]). We defined, in some detail, the principal sugars present and the subtle changes that occurred between undifferentiated cells [3] and day 12 of development. The present communication describes changes that occur in the expression of cell surface carbohydrates between days 12 and 28 of

differentiation as EBs. We employed an extensive array of lectins and anti-carbohydrate antibodies, as well as cell-type-specific antibodies to determine the carbohydrates present on 28-day cells and their correlation with the cell types present. Understanding the changes in carbohydrates that are expressed during EB formation and differentiation could allow us to develop new methods of identifying and possibly promoting differentiation of specific cell types.

Materials and methods

For information regarding the sources of fluorescein-labeled lectins and their abbreviations, see Table 1. These lectins were assayed for extent of labeling by spectrophotometry by their A_{495}/A_{280} absorbance ratio, and for retention of agglutination activity, where appropriate.

Inhibitory carbohydrate ligands and hydrolytic enzymes were used to confirm the specificity of binding of the lectins. Inhibitory ligands used in this study were: *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc) from Pfanstiehl Laboratories, Inc. (Waukegan, IL); *N*,*N*′,*N*″-triacetylchitotriose, *N*-acetyllactosamine (LacNAc), unlabeled ECA, and methyl α mannoside (Me α Man) available in this laboratory from previous studies; benzyl T antigen, α-2,3 sialyllactose, L-fucose, human milk sialyllactose (80% α2,6-linked, 20% α2,3-linked), lactose, and *N*-acetylneuraminic acid (sialic acid, Neu5Ac) from Sigma (St. Louis, MO, USA). Enzymes used to cleave specific carbohydrate moieties were *O*-glycosidase (endo-α-*N*-acetylgalactosaminidase, CalBioChem, San Diego, CA), *Aspergillus oryzae* β-galactosidase, coffee bean α-galactosidase and *Clostridium perfringens* neuraminidase (Sigma). Sodium meta periodate was purchased from J.T. Baker Chemical Company (Phillipsburg, NJ, USA). Inert blocking proteins used were bovine serum albumin (BSA, Sigma) and normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Other reagents used were Prolong Gold from Molecular Probes (Eugene, OR), 1000X Hoechst 23187 from Sigma, Triton X-100 from Research Products International (Elk Grove Village, IL), and paraformaldehyde (PFA) from Matheson Coleman and Bell Manufacturing Chemists (Norwood, OH, USA).

The following primary antibodies were used: Brachyury (mesoderm, polyclonal, rabbit) from Abcam (Cambridge, MA, USA); PDX1 (pancreas, polyclonal, rabbit) from Affinity Bioreagents (Golden, CO, USA); antibodies to the Thomsen–Friedenreich antigen (Gal β1,3 GalNAc α1—Ser/Thr, monoclonal, mouse, clone A78-G/A7) and the Tn Antigen (GalNAc α1—Ser/Thr, monoclonal, mouse, clone B1.1) from Biomeda (Foster City, CA, USA); Tuj1 (class III β-Tubulin, neurons, monoclonal, mouse) from Covance (Berkley, CA, USA); Keratin-14 (epidermal ectoderm, monoclonal, mouse, clone LL002) from LabVision Corporation (Fremont, CA, USA); and Sox3 (neural progenitors, H-135, polyclonal, rabbit), Osteocalcin (osteo-

Table 1 Lectin abbreviations and sources

Abbreviation	Lectin name
AAA[a,b]	*Anguilla anguilla* agglutinin (eel serum)
AAL[c,d]	*Aleuria aurantia* lectin (orange peel fungus)
ABA[a,b]	*Agaricus bisporus* agglutinin (edible mushroom)
ACA[c,b]	*Amaranthus caudatus* agglutinin
BanLec[e,d]	Banana lectin (*Musa acuminata*)
Con A[e,d]	Concanavalin A (jack bean)
DBA[c,b]	*Dolichos biflorus* agglutinin
DSA[e,d]	*Datura stramonium* agglutinin (jimson weed)
ECA[c,b]	*Erythrina cristagalli* agglutinin (coral tree)
EEA_1[c,b]	*Euonymus europaeus* agglutinin (spindle tree)
Gleheda[f,d]	*Glechoma hederacea* agglutinin (ground ivy)
GNA[c,b]	*Galanthus nivalis* agglutinin (snowdrop bulb)
GS $I\text{-}A_4$[e,d]	*Griffonia simplicifolia I-A*$_4$
GS $I\text{-}B_4$[c,b]	*Griffonia simplicifolia I-B*$_4$
GS II[c,b]	*Griffonia simplicifolia II*
HAA[a,b]	*Helix aspersa* agglutinin (garden snail)
HPA[a,b]	*Helix pomatia* agglutinin (edible snail)
IRL[e,d]	*Ischnoderma resinosus* lectin
LBL[a,b]	Lima bean lectin (*Phaseolus lunatus*)
LEA[c,b]	*Lycopersicon esculentum* agglutinin (tomato)
Lentil[a,b]	Lentil lectin (*Lens culinaris*)
LFA[e,d]	*Limax flavus* agglutinin (slug)
Lotus[c,d]	*Lotus tetragonolobus*
LPA[e,d]	*Limulus polyphemus* agglutinin (horseshoe crab)
MAA[a,b]	*Maackia amurensis* agglutinin
MCA[e,d]	*Maackia chinensis* agglutinin
MOA[e,d]	*Marasmius oreades* agglutinin
Morniga G[f,d]	*Morus nigra* agglutinin G (black mulberry)
PNA[a,b]	Peanut agglutinin (*Arachis hypogaea*)
PSL[e,d]	*Polyporus squamosus* lectin
PVL[g,d]	*Psathyrella velutina* lectin
PWA[a,b]	Pokeweed agglutinin (*Phytolacca americana*)
RCA[c,b]	*Ricinus communis* agglutinin (castor bean)
SBA[c,b]	Soybean agglutinin (*Glycine max*)
SNA[e,d]	*Sambucus nigra* agglutinin (black elderberry)
STL[c,b]	*Solanum tuberosum* lectin (potato)
UEA1[c,b]	*Ulex europaeus* agglutinin 1 (gorse seed)
VVA[a,b]	*Vicia villosa* agglutinin (hairy vetch)
WFA[a,b]	*Wistaria floribunda* agglutinin
WGA[c,b]	Wheat germ agglutinin (*Triticum vulgaris*)
WGA-succ[c,b]	Succinyl—wheat germ agglutinin (*Triticum vulgaris*)

[a] EY Laboratories (San Mateo, CA, USA)
[b] Purchased as FITC-labeled
[c] Vector Laboratories (Covington, LA, USA)
[d] FITC-labeled in lab according to a previously described method [2]
[e] Available from previous experiments
[f] Gift of Els Van Damme (Dept of Molecular Biotechnology, Ghent University, Ghent, Belgium)
[g] Wako Chemicals, USA (Richmond, VA, USA)

blasts, M15, polyclonal, goat), Oct 3/4 (embryonic stem cells, N-19, polyclonal, goat), and Sox17 (endoderm, S20, polyclonal, goat) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies were donkey anti mouse Cy3, donkey anti rabbit Texas Red, and donkey anti goat Texas Red purchased from Jackson Immuno-Research Laboratories, Inc.

Cryostat sections of differentiated EBs were obtained from the Center for Organogenesis, Michigan Center for Human Embryonic Stem Cell Research, University of Michigan. Cryostat sections of mouse tissues used in testing antibodies were obtained from the Center for Organogenesis, Morphology Core, University of Michigan. Cryostat sections or paraffin-embedded sections of human tissues used in testing antibodies were obtained from and sectioned by the University of Michigan Comprehensive Cancer Center Tissue Core.

Procedures for fluorescein-labeling of lectins, embryoid body formation from human embryonic stem cells, cryostat sectioning, lectin staining of differentiated cryostat sections, antibody staining, and neuraminidase treatment were previously described [2]. Lectin or antibody staining of human or mouse tissue cryostat sections proceeded as for EB cryostat sections. Singular specificities of the fucose-binding lectins (AAA, AAL, EEA1, Lotus, and UEA1) were determined by the Consortium for Functional Glycomics using a glycan array.

Microscopy and data collection

Each slide was analyzed on an Olympus BX-52 fluorescent microscope. Data was obtained by designating each EB viewed as either positive (at least a few cells within the EB stained well above background) or negative (no staining was seen above background) and calculating a percentage of EBs that were positive out of the total number viewed. Background was determined by viewing control slides (no lectin or primary antibody) under the green or red filter and comparing to positively stained slides.

Sodium periodate oxidation

Sodium meta periodate (100 mM) in 0.1 M acetate buffer with 0.15 M sodium chloride (ABS) at pH 4.5 was applied to the cryostat section for 1 h at room temperature in the dark in a moist chamber. The slide was rinsed three times in PBS for 5 min each and stained with lectin or antibody.

β-Galactosidase treatment

A. oryzae β-galactosidase, dissolved in ABS, pH 4.5 at a concentration of 300 U/ml, was applied to the cryostat section in a moist chamber for 1 h at room temperature, then rinsed and stained as above.

α-Galactosidase treatment

Coffee bean α-galactosidase was diluted 1:5 in PBS, pH 6.5, applied to the cryostat section in a moist chamber for 1.5 h at 37°C, then rinsed and stained as above.

O-Glycosidase treatment

O-Glycosidase (endo-α-*N*-acetylgalactosaminidase) at an original concentration of 1 U/ml, was diluted 1:50 in ABS, pH 5.0 and applied to the cryostat section in a moist chamber for 1 h at 30°C, then rinsed and stained as above.

Antibody staining of formalin-fixed, paraffin-embedded human pancreas sections

Slide(s) were placed on a heating block at 80°C for 10 min to melt the paraffin, removed from heat and immersed in xylene for 2 min, 2 min, and 1 min; 100% ethanol for 1 min and 1.5 min; then 95% ethanol for 30 s and water for 30 s. The slides were then wiped clean, rinsed twice with PBS, and stained with lectins or antibodies as for cryostat sections, omitting PFA treatment.

Results

In this communication we address the changes that occur in cell-surface carbohydrates during the first month of development of BG01 hESCs as EBs using lectins and anti-carbohydrate antibodies for evaluation. As controls we have employed specific carbohydrate ligands to block lectin and antibody binding, glycohydrolases to cleave lectin and antibody-specific sugar residues, and sodium periodate to chemically modify carbohydrate groups. Lectin-staining procedures included incubation with an inert blocking protein (BSA) prior to lectin treatment to prevent non-specific protein binding. For antibody staining, normal donkey serum was used for this purpose. The EBs were also stained with cell-type specific antibodies to correlate glycoconjugate and cell-type presence and locations.

Expression of α-mannosyl-binding lectins in EBs was similar on 12 and 28 day EBs. Four α-mannosyl binding lectins, BanLec, Con A, GNA, and lentil lectin showed nearly 100% binding to both 12- and 28-day EBs (Table 2). In all cases, binding was abolished in the presence of methyl α-mannoside. Periodate oxidation sharply reduced the binding of GNA to both 12- and 28-day EBs (Fig. 1), whereas Con A was unaffected.

It appears that α-galactosyl (α-Gal) residues are not present on the surface of 12- or 28-day differentiated EBs. The α-Gal-binding lectin GS I-B_4 failed to stain EBs, indicating the absence of terminal α-Gal groups [4, 5];

Table 2 EB staining data from α-Man-binding lectins

Lectin	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional information
BanLec	α-Man, 3-*O*-α-Man, branched tri-mannoside (Man α3[Man α6] Man) [34]	12[b]	10	100	0.4 M Me α Man[c]
		28	5	100	
Con A	Terminal α-Man, branched tri-mannoside [4]	12[d]	10	100	0.4 M Me α Man[c], periodate treatment[e]
		28	5	92	Periodate treatment[e]
GNA	Terminal Man α(1,3) Man > Man α(1,6) Man [31]	0[b]	20	25	
		12	10	97	0.4 M Me α Man[c], periodate treatment[c]
		28	10	98	Periodate treatment[c]
Lentil	Terminal αMan > Glc/GlcNAc [4]	12	10	100	0.4 M Me α Man[c]
		28	10	99	0.4 M Me α Man[c]

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Previous data only [2]
[c] Blocked
[d] Includes previous data [2] and new data
[e] No effect

however, MOA, which specifically recognizes Gal α1,3 Gal β1,4 GlcNAc/Glc residues [5], bound moderately well to both 12- and 28-day EBs (Table 3). MOA has also been observed to bind to LacNAc, albeit very weakly [6]. To determine if LacNAc-binding could account for the observed MOA staining, cells were pretreated with either unlabeled ECA (a LacNAc-specific lectin), α-galactosidase, β-galactosidase, or neuraminidase. Unlabeled ECA or β-galactosidase blocked MOA-binding, whereas neuraminidase enhanced binding, and α-galactosidase had no effect. Thus, we conclude that MOA bound to LacNAc, rather than α-Gal, and that terminal nonreducing α-galactosyl residues are absent from both 12- and 28-day differentiated EBs.

The presence of α-GalNAc end groups on differentiated EBs in the form of the Tn antigen and/or other terminal α-GalNAc groups is indicated by the intense staining of the EBs by numerous α-GalNAc binding lectins: SBA, HAA, and VVA all bound very strongly and consistently to both 12- and 28-day EBs (Table 4), as did Morniga G, a lectin known to bind to both the T and Tn antigens [7]. This is easily explained by high amounts of the Tn and T antigens, the latter confirmed by binding of PNA, ACA and the anti-T antibody. A few significant changes between 12 and 28 day EBs were also notable: HPA and WFA binding increased for the 28-day EBs, whereas binding by GS I-A_4, Gleheda and LBL decreased. SBA binding was drastically enhanced by treatment with neuraminidase on both 12 and 28-day cells, indicating the presence of high levels of sialylated α-GalNAc groups. On the other hand, binding by GS I-A_4 and Gleheda was unaffected by neuraminidase treatment of the 12-day cells, and only slightly enhanced by this treatment of the 28-day cells, suggesting that their α-GalNAc binding epitope (possibly α1,3-linked GalNAc) is minimally sialylated on the 28-day cells and not at all on the 12-day cells.

Sialic acid is prevalent on both 12- and 28-day EBs as revealed by the consistently high level of staining by a series of sialic acid binding lectins: LFA, LPA, MAA, MCA, PSL, PVL, SNA, and WGA. The only significant change was the increased binding by LFA, the slug lectin (Table 5), which could indicate the presence of terminal or subterminal sialyl groups. All lectins bound specifically, as demonstrated by their inhibition by specific haptenic sugars. An example of this is LFA shown in Fig. 2 as blocked by 0.1 M Neu5Ac. It appears that undifferentiated cells contain fewer Neu5Ac α2,3 Gal groups than EBs that have differentiated for at least 12 days. This is indicated by the low binding of MAA (specific for Neu5Ac α2,3 Gal/

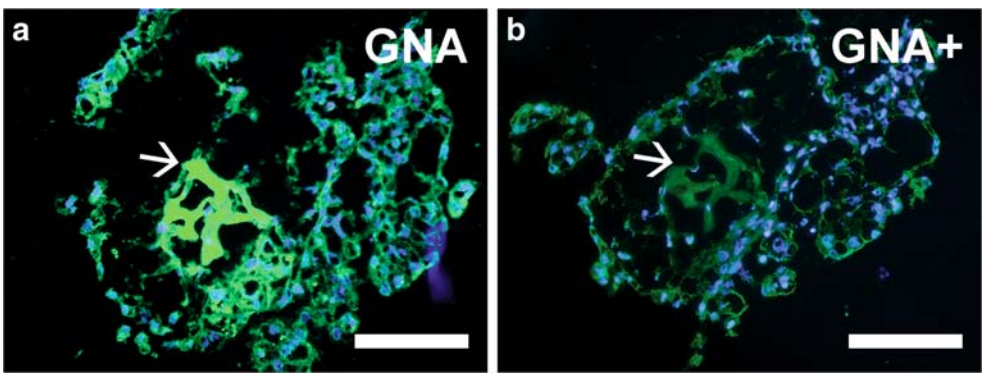

Fig. 1 **a** 28-day EB stained with GNA (FITC, *green*), **b** 28-day EB pretreated with sodium periodate, then stained with GNA (FITC, *green*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

Table 3 EB staining data from α-Gal-binding lectins

Lectin	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional information
GS I-B_4	Terminal α-Gal >>> α-GalNAc [4, 5]	12[b]	20	0	
		28	25	0	
MOA	Gal α(1,3) Gal β1,4GlcNAc/Glc >> terminal LacNAc [5, 6, 30]	12[c]	20	69	20 μg/ml ECA[d], β-galactosidase[d], neuraminidase[e], α-galactosidase[f]
		28	20	34	Neuraminidase[e], α-galactosidase[f], β-galactosidase[f]

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Previous data only [2]
[c] Includes previous data [2] and new data
[d] Blocked
[e] Enhanced
[f] No effect

GalNAc, [8]) to undifferentiated cells [2] and the high level of binding to both 12- and 28-day cells. PVL and WGA are characterized as binding to both GlcNAc and Neu5Ac [4, 9–13]; however, the absence of terminal GlcNAc groups is shown by the inability of GS II to bind. The fact that neuraminidase totally abolished the binding of PVL to the 12-day EBs [2] indicates that this lectin is probably binding to Neu5Ac residues. Since the combination of hapten and

Table 4 EB staining data from α-GalNAc-binding lectins

Lectin/Antibody	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional information
DBA	GalNAc α1,3 GalNAc > GalNAc α1,3 (Fuc α1,2) Gal >> α-GalNAc [4, 35]	12[b]	20	0	
		28	25	0	
Gleheda	Tn antigen (GalNAc α1—Ser/Thr) > GalNAc α1,3 [36]	12	20	84	Neuraminidase[c]
		28	20	40	50 mM GalNAc[d], neuraminidase[e]
GS I-A_4	GalNAc α1,3/1,6 Gal > α-GalNAc [4]	12	10	96	Neuraminidase[f]
		28	25	30	50 mM GalNAc[d], neuraminidase[e]
HAA	α or β GalNAc [37]	12	20	62	0.2 M GalNAc[d]
		28	20	73	
HPA	GalNAc α1,3 (Fuc α1,2) Gal > GalNAc α1,3 [glycan array, 4, 35]	12[g]	20	0	
		28	20	73	50 mM GalNAc[d]
LBL	GalNAc α(1,3) Gal or GalNAc α1,3 (Fuc α1,2) Gal >> GalNAc [4, 35]	12	25	25	0.1 M GalNAc[d]
		28	25	0	
MornigaG	Tn antigen > T antigen [7]	12	10	100	β-Galactosidase + 0.2 M GalNAc[d]
		28	10	100	0.2 M GalNAc[d]
SBA	GalNAc α1,3 Gal > α GalNAc [4, 35]	12[g]	10	69	50 mM GalNAc[d], neuraminidase[e]
		28	10	73	50 mM GalNAc[d], neuraminidase[e]
anti-Tn antibody	Tn antigen[h,i]	12[g]	–	89	
		28	–	78	β-Galactosidase[e]
VVA	α or β GalNAc [4, 38]	12	10	97	
		28	25	94	50 mM GalNAc[d]
WFA	GalNAc α1,6 Gal > GalNAc [4]	12	10	56	
		28	10	84	50 mM GalNAc[d]

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Previous data only [2]
[d] Blocked
[e] Enhanced
[f] No effect
[g] Includes previous data [2] and new data
[h] Specificity shown by omitting primary antibody, no staining seen with secondary antibody alone
[i] Primary antibody dilution: 1:50, secondary antibody dilution: 1:100

Table 5 EB staining data from sialic acid-binding lectins

Lectin	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional information
LFA	*N*-Acetylneuraminic acid (Neu5Ac) [4, 39]	12[b]	30	67	0.1 M Neu5Ac[c], neuraminidase[c]
		28	30	93	0.1 M Neu5Ac[c]
LPA	Neu5Ac (prefers large glycopeptides) [4, 40]	12[d]	35	100	0.1 M Neu5Ac[c], neuraminidase[c]
		28	35	94	
MAA	Neu5Ac α(2,3) Gal [8]	0[d]	10	19	
		12[e]	10	92	25 mM α2,3 sialyllactose[c]
		28	10	94	
MCA	Neu5Ac α(2,3) Gal (unpublished)	12	10	94	
		28	10	90	0.5 M Lactose[c]
PSL	Neu5Ac α 2,6 Gal β1,4 GlcNAc/Glc [32]	12[e]	20	95	0.5 M Lactose[c], neuraminidase[c]
		28	20	88	
PVL	GlcNAc > Neu5Ac α2,3 LacNAc > Neu5Ac α2,6 LacNAc [9–12]	12[e]	1	84	0.3 M GlcNAc[c], neuraminidase[c], neuraminidase + β-galactosidase[e]
		28	1	75	
SNA	Neu5Ac α2,6 Gal/GalNAc, esp. bi/triantennary chains [33]	0[d]	5	80	
		12[d]	5	100	20 mM human milk[c], neuraminidase[c]
		28	5	100	Neuraminidase[c]
WGA	$(GlcNAc\ \beta4\ GlcNAc)_{1\text{-}3,4}$ or $(Gal\ \beta4\ GlcNAc)_{1\text{-}3,4}$ > Neu5Ac [4, 13]	0[d]	5	100	40 mM chitotriose[c]
		12[b]	1	99	0.25 M LacNAc[f], neuraminidase[f]
		28	1	100	Neuraminidase + 40 mM chitotriose[c]

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Includes previous data [2] and new data
[c] Blocked
[d] Previous data only [2]
[e] Enhanced
[f] Partially blocked

neuraminidase was required to completely abolish the binding of WGA, this lectin is most probably binding to both Neu5Ac, and to internal GlcNAc such as occurs in LacNAc.

Terminal LacNAc residues are highly prevalent on both the 12 and 28-day EBs, as shown by the strong binding of DSA, ECA, LEA, PWA, and STL, as well as by WGA and WGA-succ (Table 6). Between days 12 and 28 of differentiation, the levels of bi- and tri-antennary LacNAc chains likely decrease as indicated by the decrease in binding of DSA [4, 13, 14]. As discussed above, these bi- and tri-antennary LacNAc structures may be the binding epitopes for MOA inasmuch as the staining of MOA also decreases between days 12 and 28. Consistent with the extensive binding of the aforementioned lectins to LacNAc is the strong binding of RCA and IRL to β-galactosyl (β-Gal) end groups [4, 15] on both 12- and 28-day cells (See Table 9). For both 12- and 28-day EBs, treatment with neuraminidase drastically enhanced the binding of ECA, while treatment with β-galactosidase completely abolished

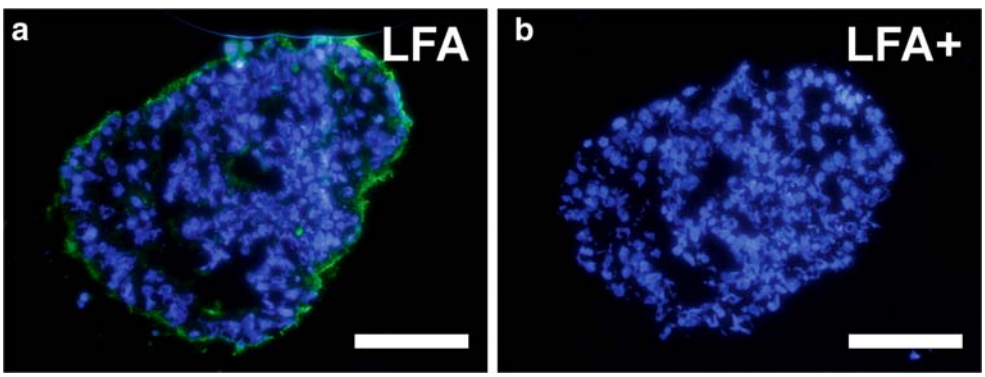

Fig. 2 28-day EBs stained with **a** LFA (FITC, *green*), **b** LFA blocked with 0.1 M neuraminic acid. Hoechst nuclear stain is shown in blue. Scale bars are 100 μm

Table 6 EB staining data from LacNAc-binding lectins

Lectin	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional information
DSA	Gal β1,4 GlcNAc (LacNAc), esp. bi/triantennary chains to Man [4, 13, 14]	12[b]	20	100	40 mM chitotriose[c], neuraminidase[d]
		28	20	45	
ECA	terminal LacNAc [4, 16]	12[e]	10	54	0.25 M LacNac[c], neuraminidase[d], β-galactosidase[c]
		28	10	69	β-galactosidase[c], neuraminidase[d]
LEA	2–3 consecutive LacNAc residues [4, 13]	12[e]	20	98	40 mM chitotriose[c], β-galactosidase[c]
		28	10	100	
MOA	Gal α(1,3) Gal β1,4 GlcNAc/Glc >>> terminal LacNAc [5, 6, 30]	12	20	69	20 μg/ml ECA[c], β-galactosidase[c], neuraminidase[d], α-galactosidase[6]
		28	20	34	neuraminidase[d], α-galactosidase[f], β-galactosidase[c]
PWA	Gal β1,4 GlcNAc β1,6 Gal or bi/triantennary [4]	12	10	71	
		28	10	93	0.4 M GlcNAc[c]
STL	GlcNAc β1,4 GlcNAc > LacNAc [4, 13, 41, 42]	12[b]	10	91	40 mM chitotriose[c]
		28	10	92	
WGA	$(GlcNAc\ \beta4\ GlcNAc)_{1\text{-}3,4}$ or $(Gal\ \beta4GlcNAc)_{1\text{-}3,4}$ > Neu5Ac [4, 13]	0[b]	5	100	40 mM chitotriose[c]
		12[e]	1	99	0.25 M LacNAc[c], neuraminidase[c]
		28	1	100	neuraminidase + 40 mM chitotriose[c]
WGA-succ	$(GlcNAc\ \beta4GlcNAc)_{1\text{-}3,4}$ or $(Gal\ \beta4GlcNAc)_{1\text{-}3,4}$ [4, 13]	12[b]	5	100	40 mM chitotriose[c]
		28	5	90	

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Previous data only [2]
[c] Blocked
[d] Enhanced
[e] Includes previous data [2] and new data
[f] No effect

binding. The results of these enzymatic treatments indicate the presence of both sialylated and non-sialylated Gal β1,4 GlcNAc (LacNAc) end groups [4, 16].

Staining by AAL, the lectin from the orange peel fungus, demonstrates the widespread presence of L-fucose on the cells ([4, 17–20], glycan array). The staining by this lectin did not change over time. However, AAA (freshwater eel agglutinin) bound neither 12- nor 28-day EBs, suggesting the absence of Fuc α1,3 GlcNAc (glycan array). UEA 1 specifically binds to α1,2-linked fucose ([4, 21–23], glycan array) and the presence of this epitope appears to increase with differentiation, since UEA1 bound to only 15% of undifferentiated cells, but bound to 70 and 80% of 12- and 28-day EBs, respectively. Lotus lectin, which binds most preferentially to terminal α1,2-linked fucosyl groups when a subterminal α1,3 fucose is also present ([24], glycan array), did not bind to 12-day EBs, but bound readily to 28-day EBs. This could indicate the appearance of this particular epitope between days 12 and 28 of differentiation or a subtle change in the carbohydrate orientation, which could allow it to bind to its less preferred epitope of terminal α1,2-linked fucose [4, 24]. The binding of UEA1 shows that this epitope is present on most of the EBs, but does not explain the inability of lotus lectin to bind to the same epitopes on 12-day cells (to which UEA1 also binds readily). EEA1 binding decreased from day 12 to day 28 of differentiation. This lectin binds most preferentially to Gal α1,3 (Fuc α1,2) Gal β1,3 GlcNAc ([23], glycan array); however, this epitope cannot be present because terminal α-Gal was shown to be absent. EEA1 also binds weakly to Gal β1,4 (Fuc α1,3) Glc/GlcNAc (glycan array) or terminal α1,2-linked fucose [22, 23], one of which must be the case here. The specificity data given here and in Table 7 was analyzed by a glycan array. See Fig. 3 for examples of staining by fucose-binding lectins of 28-day cells.

Of the GlcNAc-binding lectins tested, GS II is most specific for GlcNAc end groups [4, 25]. The fact that it did not bind to EBs indicates the absence of this epitope. PVL, WGA, and WGA-succ all bind to GlcNAc in addition to other epitopes (Table 8). Through enzymatic treatment (neuraminidase and β-galactosidase) of the sectioned EBs, we determined that these lectins bound to *internal* GlcNAc such as occur in LacNAc structures or the chitobiosyl core of *N*-linked glycans. When 12-day cells were treated successively with neuraminidase followed by β-galactosidase, the binding of GS II became evident, indicating the presence of Neu5Ac α2,3/2,6 Gal β-1,4 GlcNAc (sialyl LacNAc). Further establishing this supposition is the

Table 7 EB staining data from fucosyl-binding lectins

Lectin	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional information
AAA	Fuc α1,3 GlcNAc (glycan array)	12[b]	25	0	
		28	25	0	
AAL	L-Fucose ([4, 17–20], glycan array)	0[b]	5	100	0.3 M Fucose[c]
		12[d]	5	95	0.3 M Fucose[c]
		28	2	100	
EEA1	Gal β1,4 (Fuc α1,3)Glc/GlcNAc or	12[d]	20	49	
	Fuc α1,2Gal β1,3/4GlcNAc or α1,2	28	20	11	
	Fucose ([4, 22, 23], glycan array)				
Lotus	Fuc α1,2 Gal β1,4[Fuc α1,3]Glc/GlcNAc,	12[d]	35	0	
	other difucosyl carbohydrates >> terminal	28	20	76	0.3 M Fucose[c]
	α1,2-linked fucose ([4, 21–24], glycan array)				
UEA1	Terminal or subterminal Fuc α1,2 Gal	0[b]	25	15	
	([4, 21–23], glycan array)	12	25	70	
		28	25	80	0.3 M Fucose[c]

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Previous data only [2]
[c] Blocked
[d] Includes previous data [2] and new data

enhancing effect of these successive enzymatic treatments on the staining of the 12-day EBs by PVL.

The presence of terminal nonreducing β-galactosyl end groups is evident from the specific binding of RCA and IRL [4, 15]. Other lectins, ABA, ACA and PNA, bind most preferentially to Gal β1,3 GalNAc, the T (Thomsen–Friedenreich) antigen [4, 26–29]. Morniga G, which prefers the Tn antigen, binds secondarily to the T antigen [7]. Conversely, ACA prefers the T antigen, and secondarily binds the Tn antigen, as well as both sialylated variants [29]. Both ACA and Morniga G stained very strongly (See Table 9 for details on the β-Gal-binding lectins mentioned above). The wide prevalence of β-gal in the 12 and 28-day EBs is also indicated by the strong staining of the LacNAc-

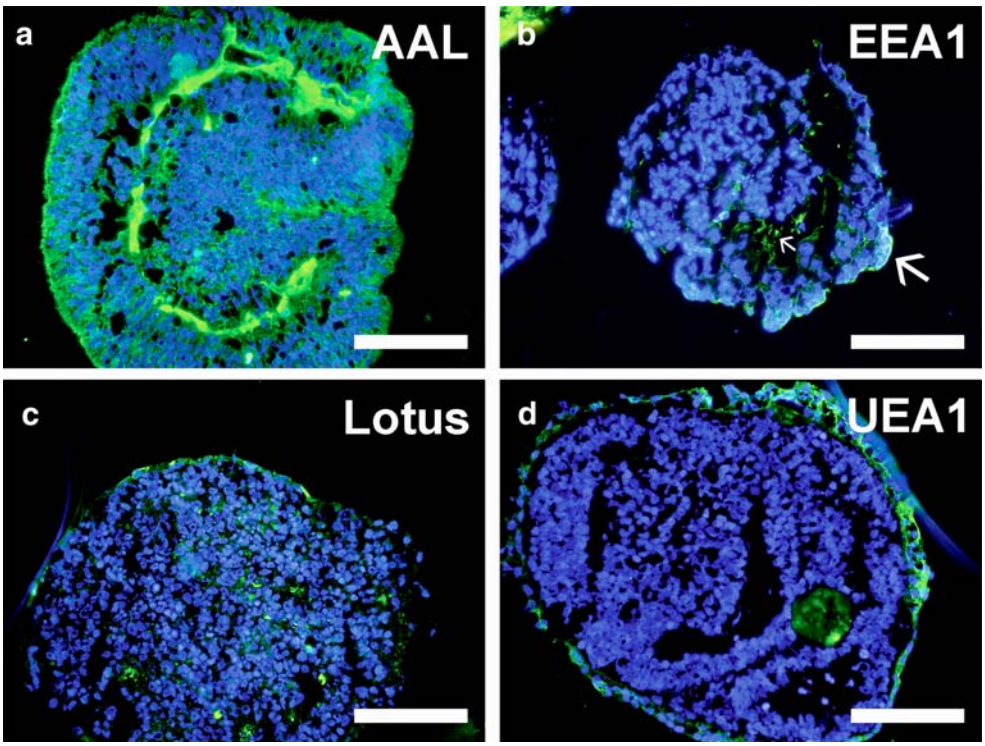

Fig. 3 28-day EBs stained with: **a** AAL, **b** EEA, **c** Lotus, or **d** UEA1 (FITC, *green*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

Table 8 EB staining data from β-GlcNAc-binding lectins

Lectin	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional Information
GS II	Terminal β GlcNAc > α GlcNAc [4, 25]	12[b]	25	0	β-Galactosidase[c], neuraminidase + β-galactosidase[c]
		28	25	0	β-Galactosidase[c], neuraminidase[c]
PVL	GlcNAc > Neu5Ac α2,3 LacNAc > Neu5Ac α2,6 LacNAc [9–12]	12[d]	1	84	0.3 M GlcNAc[d], neuraminidase[d], neuraminidase + β-galactosidase[c]
		28	1	75	
WGA	$(GlcNAc\ \beta4\ GlcNAc)_{1\text{-}3,4}$ or $(Gal\ \beta4GlcNAc)_{1\text{-}3,4}$ > Neu5Ac [4, 13]	0[e]	5	100	40 mM chitotriose[d]
		12[b]	1	99	0.25 M LacNAc[d], neuraminidase[d]
		28	1	100	Neuraminidase + 40 mM chitotriose[d]
WGA-succ	$(GlcNAc\ \beta4\ GlcNAc)_{1\text{-}3,4}$ or $(Gal\ \beta4GlcNAc)_{1\text{-}3,4}$ [4, 13]	12[b]	5	100	40 mM chitotriose[d]
		28	5	90	

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Includes previous data [2] and new data
[c] Enhanced
[d] Blocked
[e] Previous data only [2]

specific lectins, ECA, LEA, DSA, and STL. The binding of these lectins changed very little between 12 and 28 days of differentiation. Only PNA and the anti-T antigen antibody showed significantly increased binding.

Antibodies were used in this study to determine the cell types present in differentiated EBs and whether a particular lectin could be linked to a cell type. Differentiated cell types that increased from day 12 to day 28 included

Table 9 EB staining data from β-Gal-binding lectins

Lectin/Antibody	Specificity	Day	Conc (μg/ml)	% Stained[a]	Additional information
ABA	Terminal or internal Gal β(1,3) GalNAc (T-antigen) or Neu5Ac—T-antigen [26, 27]	12[b]	35	91	13 mg/ml benzyl T antigen[c], periodate treatment[c]
		28	30	93	β-galactosidase[c]
ACA	Terminal or internal T-antigen or Neu5Ac—T antigen [28, 29]	0[d]	2	100	
		12[b]	1	93	7 mg/ml benzyl T antigen[c], periodate treatment[c], neuraminidase[e]
		28	1	92	β-galactosidase[c]
IRL	Terminal β-Gal; Lactose [15]	12[d]	10	100	0.5 M Lactose[c]
		28	10	95	β-Galactosidase[c]
MornigaG	Tn antigen > T antigen [7]	12	10	100	β-Galactosidase + 0.2 M GalNAc[c]
		28	10	100	0.2 M GalNAc[c]
PNA	Terminal Gal β(1,3)GalNAc; terminal β-Gal [4]	0[d]	20	81	0.5 M Lactose[c]
		12	10	60	0.5 M Lactose[c], periodate treatment[c], neuraminidase[e]
		28	10	83	β-Galactosidase[c], neuraminidase[e]
RCA	Terminal β Gal as in Lactose/LacNAc [4]	0[d]	5	100	
		12[b]	5	60	0.5 M Lactose[c], periodate treatment[c]
		28	5	83	β-Galactosidase[c]
anti-T antigen	T antigen[f]	12[b]	–	32	PNA[g], ACA[h], ABA[h]
		28	–	59	β-Galactosidase[c], *o*-glycosidase[c], ACA[h], ABA[h], PNA[g]

[a] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[b] Includes previous data [2] and new data
[c] Blocked
[d] Previous data only [2]
[e] Enhanced
[f] Primary antibody dilution: 1:20, secondary antibody dilution: 1:100
[g] Lectin which very nearly matches the antibody staining locations
[h] Lectin which matches all antibody staining locations, but stains other locations also

Table 10 EB staining data from antibodies

Germ layer represented	Antibody	Animal/cell-type	Day	Primary dilutions[a]	% Stained[b]	Additional information
Ectoderm	Keratin 14[c]	Mouse/skin	12	1:100	8	
			28		63	LEA[d], PVL[d], Lentil[e], MornigaG[e], ABA[e], IRL[e]
	TUJ1[c]	Mouse/neurons	12	1:250	7	
			28		48	WFA[e]
	Sox3[c]	Rabbit/neural progenitors	12	1:100	31	
			28		9	
Mesoderm	Brachyury[c]	Rabbit/early mesoderm	12	1:80	14	
			28		63	MornigaG[e], WFA[e]
	Osteocalcin[c]	Goat/osteoblasts (bone)	12	1:50	0	
			28		30	Gleheda[e], GS I-A_4[e], HPA[e], PSL[e], PVL[e], DSA[e]
Endoderm	Sox17[c]	Goat/endoderm (general)	12	1:150	10	
			28		36	GNA[e], ECA[e], LEA[e], STL[e], WGA-succ[e], ABA[e]
	PDX1[c]	Rabbit/pancreatic islets	12	1:500	35	
			28		71	
Undifferentiated	Oct 3/4[c]	Goat/undifferentiated	12	1:50	69	MornigaG[e]
			28		0	

[a] Secondary antibody dilution 1:100
[b] Percentages are based on minimum count of 20 EBs from a minimum of two different slides.
[c] Specificity shown by omitting primary antibody, no staining seen with secondary antibody alone
[d] Lectin which very nearly matches the antibody staining locations
[e] Lectin which matches all antibody staining locations, but stains other locations also

epidermal ectoderm (Keratin 14), neurons (Tuj1), mesoderm (brachyury), osteoblasts (osteocalcin), pancreatic islets (PDX1), and endoderm (Sox17). Only Sox3, a neural marker that appears very early in neural development, decreased between day 12 and 28 of differentiation. Staining of Oct 3/4 showed the presence of undifferentiated cells in 12-day EBs but their absence in the 28-day EBs (See Table 10). All antibodies used were tested on their respective cell types to ensure specificity and concentration used. In some cases, mouse embryos or adult mouse cryostat sections were tested; in other cases human sections were used, *e.g.* pancreas paraffin sections for PDX1. In each case, only the tissue-type specific to the antibody was stained. To establish specificity of the secondary antibody, the primary antibody was omitted from the staining procedure. In each case, no binding was seen when the primary antibody was omitted; for example, osteocalcin was tested against its secondary antibody, Donkey anti Goat Texas Red. The result is shown in Fig. 4.

Discussion

The present communication describes cell surface carbohydrate groups and their changes during the first 28 days of development of human embryonic stem cells as EBs. Specifically, the presence of the following carbohydrate epitopes has been demonstrated on both 12- and 28-day

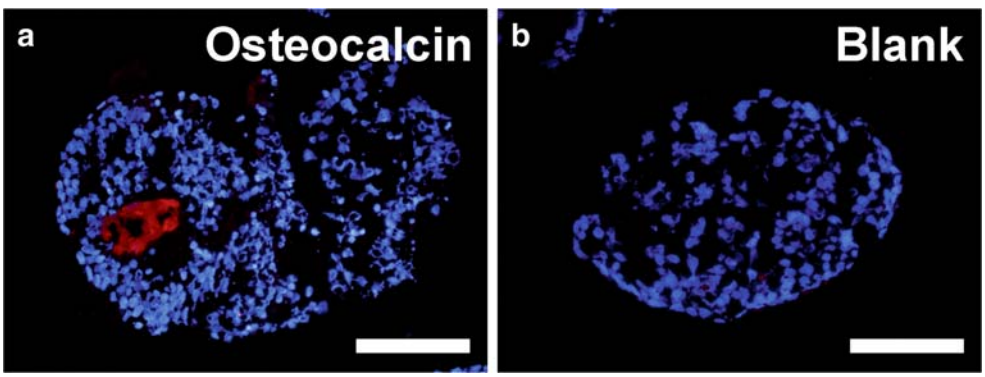

Fig. 4 **a** 28-day EB stained with Osteocalcin (Texas Red), **b** 28-day EB treated only with secondary antibody (Texas Red). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

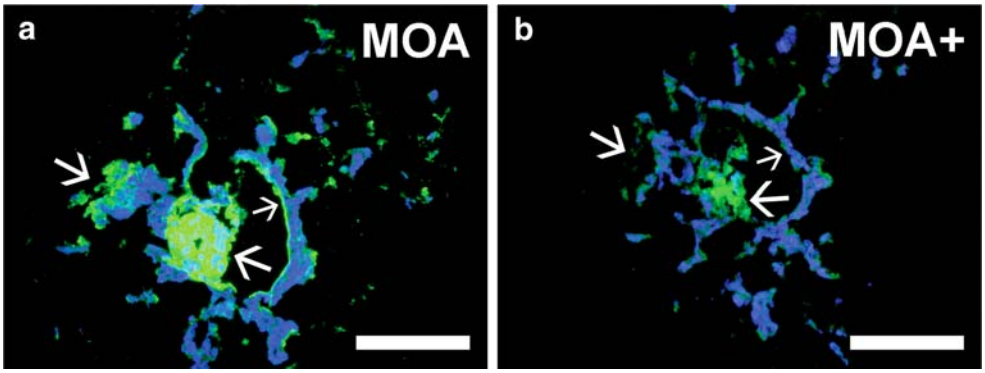

Fig. 5 **a** 12-day EB stained with MOA (FITC, *green*), **b**. 12-day EB pretreated with β-galactosidase, then stained with MOA (FITC, *green*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

EBs: 1. α-mannosyl residues, 2. sialic acid-containing glycoconjugates, including the sialyl T antigen and sialylated α-GalNAc groups, 3. α-GalNAc residues including the Tn antigen, 4. LacNAc residues, 5. fucosyl residues, and 6. other β-galactosyl residues including the T antigen. We have shown that terminal α-galactosyl residues and terminal β-GlcNAc residues are most probably absent from both 12- and 28-day differentiated EBs.

Several findings are of particular interest. Despite the apparent lack of terminal α-galactosyl groups indicated by absence of GS I-B_4-binding as well as lack of any effect of α-galactosidase treatment, MOA, which has been shown to specifically bind to Gal α1,3 Gal β1,4 GlcNAc/Glc residues, binds moderately well to both 12 and 28-day EBs. An explanation for this anomaly is the fact that MOA is known to bind weakly to LacNAc epitopes [6]. If a high density of LacNAc residues is exposed on the surface of EBs, MOA could bind with enhanced avidity [6]. This is further indicated by the recently determined x-ray structure of this lectin bound to the linear B trisaccharide epitope. As shown in that structure, the binding site of MOA is extended and shallow (as compared with most other lectins, which are narrow and deep) and interacts with all three sugars of the trisaccharide—Gal α1,3 Gal β1,4 GlcNAc [30]. It is possible, therefore, that even in the absence of α-Gal, sufficient interaction could occur with the LacNAc moiety to permit weak binding. Our data indicate the presence of this epitope (Gal β1,4 GlcNAc, LacNAc) on the EBs as shown by the very strong binding of ECA, LEA, and DSA. Additionally, preincubation of 12-day cells with unlabeled ECA blocked the binding of MOA, as did treatment with β-galactosidase (Fig. 5) or periodate. Finally, treatment of EBs with neuraminidase enhanced the binding of MOA (Fig. 6) although to a lesser extent than that shown with ECA on both 12 and 28 day EBs. These data all support the conclusions that α-galactosyl end groups are essentially absent from both 12- and 28-day differentiated EBs and that MOA-binding is the result of a high density of LacNAc end groups.

It is instructive to comment on the use of periodate on the EBs. This reagent splits vicinal hydroxyl groups present in most oligosaccharide structures making it a useful reagent for assessing the role of susceptible sugars in the binding of various lectins and antibodies to the EBs. Periodate treatment substantially decreased the binding of GNA (Fig. 1) while having no effect on the binding of Con A to 28-day EBs, establishing the difference in the binding of these two lectins: GNA binds solely to the terminal non-reducing α-Man groups [31] whereas Con A also recognizes the branched mannose trisaccharide core involved in the internal linkage region of glycoproteins [4]—an epitope which remains essentially unaffected by periodate oxidation.

In a similar fashion, periodate attacked the terminal β-Gal groups of the T-antigen drastically reducing binding by

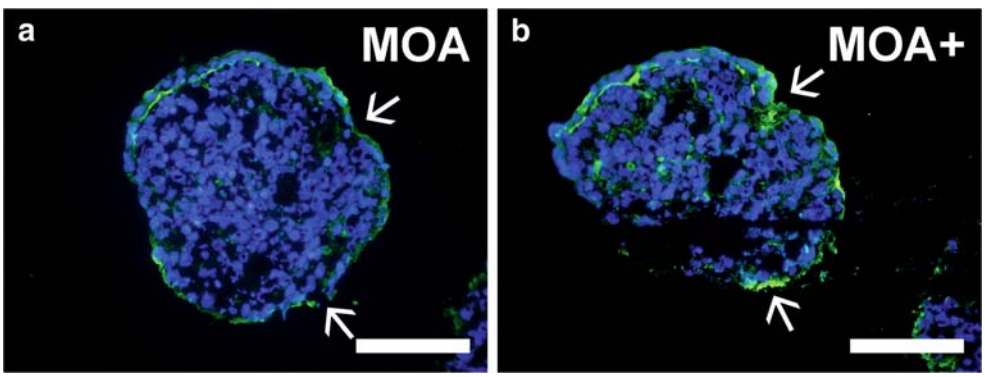

Fig. 6 **a** 28-day EB stained with MOA (FITC, *green*), **b** 28-day EB pretreated with neuraminidase, then stained with MOA (FITC, *green*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

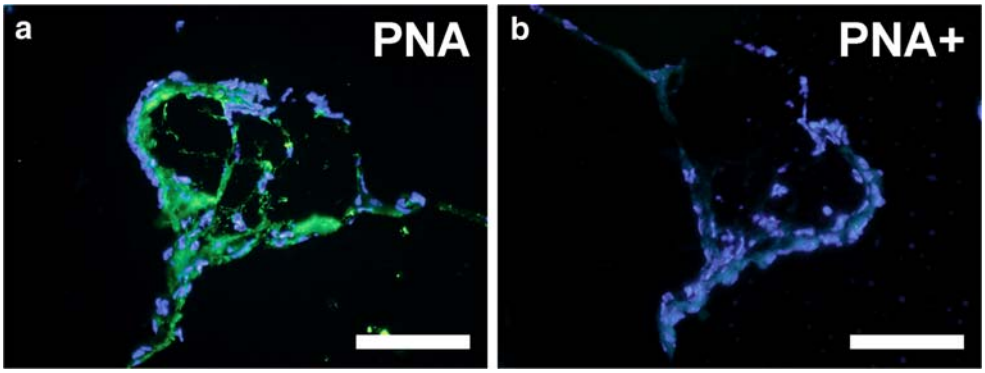

Fig. 7 **a** 12-day EB stained with PNA (FITC, *green*), **b** 12-day EB pretreated with sodium periodate, then stained with PNA (FITC, *green*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

PNA and the T antigen antibody (Fig. 7). Interestingly, periodate treatment only slightly decreased binding by two other T-binding lectins: ACA and ABA. A possible explanation is that both of these lectins can also bind to sialyl T-antigen, which, because of the hemiketal linkage at the 3′ position, is resistant to periodate oxidation except for the glyceryl tail of the sialic acid. Further supporting this explanation, treatment with neuraminidase enhanced the binding of PNA and the T-antigen antibody (Fig. 8), whereas binding by ACA and ABA was only marginally enhanced. Not surprisingly, the strong binding by MAA, MCA, PSL, and SNA, all lectins that bind sialic acid linked to Gal [8, 32, 33], confirms the extensive presence of cell surface sialylated galactosyl residues, one of which is the sialyl T antigen. The staining of PNA and the anti-T antigen antibody were nearly identical, while only the most intense staining of ACA matched this antibody (Fig. 9). Additionally, *O*-glycosidase, an enzyme that cleaves carbohydrate chains linked to Ser/Thr amino acyl groups of proteins, abolished antibody binding to the T antigen while having no effect on the binding of ABA or ACA. This could indicate that sialylated chains are unaffected by this enzyme, while other chains are cleaved, further confirming the presence of the sialyl T antigen.

Some changes in the staining of EBs between 12 and 28 days are quite remarkable. Lotus lectin did not bind to 12-day EBs, yet binds well to the 28-day EBs, indicating the possible appearance of Fuc α1,2 Gal β1,4 (Fuc α1,3) GlcNAc (Lewis y) groups ([4, 21–24], glycan array). Subtle differences among lectins of similar nominal specificity could have a large effect on their ability to bind to cells. The occurrence of subterminal α1,3 linked fucosyl group

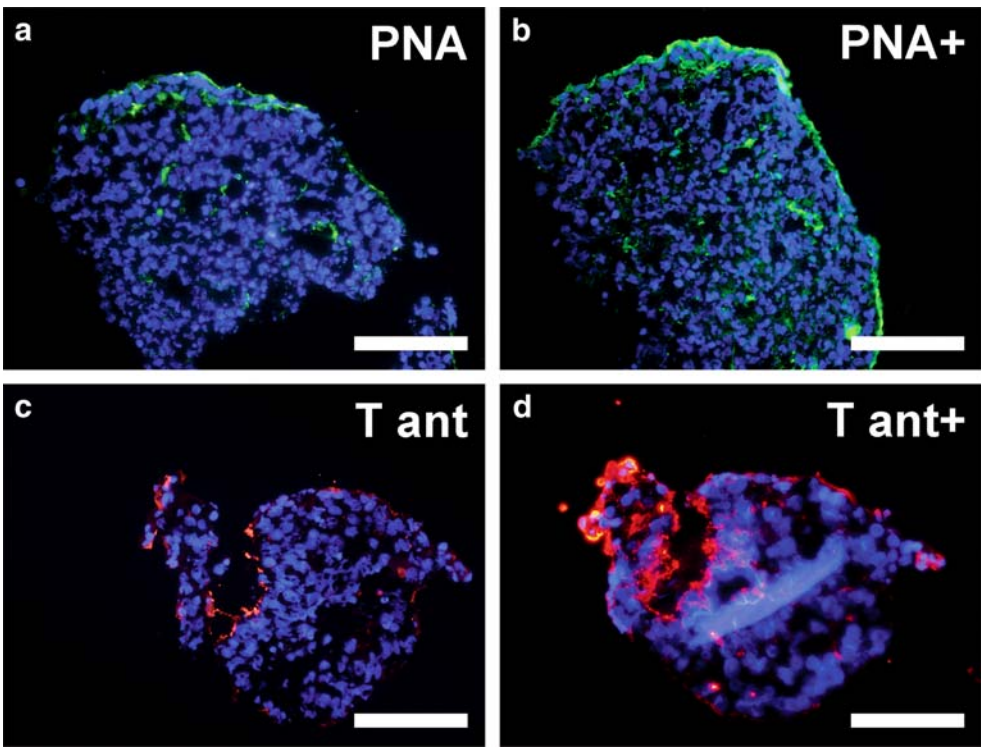

Fig. 8 **a** 28-day EB stained with PNA (FITC, *green*), **b** 28-day EB pretreated with neuraminidase, then stained with PNA (FITC, *green*), **c** 28-day EB stained with anti-T antigen antibody (Cy3, *Red*), **d** 28-day EB pretreated with neuraminidase, then stained with anti-T antigen antibody (Cy3, *Red*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

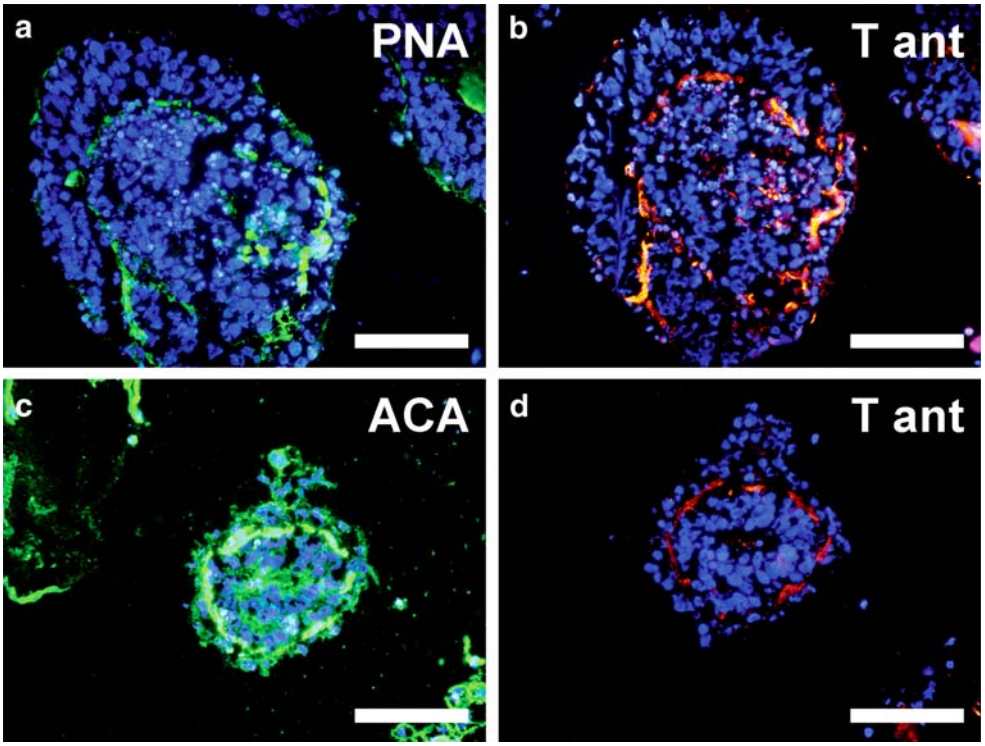

Fig. 9 Matching stains of 28-day EBs by: **a** PNA (FITC, *green*) and **b** anti-T antigen antibody (Cy3, *Red*); **c** ACA (FITC, *green*) and **d** anti-T antigen antibody (Cy3, *Red*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

adjacent to a terminal α1,2-linked fucosyl group might significantly enhance the binding of lotus lectin, but have no effect on the binding of UEA1.

Similarly, slight changes in α-GalNAc residues' environment might have a significant effect on the binding capability of the α-GalNAc-specific lectins. HPA, a lectin specific for various α-GalNAc-containing epitopes, binds to 28-day EBs, but not to 12-day EBs, whereas lectins with similar specificities—Gleheda and GS I-A_4—show decreased binding between 12 and 28 days. We have no

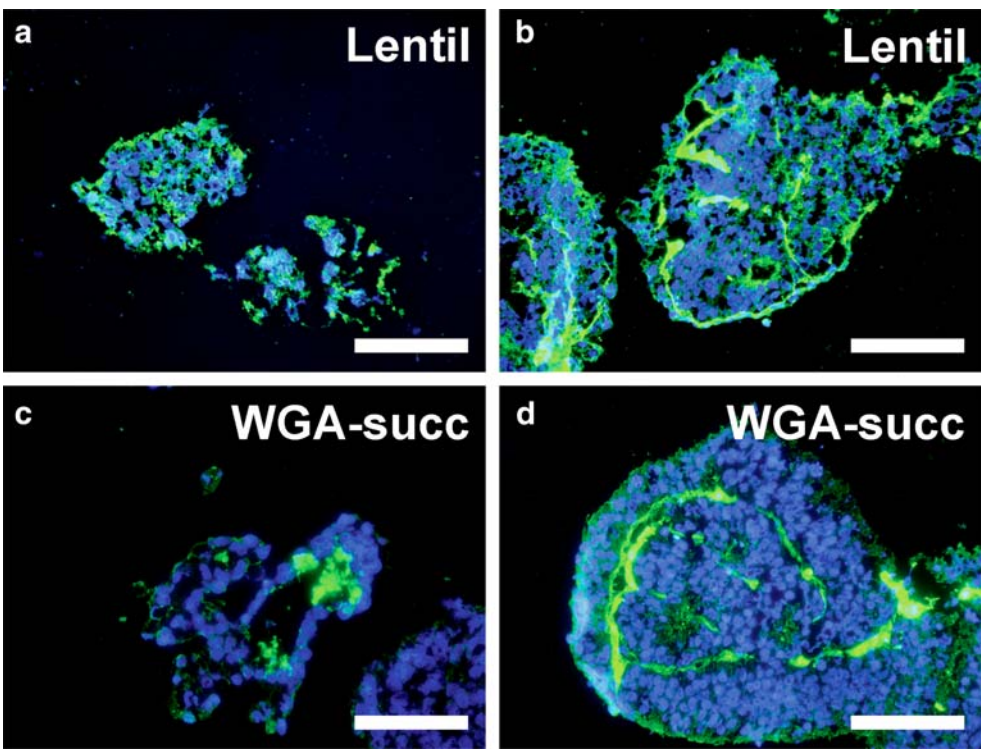

Fig. 10 **a** 12-day EBs stained with Lentil lectin (FITC, *green*), **b** 28-day EBs stained with Lentil lectin (FITC, *green*), **c** 12-day EBs stained with WGA-succ (FITC, *green*), **d** 28-day EBs stained with WGA-succ (FITC, *green*). Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

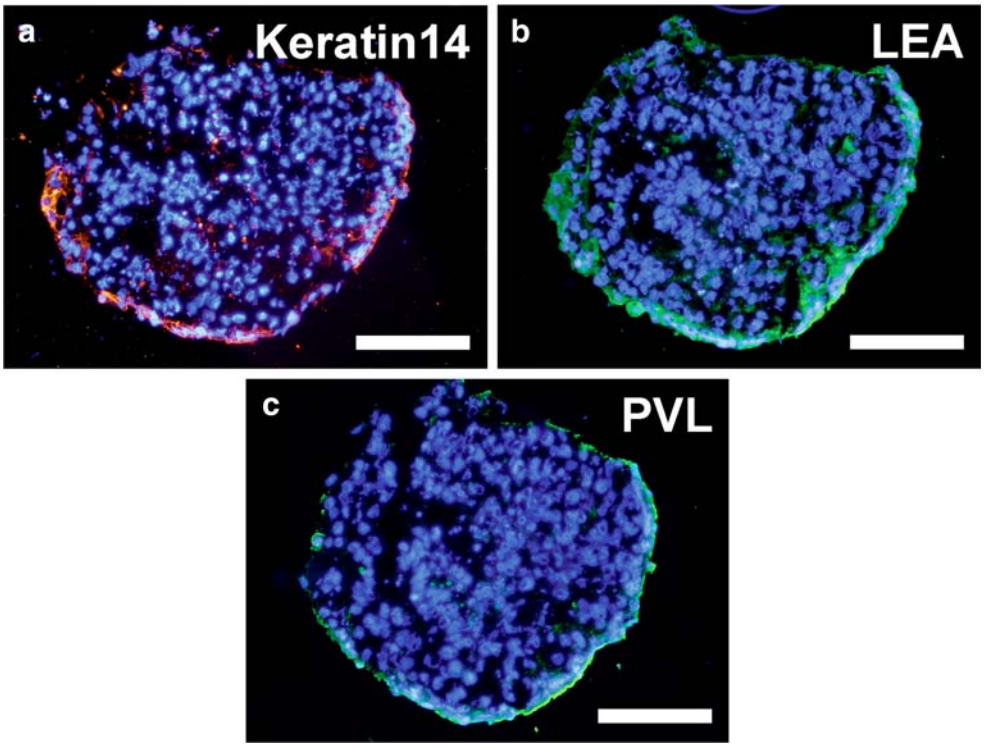

Fig. 11 Matching stains of 28-day EBs by: **a** Keratin14 antibody (Cy3, *Red*), **b** LEA (FITC, *green*) and **c** PVL (FITC, *green*). Hoechst nuclear stain is shown in blue. Scale bars are 100 μm

definitive explanation for the cause of these differences. Analysis of HPA specificity by glycan array binding revealed its preference for GalNAc α1,3 (Fuc α1,2) Gal over other α-GalNAc structures, but as not all known α-GalNAc structures are included in the array, we cannot completely define or rule out other options for binding specificity. Additionally, LBL and DBA bind only very minimally or not at all to the EBs, indicating the absence of GalNAc α1,3 (Fuc α1,2) Gal. Another possible explanation for the different binding of HPA might be the alteration of structures adjacent to the glycan, unmasking binding sites for HPA in the 28-day cells. WFA, a lectin specific for GalNAc α1,6 Gal, also increased its staining slightly between 12 and 28 days, and such α1,6-linked GalNAc residues may also enhance the binding of HPA. SBA binding is strongly enhanced by neuraminidase treatment of both 12 and 28-day cells, indicating that α-GalNAc in some form is highly sialylated. Binding of GS I-A_4 and Gleheda is unaffected by neuraminidase treatment of the 12-day cells, but slightly enhanced on the 28-day cells, indicating that their binding epitope is only minimally sialylated on the 28-day cells and not at all on the 12-day cells. When these two lectins, are used to stain adjacent cell sections, they bind in the same pattern on the EBs, suggesting that they are binding to the same epitopes. Further supporting this idea is the fact that the binding of GS I-A_4 and Gleheda decreases in a similar fashion between 12 and 28 days of differentiation. This could be due to the loss of α1,3-linked GalNAc or some other epitope to which both bind.

Changes in the *pattern* of lectin binding to EBs may be important. A few lectins (HPA, LBL, and lotus) bind to either 12- or 28-day EBs (but not both), while other lectins

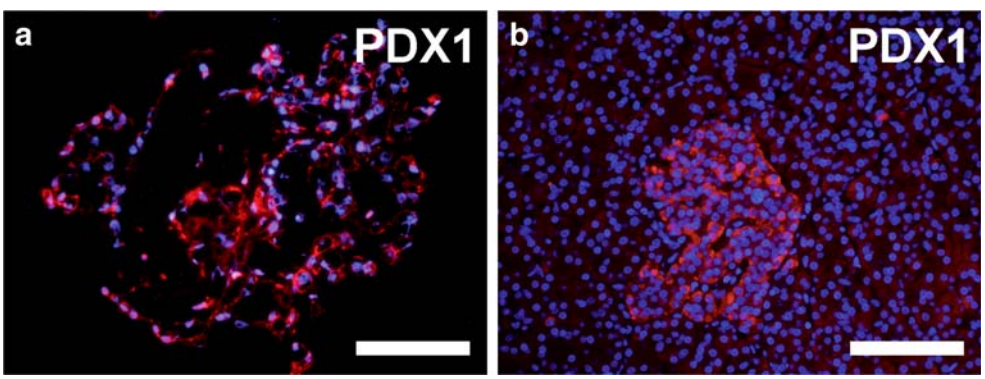

Fig. 12 **a** 28-day EB stained with PDX1 antibody (Texas Red), **b** human pancreatic islet stained with PDX1 antibody. Hoechst nuclear stain is shown in *blue*. Scale bars are 100 μm

(lentil, MCA, PWA, WGA-succ) stain nearly the same percentage of EBs of both age groups, but in different patterns. Specifically, lentil lectin changes from staining consistently throughout the EBs to producing trails in each EB, indicating a high concentration of α-mannosyl residues in these trails. In contrast, MCA, PWA, and WGA-succ change from staining large clumps of cells to staining edges and trails similar to those stained by the lentil lectin (Fig. 10). Greatly differing patterns of binding of a particular lectin at two stages of differentiation might indicate a relationship between cell-type and glycan presence. Such a relationship likely indicates a progression of differentiation guided by cell–cell interactions involving these glycans. In any event, movements in the concentrations of α-mannosyl groups (lentil), Neu5Ac α2,3 Gal (MCA), and LacNAc (PWA, WGA-succ) groups from one area to another are apparent.

Finally, we used antibodies to identify some of the cell types and germ layers present in 28-day EBs. Three antibodies were used to identify the ectoderm: Keratin14 for epidermal ectoderm, Tuj1, for differentiated neurons, and Sox3 for neural progenitors. Each of these antibodies stained a different percentage of the 12 and 28-day EBs. Keratin, Tuj1 and Sox3 stained 8, 7, and 31% respectively, of the 12-day EBs while staining 63, 48 and 9% respectively, of the 28-day EBs. This increase in Keratin14 and TUJ1 was expected, as was the decrease in Sox3 since neurons are more fully developed after 28-days and express Sox3 in much smaller amounts. Keratin14 bound to nearly the same cell-locations as PVL and LEA (See Fig. 11). Other lectins, lentil, morniga G, and UEA1, bound to these locations as well as others. This indicates that some epidermal cells contain α-mannosyl residues, the Tn antigen, and α-fucosyl residues, respectively, whereas most contain Neu5Ac and LacNAc.

We used brachyury (early mesoderm) and osteocalcin (osteoblasts) antibodies to identify the cells of mesoderm origin. These antibodies stained 14 and 0% of the 12-day cells and 63 and 30% of the 28-day cells, respectively, indicating a large increase in these epitopes between 12 and 28 days of differentiation. Several lectins appear to stain the same locations as osteocalcin in addition to other locations. These include Gleheda, GS I-A_4, HPA, PSL, PVL, and DSA. The binding of these lectins suggests the presence of α-GalNAc (Gleheda, GS I-A_4, and HPA), Neu5Ac (PSL), and LacNAc (PVL and DSA), on osteoblasts in 28-day EBs. The significance of these observations is yet unknown.

Antibodies used for the detection of endoderm were Sox17 and PDX1. Sox17 is a general endoderm antibody and PDX1 is specific for pancreatic islets. Few endoderm cells were found in either 12 or 28-day EBs, given that only 10% of the 12-day EBs and 36% of the 28-day EBs were stained by Sox17. PDX1, however, stained 35% of the 12-day EBs and 71% of the 28-day EBs, indicating a prevalence of pancreatic development in the endoderm derivatives. This unexpected result was confirmed by numerous blocking experiments and positive controls (see Fig. 12).

Finally, using Oct 3/4 to identify undifferentiated cells, we showed that 69% of 12-day EBs contained undifferentiated cells while none of the 28-day EBs did. This shift from undifferentiated to differentiated cell types could help to account for the changes observed in lectin binding; however, we cannot specifically account for all such changes.

Acknowledgements We thank Sue O'Shea for helpful advice and critical reading of this manuscript and Crystal Pacut for growing the BG01 hESCs and supplying us with cryostat sections. We also thank the Consortium for Functional Glycomics for the use of their glycan array on our fucose-binding lectins. Finally, we thank the NIH for funding this project with grants GM-29470 and GM-069985 (This research is supported (in part) by the National Institutes of Health through the University of Michigan's Cancer Center Support Grant (5 P30 CA46592)).

References

1. Haltiwanger, R.S., Lowe, J.B.: Role of glycosylation in development. Annu. Rev. Biochem. **73**, 491–537 (2004)
2. Wearne, K.A., Winter, H.C., O'Shea, K., Goldstein, I.J.: Use of lectins for probing differentiated embryonic stem cells for carbohydrates. Glycobiology **16**, 981–990 (2006)
3. Venable, A., Mitalipova, M., Lyons, I., Jones, K., Shin, S., Pierce, M., Stice, S.: Lectin binding profiles of SSEA-4 enriched pluripotent human embryonic stem cell surfaces. BMC Dev. Biol. **5**, 5–15 (2005)
4. Goldstein, I.J., Poretz, R.D.: Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. In: Liener, I.E., Sharon, N., Goldstein, I.J. (eds.) The Lectins: Properties, Functions and Applications in Biology and Medicine, pp. 35–247. Academic Press Inc., Orlando (1986)
5. Kirkeby, S., Winter, H.C., Goldstein, I.J.: Comparison of the binding properties of the mushroom *Marasmius oreades* lectin and *Griffonia simplicifolia* I-B_4 isolectin to αgalactosyl carbohydrate antigens in the surface phase. Xenotransplantation **11**, 254–261 (2004)
6. Winter, H.C., Mostafapour, K., Goldstein, I.J.: The mushroom *Marasmius oreades* lectin is a blood group type B agglutinin that recognizes the Galα1,3Gal and Galα1,3Galβ1,4GlcNAc porcine xenotransplantation epitopes with high affinity. J. Biol. Chem. **277**, 14996–15001 (2002)
7. Singh, T., Wu, J.H., Peumans, W.J., Rougé, P., Van Damme, E.J. M., Wu, A.M.: Recognition profile of *Morus nigra* agglutinin (Morniga G) expressed by monomeric ligands, simple clusters and mammalian polyvalent glycotopes. Mol. Immunol. **44**, 451–462 (2007)
8. Yamamoto, K., Konami, Y., Irimura, T.: Sialic acid-binding motif of *Maackia amurensis* lectins. J. Biochem. **121**, 756–761 (1997)
9. Endo, T., Ohbayashi, H., Kanazawa, K., Kochibe, N., Kobata, A.: Carbohydrate binding specificity of immobilized *Psathyrella velutina* lectin. J. Biol. Chem. **267**, 707–713 (1992)
10. Kochibe, N., Matta, K.L.: Purification and Properties of an *N*-acetylglucosamine-specific lectin from *Psathyrella velutina* mushroom. J. Biol. Chem. **264**, 173–177 (1989)

11. Ueda, H., Kojima, K., Saitoh, T., Ogawa, H.: Interaction of a lectin from *Psathyrella velutina* mushroom with *N*-acetylneuraminic acid. FEBS Lett. **448**, 75–80 (1999)
12. Ueda, H., Matsumoto, H., Takahashi, N., Ogawa, H.: *Psathyrella velutina* mushroom lectin exhibits high affinity toward sialoglycoproteins possessing terminal *N*-acetylneuraminic acid α2,3-linked to penultimate galactose residues of trisialyl *N*-glycans. J. Biol. Chem. **277**, 24916–24925 (2002)
13. Kawashima, H., Sueyoshi, S., Li, H., Yamamoto, K., Osawa, T.: Carbohydrate binding specificities of several poly-*N*-acetyllactosamine-binding lectins. Glycoconj. J. **7**, 323–334 (1990)
14. Crowley, J.F., Goldstein, I.J., Arnarp, J., Löngren, J.: Carbohydrate binding studies on the lectin from *Datura stramonium* seeds. Arch. Biochem. Biophys. **231**, 524–533 (1984)
15. Kawagish, H., Mizuno, T.: Purification and properties of a β-galactosyl-specific lectin from the fruiting bodies of *Ischnoderma resinosus*. FEBS Lett. **227**, 99–102 (1988)
16. Teneberg, S., Ångström, J., Jovall, P-Å., Karlsson, K-A.: Characterization of binding of Galβ4GlcNAc-specific lectins from *Erythrina cristagalli* and *Erythrina corallodendron* to glycosphingolipids. J. Biol. Chem. **269**, 8554–8563 (1994)
17. Fukumori, F., Takeuchi, N., Hagiwara, T., Ohbayashi, H., Endo, T., Kochibe, N., Nagata, Y., Kobata, A.: Primary structure of a Fucose-specific lectin obtained from a mushroom, *Aleuria aurantia*. J. Biochem. **107**, 190–196 (1990)
18. Kochibe, N., Furukawa, K., Purification and properties of a novel fucose-specific hemagglutinin of *Aleuria aurantia.* Biochemistry **19**, 2841–2846 (1980).
19. Wimmerova, M., Mitchell, E., Sanchez, J.-F., Gautier, C., Imberty, A.: Crystal structure of a fungal lectin, J. Biol. Chem. **278**, 27059–27067 (2003)
20. Yamashita, K., Kochibe, N., Ohkura, T., Ueda, I., Kobata, A.: Fractionation of L-Fucose-containing oligosaccharides on immobilized *Aleuria aurantia* lectin. J. Biol. Chem. **260**, 4688–4693 (1985)
21. Moreno, F.B.M.B., Martil, D.E., Cavada, B.S., Filguiera de Azevedo, Jr. W.: Crystallization and preliminary X-ray diffraction analysis of an anti-H(O) lectin from *Lotus tetragonolobus* seeds. Acta Crystallograph. Sect. F. Struct. Biol. Cryst. Commun. **62**, 680–683 (2006)
22. Petryniak, J., Duś, D., Podwińska, J.: Agglutination of murine and guinea pig peritoneal cells by α-L-fucose-binding lectin: *Evonymus europaea*. Eur. J. Immunol. **13**, 459–464 (1983)
23. Petryniak, J., Goldstein, I.J.: Immunochemical studies on the interaction between synthetic glycoconjugates and α-L-fucosyl binding lectins. Biochemistry **25**, 2829–2838 (1986)
24. Pereira, M.E.A., Kabat, E.A.: Specificity of Purified Hemagglutinin (Lectin) from *Lotus tetragonolobus*. Biochemistry **13**, 3184–3192 (1974)
25. Iyer, P.N.S., Wilkinson, K.D., Goldstein, I.J.: An *N*-acetyl-D-glucosamine binding lectin from *Bandeiraea simplicifolia* seeds. Arch. Biochem. Biophys. **177**, 330–333 (1976)
26. Nakamura-Tsuruta, S., Kominami, J., Kuno, A., Hirabayashi, J.: Evidence that *Agaricus bisporus* agglutinin (ABA) has dual sugar-binding specificity. Biochem. Biophys. Res. Commun. **347**, 215–220 (2006)
27. Wu, A.M., Wu, J.H., Herp, A., Liu, J.-H.: Effect of polyvalencies of glycotopes on the binding of a lectin from the edible mushroom, *Agaricus bisporus*. Biochem. J. **371**, 311–320 (2003)
28. Boland, C.R., Chen, Y.-F., Rinderle, S.J., Resau, J.H., Luk, G.D., Lynch, H.T., Goldstein, I.J.: Use of the lectin from *Amaranthus caudatus* as a histochemical probe of proliferating colonic epithelial cells. Cancer Res. **51**, 657–665 (1991)
29. Rinderle, S.J., Goldstein, I.J., Matta, K.L., Ratcliffe, R.M.: Isolation and characterization of amaranthin, a lectin present in the seeds of *Amaranthus caudatus*, that recognizes the T- (or cryptic T)-antigen. J. Biol. Chem. **264**, 16123–16131 (1989)
30. Grahn, E., Askarieh, G., Rocklöv, Å.H, Tateno, H., Winter, H.C., Goldstein, I.J., Krengel, U.: Crystal structures of the *Marasmius oreades* mushroom lectin in complex with a xenotransplantation epitope. J. Mol. Biol. **369**, 710–721 (2007)
31. Shibuya, N., Goldstein, I.J., Van Damme, E.J.M., Peumans, W.J.: Binding properties of a mannose-specific lectin from the snowdrop (*Galanthus nivalis*) bulb. J. Biol. Chem. **263**, 728–734 (1988)
32. Mo, H., Winter, H.C., Goldstein, I.J.: Purification and characterization of a Neu5Acα2-6Galβ1,4Glc/GlcNAc-specific lectin from the fruiting body of the polypore mushroom *Polyporus squamosus*. J. Biol. Chem. **275**, 10623–10629 (2000)
33. Shibuya, N., Goldstein, I.J., Broekaert, W.F., Nsimba-Lubaki, M., Peeters, B., Peumans, W.J.: The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(α2-6)Gal/GalNAc sequence. J. Biol. Chem. **262**, 1596–1601 (1987)
34. Mo, H., Winter, H.C., Van Damme, E.J.M., Peumans, W.J., Misaki, A., Goldstein, I.J.: Carbohydrate binding properties of banana (*Musa acuminata*) lectin. Eur. J. Biochem. **268**, 2609–2615 (2001)
35. Hammarström, S., Murphy, L.A., Goldstein, I.J., Etzler, M.E., Carbohydrate binding specificity of four *N*-acetyl-D-galactosamine-"specific" lectins: *Helix pomatia* A hemagglutinin, soy bean agglutinin, lima bean lectin, and *Dolichos biflorus* lectin. Biochemistry **16**, 2750–2755 (1977)
36. Singh, T., Wu, J.H., Peumans, W.J., Rougé, P., Van Damme, E.J. M., Avarez, R.A., Blixt, O.: Carbohydrate specificity of an insecticidal lectin isolated from the leaves of *Glechoma hederacea* (ground ivy) towards mammalian glycoconjugates. Biochem. J. **393**, 331–341 (2006)
37. Ishiyama, I., Uhlenbruck, G., Hermann, G.: Isolation of an anti-A-agglutinin from *Helix aspersa.* Blut **24**, 178–179 (1972)
38. Grubhoffer, L., Ticha, M., Kocourek, J.: Isolation and properties of a lectin from the seeds of hairy vetch (*Vicia villosa* Roth). Biochem. J. **195**, 623–626 (1981)
39. Knibbs, R.N., Osborne, S.E., Glick, G.D., Goldstein, I.J.: Binding determinants of the sialic acid-specific lectin from the slug *Limax flavus*. J. Biol. Chem. **268**, 18524–18531 (1993)
40. Muresan, V., Iwanij, V., Smith, Z.D.J., Jamieson, J.D.: Purification and use of limulin: a sialic acid-specific lectin. J. Histochem. Cytochem. **30**, 938–946 (1982)
41. Ciopraga, J., Ångström, J., Bergström, J., Larsson, T., Karlsson, N., Motas, C., Gozia, O., Teneberg, S.: Isolectins from *Solanum tuberosum* with different detailed carbohydrate binding specificities: unexpected recognition of lactosylceramide by *N*-acetyllactosamine-binding lectins. J. Biochem. **128**, 855–867 (2000)
42. Matsumoto, I., Jimbo, A., Mizuno, Y., Seno, N., Jeanloz, R.W.: Purification and characterization of potato lectin. J. Biol. Chem. **258**, 2886–2891 (1983)