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Discovery of novel differentiation markers in the early stage of chondrogenesis by glycoform-focused reverse proteomics and genomics

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ABSTRACT

Background: Osteoarthritis (OA) is one of the most common chronic diseases among adults, especially the elderly, which is characterized by destruction of the articular cartilage. Despite affecting more than 100 million individuals all over the world, therapy is currently limited to treating pain, which is a principal symptom of OA. New approaches to the treatment of OA that induce regeneration and repair of cartilage are strongly needed.
Methods: To discover potent markers for chondrogenic differentiation, glycoform-focused reverse proteomics and genomics were performed on the basis of glycoblotting-based comprehensive approach.
Results: Expression levels of high-mannose type *N*-glycans were up-regulated significantly at the late stage of differentiation of the mouse chondroprogenitor cells. Among 246 glycoproteins carrying this glycotype identified by ConA affinity chromatography and LC/MS, it was demonstrated that 52% are classified as cell surface glycoproteins. Gene expression levels indicated that mRNAs for 15 glycoproteins increased distinctly in the earlier stages during differentiation compared with Type II collagen. The feasibility of mouse chondrocyte markers in human chondrogenesis model was demonstrated by testing gene expression levels of these 15 glycoproteins during differentiation in human mesenchymal stem cells.
Conclusion: The results showed clearly an evidence of up-regulation of 5 genes, ectonucleotide pyrophosphatase/phosphodiesterase family member 1, collagen alpha-1(III) chain, collagen alpha-1(XI) chain, aquaporin-1, and netrin receptor UNC5B, in the early stages of differentiation.
General significance: These cell surface 5 glycoproteins become highly sensitive differentiation markers of human chondrocytes that contribute to regenerative therapies, and development of novel therapeutic reagents.

1. Introduction

Human osteoarthritis (OA), the most common form of joint disease, is characterized by degeneration of the articular cartilage [1–3]. Currently, OA is recognized as a major disease that causes disability in the elderly; the prevalence of this disease is expected to increase dramatically over the next 20 years with population aging. Currently available pharmacological therapies are mainly used for pain relief, and include acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxgenase-2 inhibitors, glucocorticoids, and opioids. Other palliative drugs affecting various targets such as catabolic enzymes or cytokine-activated signaling cascades are currently in development, but a curative treatment to induce regeneration and repair of cartilage is still lacking.

Mesenchymal stem cells (MSCs) in various adult tissues, such as bone marrow, adipose tissue, and synovial fluid, have multipotency and self-renewal capabilities, including induction to undergo chondrogenic differentiation [4–8]. Especially, cells from the synovial membrane have the ability to differentiate into chondrocytes [6], indicating that they potentially have a physiological role in the repair and degeneration of the articular cartilage. Therefore, it is expected that medications induce proliferation and differentiation of MSCs *in vitro* and *in vivo* will contribute to repair and regeneration of damaged cartilage. Advent of new sensitive markers that contribute to development of novel therapeutic reagents to induce differentiation of MSCs are strongly required for improving quality of life of patients suffering OA.

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Protein glycosylation is one of the most important posttranslational modifications in cell surface proteins and extracellular matrix proteins, which is considered to have a variety of biological functions, including enhancement of protein stability, controlling cell-to-cell communication and adhesion [9]. Recently, it has been well documented that glycan modifications of proteins greatly contribute to the pathogenesis of many diseases [10,11]. One of the characteristics of cartilage is that chondrocytes exist in the extracellular matrix. It is also well known that glycoproteins are abundant on the cell surface and in cartilage extracellular matrix. Recently, we reported that some high-mannose type *N*-glycan levels are decreased significantly both in human OA cartilage and in degraded mouse cartilage [12], strongly suggesting an association between *N*-glycans of cell surface glycoproteins and the pathogenesis of OA. Pabst et al. [13] also demonstrated that the levels of glycophenotype of primary human chondrocytes were altered by inflammatory cytokines. These means clearly that proteins with such characteristic *N*-glycoforms might become novel chondrogenic cell markers. However, glycoproteins bearing high-mannose type *N*-glycans in cartilage and their functional roles in the process of cartilage degradation have not yet been uncovered. Glycoproteomics is a new potential strategy among focused proteomics approaches for the characterization of plasma membrane proteins [14]. This approach has facilitated comprehensive identification of cell surface glycoproteins with low abundance and insolubility by hydrazide chemistry [15,16] and lectin affinity chromatography [17]. However, determination of the total glycan structures from whole cellular glycoproteins had long remained to be a challenging and extremely difficult task due to the lack of general platforms for high throughput glycomics [18]. We have developed a standardized protocol of glycomics based on the simple chemical enrichment method, namely glycoblotting method [19] that allows for rapid and large-scale enrichment analysis of human serum glycans [20–25]. We also recently demonstrated the versatility of this glycoblotting protocol for the monitoring and characterization of the processes of dynamic cellular differentiation of mouse embryonic carcinoma cells and mouse embryonic stem cells into cardiomyocytes or neural cells [26]. In the present study, we discovered novel chondrogenic differentiation markers through high throughput glycoproteomics uncovering proteins displaying characteristic glycan structures, notably a combination of glycoblotting-based quantitative cellular *N*-glycomics and glycoform-focused reverse proteomics/genomics.

2. Experimental section

2.1. Cell culture and chondrogenic differentiation

ATDC5 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (Sigma, St. Louis, MO) containing 5% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Life Technologies), 100 units/mL penicillin (Life Technologies), and 100 μg/mL streptomycin (Life Technologies). The cells were seeded into 6-well tissue culture plates at a density of 1.0×10^5 cells and cultured in the above medium supplemented with 10 μg/mL transferrin (Roche Diagnostics Co., Basel, Switzerland). For induction of chondrogenesis, 10 μg/mL bovine insulin (Sigma) was reacted to the sub-confluent cells. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every other day.

Human MSCs were obtained from Lonza Walkersville, Inc. (Walkersville, MD). Cell culture and chondrogenic differentiation of human MSCs were performed according to the manufacturer's protocols. Human MSCs were expanded in monolayer culture by passaging twice in MSC basal medium (MSCBM) with MSC growth supplement (MCGS), L-glutamine, and GA-1000. For induction of chondrogenic differentiation, human MSCs were suspended in chondrogenic basal medium with ITS supplement (containing insulin, transferrin, and selenite), dexamethasone, ascorbate, sodium pyruvate, proline, GA-1000, L-glutamine, and 10 ng/mL TGF-β3. The seeding cell number for human MSCs was 2.5×10^5 cells/15 mL polypropylene culture tube (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and the cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every other day.

2.2. Alcian blue staining

Accumulation of glycosaminoglycans associated with chondrocyte differentiation was assessed by staining with Alcian blue. Differentiating ATDC5 cells were rinsed with PBS (Life Technologies) and fixed with 95% methanol (Wako Pure Chemical, Osaka, Japan) for 20 min. They were then stained with 0.1% Alcian blue 8GX (Merck Chemicals, Darmstadt, Germany) in 0.1 M HCl (Nacalai Tesque, Kyoto, Japan) overnight.

Differentiating human MSCs were rinsed with PBS (Life Technologies) and fixed with 4% formaldehyde (Wako Pure Chemical). The formaldehyde-fixed cells were embedded in paraffin and sectioned. The paraffin-embedded sections were rinsed with *p*-xylene (Wako Pure Chemical), ethanol (Wako Pure Chemical), and 3% acetic acid (Wako Pure Chemical) and stained with 10 mg/mL Alcian blue 8GX in 3% acetic acid for 30 min.

2.3. Glycoblotting-based quantitative cellular N-glycomics

Release of total *N*-glycans was carried out directly using whole-cell lysates as follows. After inducing differentiation, ATDC5 cells were scraped in PBS containing 10 mM EDTA (Wako Pure Chemical) at various time points. The scraped cells were washed three times with PBS containing 10 mM EDTA and lysed by incubation with 1% Triton X-100 for 1 h on ice. The lysates were centrifuged at 15,000 rpm for 10 min at 4 °C, and the obtained supernatant was added to cold acetone (1:4) to precipitate proteins. The precipitates were collected by centrifugation at 12,000 rpm for 15 min at 4 °C followed by serial washing with acetonitrile. The resulting precipitates were dissolved in 50 μL of 100 mM ammonium bicarbonate containing 0.04% of 1-propanesulfonic acid, 2-hydroxyl-3-myristamido followed by incubation at 60 °C for 10 min. The solubilized proteinaceous materials were reduced by 10 mM DTT at 60 °C for 30 min followed by alkylation with 20 mM iodoacetamide in the dark at room temperature for 30 min. The mixture was then treated with 800 units of trypsin (Sigma-Aldrich, Cat. No. T0303, St. Louis, MO) at 37 °C overnight followed by heat inactivation of the enzyme at 90 °C for 10 min. After cooling to room temperature, *N*-glycans of glycopeptides were released from trypsin-digested samples by incubation with 2.5 units of peptide-*N*-glycosidase F (Roche Applied Science, Basel, Switzerland) at 37 °C overnight. The sample mixture was then dried by vacuum centrifugation and stored at −20 °C until use.

Glycoblotting of *N*-glycans was performed according to the procedure described previously [20]. BlotGlyco H beads (50 μL) (10 mg/mL suspension; Sumitomo Bakelite Co., Ltd.) were aliquoted onto the wells of a MultiScreen Solvinert filter plate (Millipore, Billerica, MA). Peptide-*N*-glycosidase F-digested samples were added with an internal standard (A2 amide glycan, $Hex_5(HexNAc)_4(NeuAcAmide)_2$, prepared from egg yolks) to the wells followed by addition of 360 μL of 2% acetic acid in acetonitrile. The plates were incubated at 80 °C for 100 min to specifically capture total glycans in sample mixtures onto beads via stable hydrazone bonds. The plate was washed with 200 μL of 2 M guanidine HCl in ammonium bicarbonate followed by washing with the same volume of water and 1% triethylamine in methanol (MeOH). Each washing step was performed twice. Unreacted hydrazide functional groups on beads were capped by incubation with 10% acetic anhydride in MeOH for 30 min at room temperature. Then, the solvent was removed under vacuum, and the beads were serially washed with 2 × 200 μL of 10 mM HCl, MeOH, and dioxane,

respectively. On-bead methyl esterification of carboxyl groups in sialic acids [27] was carried out by incubation with 100 mM 3-methyl-1-*p*-tolyltriazene in dioxane at 60 °C for 80 min. Then, the beads were serially washed with 200 μL of dioxane, water, MeOH, and water. The glycans blotted on beads were subjected to *trans*-iminization reaction with N^{α}-((aminooxy)acetyl)tryptophanylarginine methyl ester (aoWR, aminooxy-functionalized peptide reagent) for 70 min at 80 °C. WR-tagged glycans were eluted by adding 100 μL of water and then purified on a Mass PREP™ hydrophilic interaction chromatography (HILIC) μElution Plate (Waters, Manchester, UK) according to the manufacturer's protocol. Each purified aoWR-derivatized sample was dried and diluted with 5 μL of 2,5-dihydroxybenzoic acid (DHB; 10 mg/mL in 30% acetonitrile; Bruker Daltonics, Bremen, Germany), and an aliquot (1 μL) was deposited on the stainless steel target plate. The analytes were then subjected to MALDI-TOF-MS analysis using an Ultraflex time-of-flight mass spectrometer II (Bruker Daltonics) in reflector, positive ion mode typically summing 1000 shots. The detected *N*-glycan peaks in MALDI-TOF-MS spectra were picked using the software FlexAnalysis version 2.0 (Bruker Daltonics). The intensities of the isotopic peaks of each glycan were normalized relative to 1.5 pmol of internal standard (A2 amide glycan) in each status. The glycan structures were estimated by input of peak masses into the GlycoMod Tool (Swiss Institute of Bioinformatics) and GlycoSuite (Proteome Systems). All glycomic analyses were performed three times and the average values and standard deviation were summarized in Supplementary Table S-IV and V, respectively.

2.4. Enrichment and identification of high-mannose type N-glycopeptides

Enrichment of high-mannose type *N*-glycopeptides was carried out directly using whole-cell lysates as follows. After inducing differentiation, ATDC5 cells were scraped in PBS containing 10 mM EDTA at various time points. The scraped cells were washed three times with PBS containing 10 mM EDTA and lysed by incubation with 1% Triton X-100 for 1 h on ice. The lysates were centrifuged at 15,000 rpm for 10 min at 4 °C, and the obtained supernatant was added to cold acetone (1:4) to precipitate proteins. The precipitates were dissolved in 500 mM Tris–HCl (pH 8.5) containing 1 mM EDTA, 8 M urea, and 0.1% SDS. The solubilized proteinaceous materials were reduced by 40 mM DTT at 37 °C for 90 min followed by alkylation with 60 mM iodoacetamide by incubation in the dark at room temperature for 30 min. The mixture was then treated with 50 μg of trypsin (Promega, Madison, WI) at 37 °C overnight. The digested proteins were applied to a concanavalin A (ConA)-agarose (Seikagaku Co., Tokyo, Japan) column equilibrated with a solution of 1.5 M NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 100 mM Tris–HCl buffer (pH 7.5). After the column was washed with equilibrated buffer, the high-mannose type *N*-glycopeptides were eluted with buffer containing 10 mM methyl α-D-glucopyranoside followed by elution with buffer containing 10 mM methyl α-D-mannopyranoside. The eluate was then

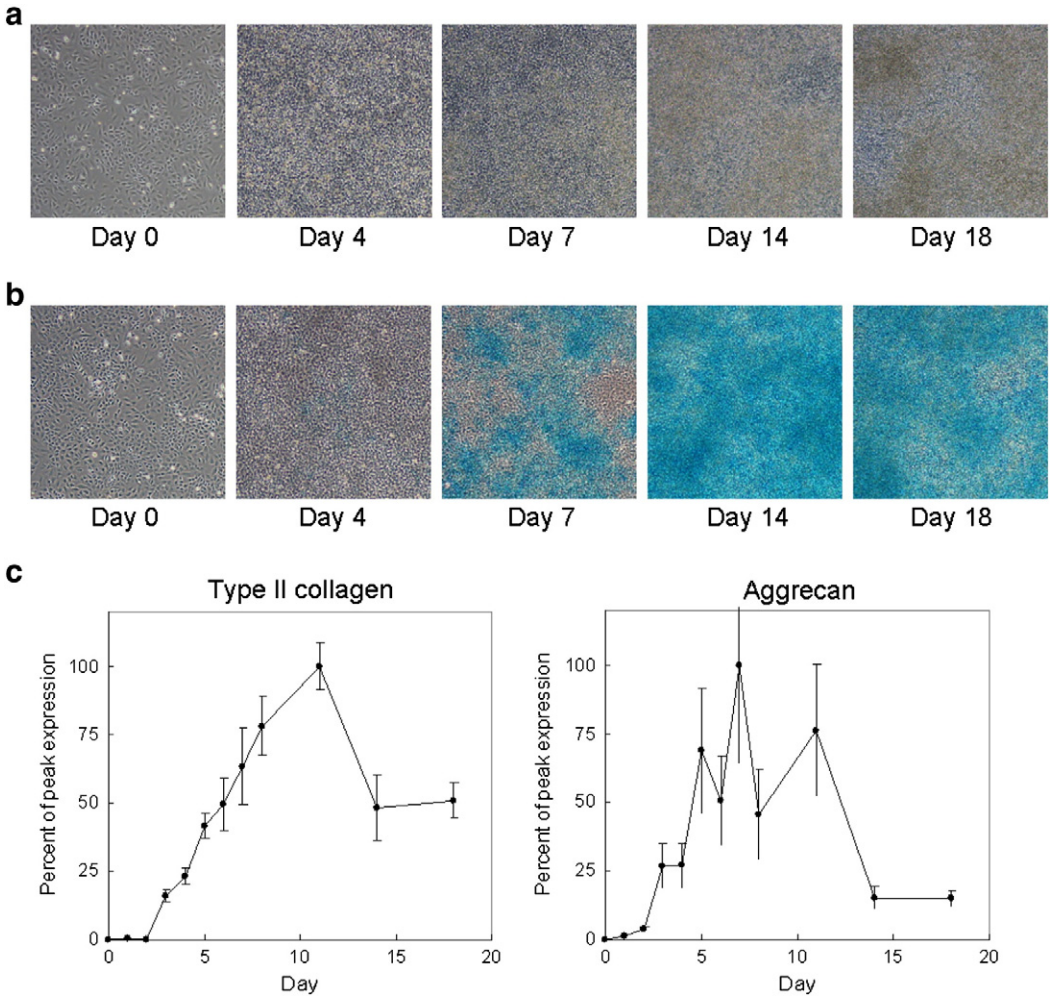
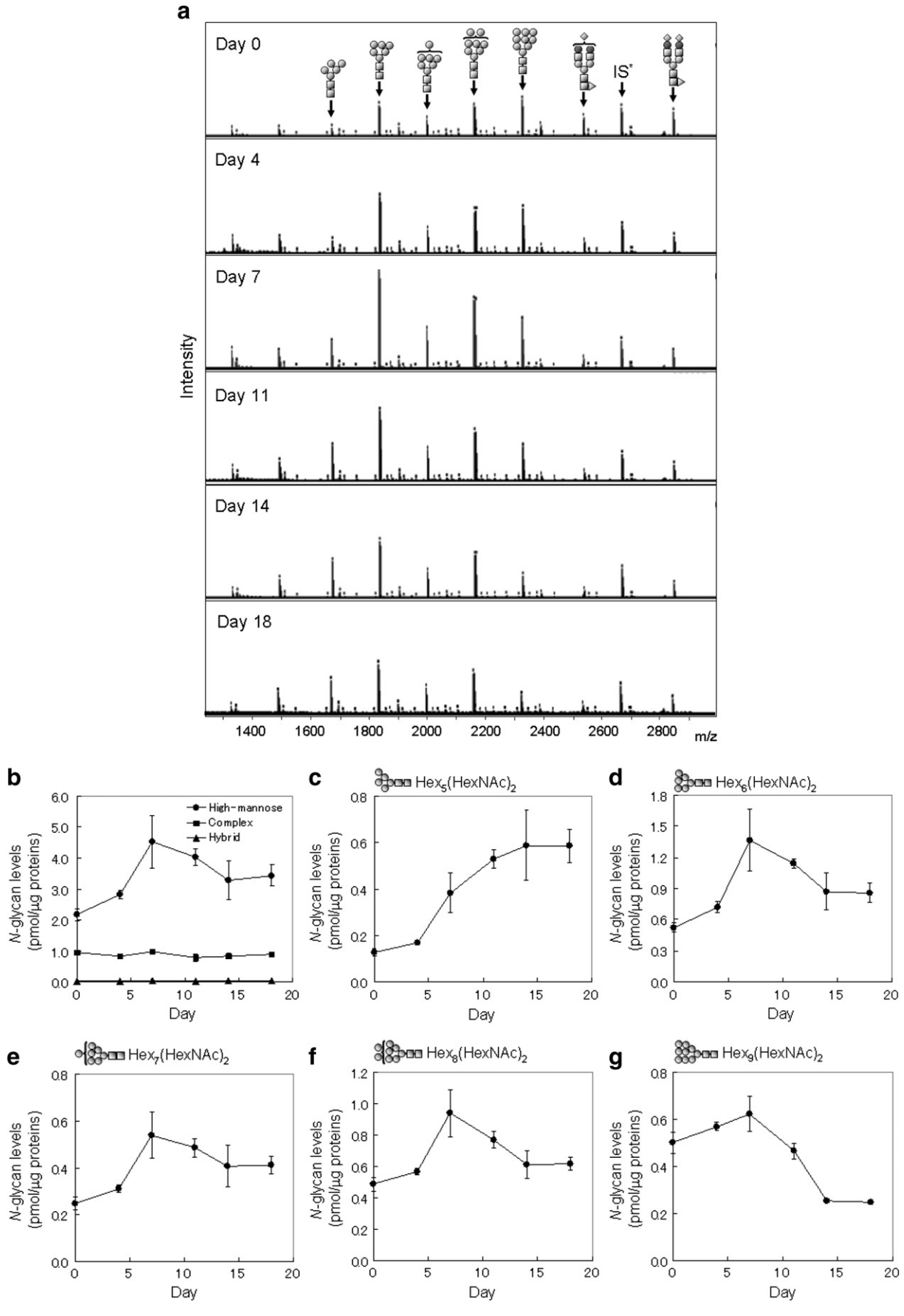


Fig. 1. Chondrogenic differentiation of ATDC5 cells. (a) ATDC5 cells formed cartilage nodules through cellular proliferation and condensation after induction with insulin. (b) Cultured ATDC5 cells were stained with Alcian blue. The Alcian blue-positive area was observed on Day 7 and increased after Day 14. (c) Type II collagen and aggrecan expression was analyzed by qPCR in biological triplicate. Levels of both transcripts increased after the cells reached confluence and peaked at Days 7–11.


a
Day 0
Day 4
Day 7
Day 11
Day 14
Day 18
IS*
Intensity
1400 1600 1800 2000 2200 2400 2600 2800 m/z
b
High-mannose
Complex
Hybrid
N-glycan levels (pmol/μg proteins)
Day
c
Hex5(HexNAc)2
d
Hex6(HexNAc)2
e
Hex7(HexNAc)2
f
Hex8(HexNAc)2
g
Hex9(HexNAc)2

dried by vacuum centrifugation, dissolved in 100 mM NH_4HCO_3, and incubated with 2.5 units of peptide-*N*-glycosidase F at 37 °C overnight. The sample was then dried by vacuum centrifugation and stored at −20 °C until use.

The high-mannose type *N*-glycopeptides were analyzed using a NanoFrontier (L) system (Hitachi High-Technologies, Tokyo, Japan), consisting of a capillary HPLC system based on a nanoGR generator and electrospray ionization linear ion trap time-of-flight mass spectrometry (ESI-LIT-TOFMS). Linear ion trap-time of flight (LIT-TOF) and collision-induced dissociation (CID) modes were used for MS detection and peptide fragmentation. The reverse-phase nano-capillary columns (NTCC-360/75-3) were prepared by Nikkyo Technos (Tokyo, Japan). The separated peptides were then ionized with a capillary voltage of 1600 V. The ionized peptides were detected at a detector potential of 2000 V. The mobile phases consisted of 2% acetonitrile (0.2% formic acid) in water (A) and 98% acetonitrile (0.2% formic acid) in water (B). After loading peptides onto the column, the mobile phase was held at 95% A and 5% B for 6 min. Exponential gradient elution was performed by increasing the mobile phase composition from 5% to 50% B over 55 min at a flow rate of 100 nL/min. Raw data were converted into the Mascot format using the Data Processing software (Hitachi High-Technologies), and analyzed subsequently using MASCOT ver. 2.2 (Matrix Science K.K., Tokyo, Japan) against the Swiss-Prot protein database(mouse, version 57.4). The following dynamic modifications were considered: carbamidomethylation of cysteine, oxidation of methionine, and PNGase F-catalyzed conversion of asparagine to aspartic acid at the site of carbohydrate attachment.

2.5. Real-time qPCR

Total RNA was prepared from ATDC5 cells and human MSCs cultured in differentiation medium using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. After reverse transcription using SuperScript III First-Strand Synthesis SuperMix (Life Technologies), real-time qPCR was performed with the 7500 Real-Time PCR System using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). For primer sequences, see Supplementary Table S-I and II.

2.6. Microarray analysis

Using the total RNA as templates, cyanine 3-labeled cRNA targets were prepared and purified with Quick Amp Labeling kit according to the "One-Color Microarray-Based Gene Expression Analysis" protocol provided by the manufacturer (Agilent Technologies). The resultant cRNA targets were hybridized to Whole Mouse Genome Oligo Microarray (Agilent Technologies, 41,174 probes). After hybridization, the microarray slide was washed, dried, and scanned on an Agilent DNA microarray scanner (Agilent Technologies), and hybridization images were quantified using Feature Extraction Software 9.1 (Agilent Technologies). Data analyses were conducted with GeneSpring GX 7.3.1 (Agilent Technologies).

3. Results

3.1. Entire N-glycan profiling during chondrogenic differentiation of ATDC5 cells

We performed glycoblotting for comprehensive analysis of *N*-glycan alterations during chondrogenic differentiation of ATDC5 cells, a mouse chondroprogenitor cell line to serve as a common model for studying chondrogenic differentiation after insulin induction [28]. As shown in Fig. 1a, ATDC5 cells were grown to confluence and formed into cartilage nodules through cellular condensation in the presence of insulin (10 μg/mL). To confirm the maturation of cartilage nodules, Alcian blue staining, a representative method to detect cartilage matrix, was performed. As shown in Fig. 1b, Alcian blue-positive cells were observed on Day 7 and increased in number after Day 14. Moreover, the levels of expression of the major cartilage matrix proteins, type II collagen and aggrecan, were confirmed by quantitative polymerase chain reaction (qPCR) (Fig. 1c). Both were increased after Day 3 and peaked at Day 7-11. As described above, we confirmed that ATDC5 cells could be differentiated into mature chondrocytes after Day 7 of induction with insulin.

Whole *N*-glycans obtained from ATDC5 cells with or without induction into chondrocytes were profiled by glycoblotting-based high-throughput MALDI-TOF mass spectrometry (Fig. 2). As summarized in Table 1, 45 kinds of glycoforms were detected and quantified reproducibly. High-mannose type *N*-glycans were the major components

Table 1
Glycoforms detected during chondrogenic differentiation of ATDC5 cells.

Peak no.	m/z	Composition
1	1324.55	$Hex_2(HexNAc)_2dHex_1$
2	1340.55	$Hex_3(HexNAc)_2$
3	1486.61	$Hex_3(HexNAc)_2dHex_1$
4	1502.60	$Hex_4(HexNAc)_2$
5	1543.63	$Hex_3(HexNAc)_3$
6	1648.66	$Hex_4(HexNAc)_2dHex_1$
7	1664.65	$Hex_5(HexNAc)_2$
8	1689.69	$Hex_3(HexNAc)_3dHex_1$
9	1705.68	$Hex_4(HexNAc)_3$
10	1746.71	$Hex_3(HexNAc)_4$
11	1810.71	$Hex_5(HexNAc)_2dHex_1$
12	1826.71	$Hex_6(HexNAc)_2$
13	1851.74	$Hex_4(HexNAc)_3dHex_1$
14	1867.73	$Hex_5(HexNAc)_3$
15	1892.76	$Hex_3(HexNAc)_4dHex_1$
16	1908.76	$Hex_4(HexNAc)_4$
17	1949.79	$Hex_3(HexNAc)_5$
18	1988.76	$Hex_7(HexNAc)_2$
19	2010.79	$Hex_4(HexNAc)_3(NeuAc)_1$
20	2029.79	$Hex_6(HexNAc)_3$
21	2054.82	$Hex_4(HexNAc)_4dHex_1$
22	2070.81	$Hex_5(HexNAc)_4$
23	2095.84	$Hex_3(HexNAc)_5dHex_1$
24	2150.81	$Hex_8(HexNAc)_2$
25	2156.85	$Hex_4(HexNAc)_3dHex_1(NeuAc)_1$
26	2172.84	$Hex_5(HexNAc)_3(NeuAc)_1$
27	2191.84	$Hex_7(HexNAc)_3$
28	2216.87	$Hex_5(HexNAc)_4dHex_1$
29	2257.90	$Hex_4(HexNAc)_5dHex_1$
30	2298.92	$Hex_3(HexNAc)_6dHex_1$
31	2312.86	$Hex_9(HexNAc)_2$
32	2318.90	$Hex_5(HexNAc)_3dHex_1(NeuAc)_1$
33	2334.90	$Hex_6(HexNAc)_3(NeuAc)_1$
34	2359.93	$Hex_4(HexNAc)_4dHex_1(NeuAc)_1$
35	2375.92	$Hex_5(HexNAc)_4(NeuAc)_1$
36	2419.95	$Hex_5(HexNAc)_5dHex_1$
37	2521.98	$Hex_5(HexNAc)_4dHex_1(NeuAc)_1$
38	2537.98	$Hex_6(HexNAc)_4dHex_1$
39	2563.01	$Hex_4(HexNAc)_5dHex_1(NeuAc)_1$
40	2681.03	$Hex_5(HexNAc)_4(NeuAc)_2$
41	2684.03	$Hex_6(HexNAc)_4dHex_1(NeuAc)_1$
42	2827.09	$Hex_5(HexNAc)_4dHex_1(NeuAc)_2$
43	3192.22	$Hex_6(HexNAc)_5dHex_1(NeuAc)_2$
44	3351.28	$Hex_6(HexNAc)_5(NeuAc)_3$
45	3497.34	$Hex_6(HexNAc)_5dHex_1(NeuAc)_3$

Hex, hexose; dHex, deoxyhexose; HexNAc, *N*-acetylhexosamine; NeuAc, *N*-acetylneuraminic acid.

Fig. 2. *N*-glycan profiling by glycoblotting during chondrogenic differentiation of ATDC5 cells. (a) All samples derived from Day 0 to Day 18 after induction with insulin were measured by MALDI-TOF-MS in biological triplicate. Forty-five glycans shown in Table 1 were profiled. (b) Relative expression levels of high-mannose, hybrid, and complex *N*-glycan subtypes are shown. High mannose type *N*-glycans are major components and the expression levels increased throughout chondrogenic differentiation. (c – g) Relative expression levels of each high-mannose type *N*-glycan are shown. The signal intensity was normalized relative to an internal standard.

Table 2
Gene expression levels of glycosyltransferases during chondrogenic differentiation by microarray analysis.

No.	Gene symbol	Fold change				Annotation
		Day 0	Day 3	Day 5	Day 7	
1	Gcs1	1	1.08	1.10	0.88	–
2	Ganab	1	2.04	1.86	1.52	↑ (upregulated)
	Prkcsh	1	1.28	1.36	1.15	–
3	Ugcgl 1	1	3.06	2.13	1.39	↑ (upregulated)
	Ugcgl 2	1	0.89	1.02	0.82	–
4	Manea	1	1.32	1.41	2.23	↑ (upregulated)
5	Man1b1	1	0.93	0.85	0.78	–
6	Man2c1	1	1.36	1.35	1.28	–
7	Man1a	1	0.46	0.59	0.57	↓ (downregulated)
	Man1a2	1	0.69	0.47	0.29	↓ (downregulated)
	Man1c1	1	0.97	0.75	0.44	↓ (downregulated)
8	Mgat1	1	0.78	0.67	0.64	↓ (downregulated)

with the peak expression twice on Day 7 compared with undifferentiated state (Fig. 2b). The expression pattern of high-mannose type *N*-glycan was similar to the results of analysis of chondrogenic differentiation markers (Fig. 1b–c). These findings clearly suggested that expression levels of high-mannose type *N*-glycans were significantly accompanied by chondrogenic differentiation. Fig. 2c–g shows the expression level of each high-mannose type *N*-glycan. The amount of $Hex_5(HexNAc)_2$ increased by fivefold throughout chondrogenic differentiation and retained at the elevated level on mature chondrocytes (Fig. 2c). Those of $Hex_6(HexNAc)_2$, $Hex_7(HexNAc)_2$, and $Hex_8(HexNAc)_2$ increased and peaked on Day 7 (Fig. 2d–f). On the other hand, $Hex_9(HexNAc)_2$ was downregulated during maturation into chondrocytes. These results indicated that conversion of $Hex_9(HexNAc)_2$ into $Hex_5(HexNAc)_2$ was promoted and that $Hex_5(HexNAc)_2$ accumulated eventually in mature chondrocytes while other high-mannose type *N*-glycanss decreased. We next confirmed the gene expression level of glycohydrolases and glycosyltransferases related to the synthesis of a series of *N*-glycans (Table 2, Fig. 3). The levels of mRNA for *Ganab* and *Ugcgl 1*, which are enzymes involved in converting into $Hex_8(HexNAc)_2$, were upregulated and peaked on Day 3. However, those of *Man1a* and *Man1c*, which convert $Hex_8(HexNAc)_2$ into $Hex_5(HexNAc)_2$, were gradually downregulated. *Mgat1*, which is a key enzyme responsible for switching from high-mannose type *N*-glycans to other hybrid/complex type *N*-glycans, was also downregulated. These results of qPCR analysis showed that both trimming of mannose residues of high-mannose type *N*-glycans and conversion from these glycans into other *N*-glycan types were suppressed during chondrogenic differentiation following activation of biosynthesis of the high-mannose type *N*-glycans at the early stages of differentiation. This finding was strongly correlated with those of quantitative *N*-glycan analysis (Fig. 2).

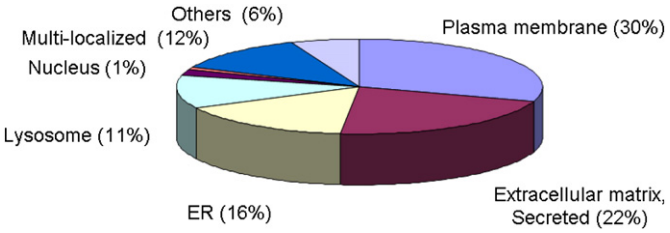


Fig. 4. Subcellular locations of the 246 glycoproteins identified. The identified *c*-glycoproteins were classified based on localization in the cell using information from the UniProt database (http://www.uniprot.org/).

3.2. Identification of high-mannose type N-glycopeptides

Driven by the observed unique *N*-glycan profile in chondrogenic differentiation of ATDC5 cells, proteins that carry high-mannose type oligosaccharides were investigated. Concanavalin A (ConA) was used as an affinity reagent to selectively recover the glycopeptides with high-mannose type oligosaccharides [29,30]. Following the enrichment of glycopeptides of interest in a crude tryptic digest of the chondrogenic differentiated cells, the ConA-bound fraction was further incubated with peptide-*N*-glycosidase F and examined by LC/MS to identify the peptides. The *N*-glycosylation sites were assessed by conversion of asparagine into aspartic acid residues at the position of glycan attachment caused by the peptide-*N*-glycosidase F-catalyzed deglycosylation reaction. The consensus amino acid sequence of *N*-glycosylation (Asn-X-Ser/Thr) was also confirmed. Supplementary Table S-III shows a list of 246 identified *N*-glycoproteins and 434 *N*-glycopeptides (including 460 *N*-glycosylation sites). Further, we analyzed the subcellular localization of the identified proteins using information from the UniProt database (http://www.uniprot.org/). Of the total of 246 identified proteins, 74 proteins (30%) were localized at the plasma membrane and 53 proteins (22%) were localized to the extracellular matrix or were secreted (Fig. 4).

In this study, glycoform-focused reverse proteomics of the chondrogenic differentiated cells revealed many proteins carrying

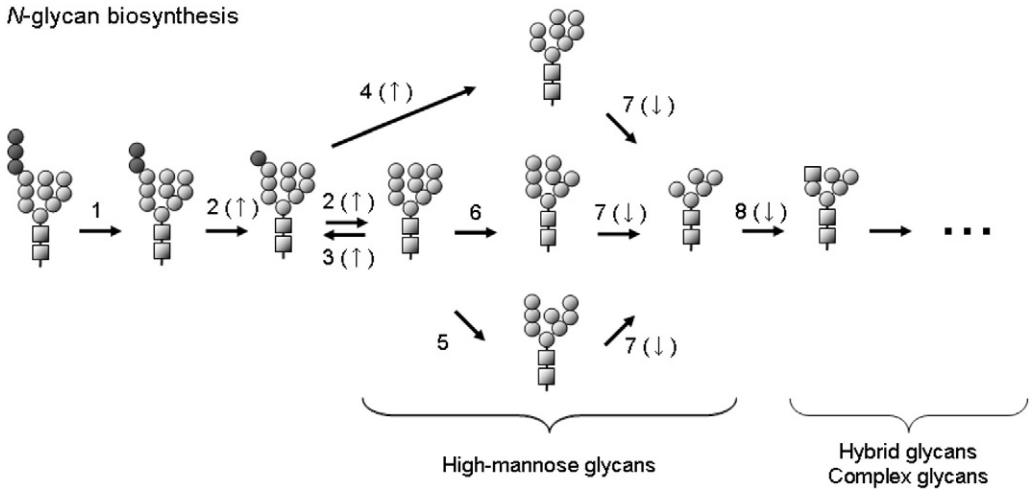


Fig. 3. Biosynthesis of high-mannose type *N*-glycans according to alterations of the glycohydrolases and glycosyltransferases. The levels of mRNA for the glycosyltransferases during chondrogenic differentiation by microarray analysis were shown in Table 2. Up- and downregulated glycosyltransferases are indicated by upward and downward pointing arrows, respectively. The numbers in figure correspond to those of each glycosyltransferase shown in Table 2.

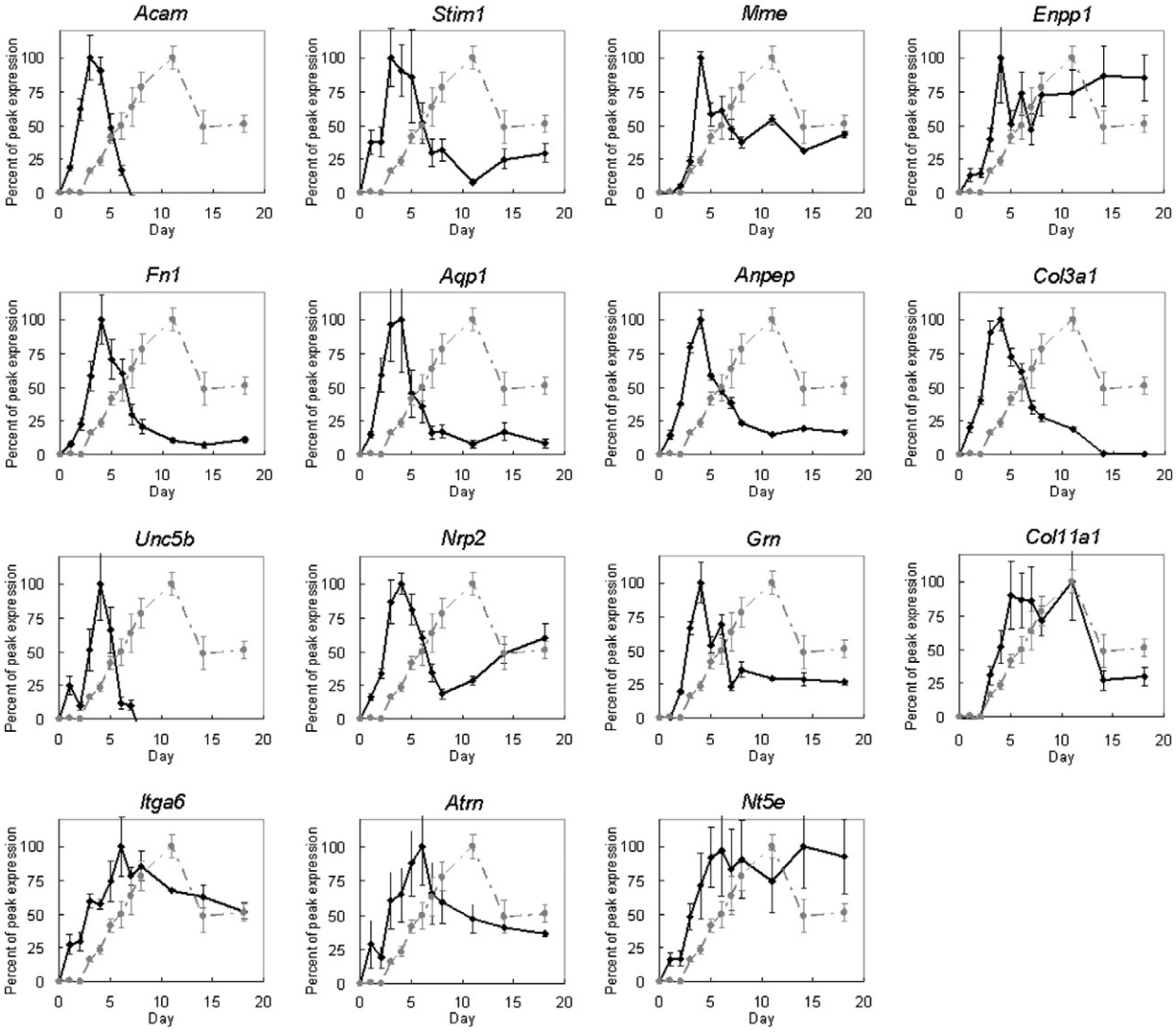


Fig. 5. Gene expression profiles of 15 differentiation marker candidates with increasing transcript levels at the early stages of chondrogenic differentiation in ATDC5 cells. The solid lines indicate gene expression profiles of each of the 15 differentiation marker candidates and the dotted line indicates the gene expression profile of Type II collagen. The profiles were performed in biological triplicate.

high-mannose type *N*-glycans. Interestingly, 52% of the identified glycoproteins were cell surface proteins, which were easily detectable by flow cytometry or immunochemistry, and may include some differentiation markers.

3.3. Gene expression analysis of the identified glycoproteins

Quantitative and qualitative alterations of high-mannose type *N*-glycans are significantly correlated to chondrogenic differentiation. To investigate whether expression of the identified high-mannose glycoproteins is regulated at the mRNA level during differentiation, we performed gene expression analysis of the identified proteins. A total of 246 candidates were preliminary screened by microarray analysis, and genes up-regulated at the earlier stages of chondrogenic differentiation compared with Type II collagen were selected (Supplementary Table S-VI). Then, we focused on cell surface proteins by using information from the UniProt database. Finally, qPCR were performed to validate the expression of the selected genes. The results identified 15 genes showing peak expression at the earlier stages of chondrogenic differentiation compared with Type II collagen (Fig. 5, Table 3).

3.4. Gene expression analysis of 15 differentiation marker candidates during human chondrogenic differentiation

To evaluate the applicability of 15 marker candidates for human chondrogenic differentiation, gene expression analysis was performed in a human MSC model. MSCs have the ability to differentiate into cells of the chondrogenic lineage. Human MSCs were cultured and differentiated into chondrocytes. To confirm the chondrogenic differentiation of the cells, Alcian blue staining was performed. As shown in Fig. 6a, Alcian blue-positive cells on extracellular matrices were observed after Day 7 of induction. Moreover, the expression of the major cartilage matrix proteins type II collagen was confirmed to be induction-dependent as well as in mouse ATDC5 by qPCR. In the human chondrogenesis model, five of the 15 genes—*Aqp1*, *Col3a1*, *Col11a1*, *Enpp1*, and *Unc5b*—showed distinct up-regulation of expression at the mRNA level in the earlier stage of differentiation compared with Type II

Table 3
Fifteen cell surface *N*-glycoproteins showing increased expression during chondrogenic differentiation.

Gene symbol	Swiss-prot number	Protein name	Sequence	Mascot score[a]	Subcellular location[b]
Nt5e	5NTD_MOUSE	5′-nucleotidase	LDNYSTQELGR	59	plasma membrane
Acam	ACAM_MOUSE	Adipocyte adhesion molecule	HVYNNLTEEQK	87	plasma membrane
Anpep	AMPN_MOUSE	Aminopeptidase N	FTCNQTTDVIIIHSK	57	plasma membrane
			KLNYTLK	53	
			LNYTLK	36	
			SGQEDHYWLDVEKNQSAK	68	
Aqp1	AQP1_MOUSE	Aquaporin-1	NQTLVQDNVK	45	plasma membrane
Atrn	ATRN_MOUSE	Attractin	IDSTGNVTNELR	107	plasma membrane
Col3a1	CO3A1_MOUSE	Collagen alpha-1(III) chain	LLSSRASQNITYHCK	57	Extracellular matrix, Secreted
Col11a1	COBA1_MOUSE	Collagen alpha-1(XI) chain	VYCNFTAGGETCIYPDK	109	Extracellular matrix, Secreted
			NTSEDILYGNK	45	
Enpp1	ENPP1_MOUSE	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1	VYNGSVPFEER	48	plasma membrane
Fn1	FINC_MOUSE	Fibronectin	DQCIVDDITYNVNDTFHK	114	Extracellular matrix, Secreted
			HEEGHMLNCTCFGQGR	67	
			LDAPTNLQFVNETDR	121	
			WTPLNSSTIIGYR	81	
			NYTDCTSEGR	71	
Grn	GRN_MOUSE	Granulins Grn	NYTTDLLTK	45	Secreted
Itga6	ITA6_MOUSE	Integrin alpha-6	LWNSTFLEEYSK	77	plasma membrane
Mme	NEP_MOUSE	Neprilysin	SCINESAIDSR	98	plasma membrane
			EIANATTKPEDR	72	
Nrp2	NRP2_MOUSE	Neuropilin-2	NFTSPNGTIESPGFPEK	85	plasma membrane
Stim1	STIM1_MOUSE	Stromal interaction molecule 1	LAVTNTTMTGTVLK	117	plasma membrane
Unc5b	UNC5B_MOUSE	Netrin receptor UNC5B	LSDTANYTCVAK	55	plasma membrane

[a] $P < 0.05$ by Mascot Search.
[b] Subcellular localizations of the identified proteins using information from the UniProt database (http://www.uniprot.org/).

collagen (Fig. 6b). These results clearly indicated that these 5 genes (*Aqp1*, *Col3a1*, *Col11a1*, *Enpp1*, and *Unc5b*) are highly sensitive markers during human chondrocyte differentiation.

4. Discussion

Comprehensive *N*-glycan profiling by a glycoblotting method was first performed on chondrogenic differentiation of mouse ATDC5 cells. By quantitative monitoring of the 45 *N*-glycans during cell differentiation, we clarified that high-mannose type *N*-glycans increased specifically during chondrocyte maturation (Fig. 2, Table 1). The increases in this type of *N*-glycans were corroborated by gene expression analysis of glycohydrolases and glycosyltransferases (Fig. 3, Table 2). On the other hand, considering that ATDC5 cells differentiate into osteocytes after chondrogenic differentiation with insulin [31], it seems that the alterations of high-mannose type *N*-glycans observed here may be influenced by osteogenic differentiation. However, it was also reported that an elevation of alkaline phosphatase activity and expression of Type X collagen mRNA induced during osteogenic differentiation of ATDC5 cells are first observed during Days 16–20 after induction of insulin [31]. In the present study, the alterations of high-mannose type *N*-glycans were observed during Days 7–14, indicating that there must be little effect of osteogenic differentiation on the alteration of high-mannose type *N*-glycans. Moreover, Heiskanen et al. [32] demonstrated that the levels of high-mannose type *N*-glycans on osteoblasts differentiated from human MSCs were lower than those of human MSCs. In addition, we revealed previously that no significant change in the expression level of high-mannose type *N*-glycans was observed during mouse cardiac and neural differentiation while increase in other glycotypes such as complex type *N*-glycans carrying fucose or bisect-type GlcNAc residues was detected [26]. Therefore, it is concluded that the increases in high-mannose *N*-glycans observed here may have specificity in the chondrogenic differentiation. Interestingly, it was uncovered that the level of a key high-mannose type *N*-glycan, $Hex_5(HexNAc)_2$, decreased significantly in mouse and human OA cartilage [12]. As anticipated, the gene expression of *Mgat1*, which converts specifically this glycoform into other hybrid and/or complex type *N*-glycans, was significantly upregulated in degraded mouse cartilage, and mouse chondrocytes with suppressed *Mgat1* expression exhibited drastically reduced expression levels of matrix metalloprotease 13 and aggrecanase 2 mRNA following stimulation with interleukin-1α [12]. These observations suggest clearly that the alteration of high-mannose type *N*-glycans is attributable to gene expression of *Mgat1*, which regulates the expression of key proteases, matrix metalloprotease 13 and aggrecanase 2, in a cartilage degradation process. Further, Pabst et al. also demonstrated that the levels of high-mannose type *N*-glycans of primary human chondrocytes decrease under inflammatory cytokines, IL-1β and TNF-α[13]. Moreover, it was also demonstrated that mouse neural cells differentiated from embryonic stem cells induce upregulation of various bisect-type *N*-glycans during cell differentiation [26]. Taking into account all these results, it was strongly suggested that alteration and accumulation of high-mannose type *N*-glycans might have essential roles in the homeostatic and functional maintenance of chondrocytes and an association with the pathogenesis of OA.

By performing proteomics focusing on the glycoproteins bearing high-mannose type *N*-glycans during chondrogenic differentiation, we identified 246 glycoproteins (Supplementary Table S-III) that included cartilage-specific extracellular matrix proteins (e.g., aggrecan core protein and collagen α-1(II) chain). It was expected that most of the identified glycoproteins, in which 52% were plasma membrane residences (Fig. 4), should have important roles in maintenance of chondrocytes because decrease of high-mannose type *N*-glycans was observed both in mouse and human OA cartilage [12]. Recently, proteomics based on differential gel electrophoresis (DIGE) revealed alteration of 39 proteins during chondrogenic differentiation of human umbilical cord stromal MSCs [33]. Although five of these 39 proteins (12.8%) were cell surface proteins, it seems likely that they are not candidates of chondrocyte specific markers during cell differentiation. Merit of our strategy to discover cells specific marker is evident because most *N*-glycans are distributing on cell surfaces as multiple branching structures involved in the glycoproteins and have essential functions in cellular adhesion. In addition, rapid structural alterations in carbohydrate moieties of the glycoproteins appear to be beneficial for rapid cell proliferation and differentiation that need to exchange cell surface receptors/ligands at each stage during differentiation [34]. On the other

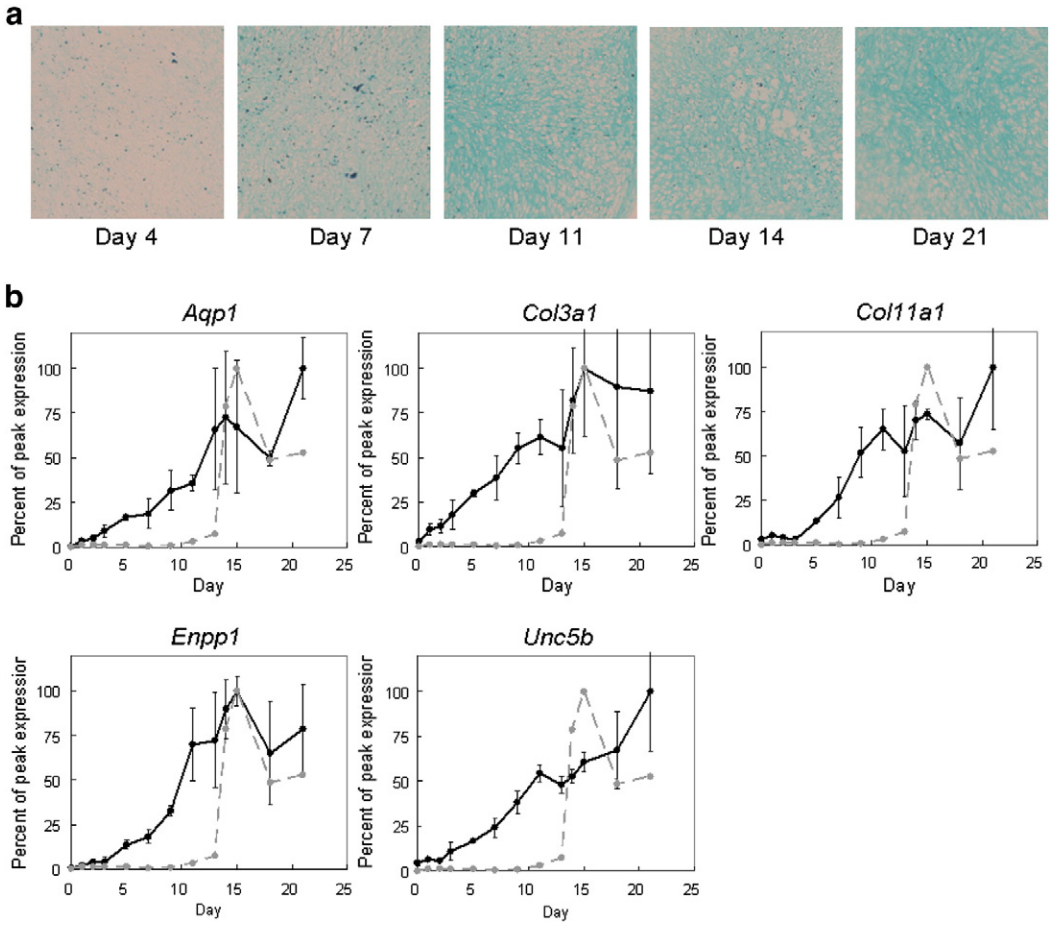


Fig. 6. Chondrogenic differentiation of human MSCs and gene expression profiles of five differentiation marker candidates. (a) Human MSCs differentiated after induction with TGF-β3 were stained with Alcian blue. The Alcian blue-positive area was observed on Day 7 and increased after Day 11. (b) Gene expression levels of five differentiation marker candidates are shown according to the time after induction of differentiation in comparison with Type II collagen. The profiles were performed in biological triplicate. The solid lines indicate gene expression profiles of each of the five differentiation marker candidates and the dotted line indicates the gene expression profile of Type II collagen.

hand, it is clear that proteins may alter only the expression levels without any drastic changes in the primary amino acid sequence directly regulated by genetic information. Approach focusing on the glycoforms altered specifically during cell differentiation apparently facilitated the process to enrich potential glycoprotein markers from a large set of tryptic peptides of whole cellular proteins. In the present study, although ConA may become a suited tool for the enrichment of glycopeptides bearing high-mannose type *N*-glycans, it is noteworthy that other options such as reverse glycoblotting method focusing sialic acid residues [35,36] would expand glycoform-focused reverse proteomics and genomics approach.

The gene expression levels of the identified glycoproteins on mouse ATDC5 cells revealed that 15 cell surface glycoproteins showed an enhanced expression during chondrogenic differentiation with a peak at the earlier stages of differentiation compared with Type II collagen (Fig. 5). These genes may have several functional roles in development and are expected to be useful as markers indicating the initial stage of differentiation. Five of these 15 genes—*Col3a1*, *Col11a1*, *Enpp1*, *Fn1*, and *Itga6*—have already been reported to be upregulated in chondrogenic differentiation of ATDC5 cells or human MSCs [37–42]. Although the significance of the other 12 genes to chondrogenic differentiation is still unclear, we uncovered that 5 genes—*Aqp1*, *Col3a1*, *Col11a1*, *Enpp1*, and *Unc5b*—are significantly enhanced expression levels at the earlier stages of chondrogenic differentiation of human MSCs compared with Type II collagen (Fig. 6). As the expression profiles of these 5 genes were quite similar in both human and mouse cases, we may suggest that they have essential roles in the maintenance of chondrocytes and potential for use as chondrocyte specific differentiation markers.

Two proteins, COL3A1 and COL11A1, are well-known cartilage collagens. COL3A1 is expressed in the superficial and deepest area of articular cartilage along with type I collagen. The deepest zone contains ellipsoidal chondrocytes that synthesize lubricin and types I, II, and III collagen fibers. COL11A1 exists in transitional and deep area of articular cartilage [2]. ENPP1 is an enzymatic generator of pyrophosphate in differentiated chondrocytes. The regulation of extracellular pyrophosphate levels is necessary for appropriate mineral homeostasis in cartilage. In aging and OA cartilage, a dysregulated increase in pyrophosphate elaboration in chondrocytes promotes calcium pyrophosphate dihydrate crystal deposition [43]. AQP1 is a well-characterized membrane glycoprotein that belongs to the aquaporin family and is selectively permeated by water driven by osmotic gradients. Cell adhesion to type II collagen-coated plates was significantly reduced by AQP1 deletion. AQP1-mediated plasma membrane water permeability plays an important role in chondrocyte migration and adhesion [44]. UNC5B, netrin receptor, is highly expressed in the brain and has functions in axon growth of neurons and angiogenesis. It is also expressed at lower levels in developing cartilage. The level of UNC5B transcript increases in mouse embryos from day 10.5 in the cartilaginous primordia of many bones and cartilage [45] while its function during development remains to be unclear. All differentiation marker candidates found in this study are present in cartilage. Considering that some of these molecules may be related closely to the pathogenesis of OA, they are

expected to become not only differentiation markers but biomarkers and therapeutic targets.

Proteomic approaches in cartilage biology and OA research have uncovered many cartilage- and OA-related protein markers [46]. The increase or decrease in the levels of the identified proteins could have an important role in to the pathology of OA but most of those proteins were already known to be present in the extracellular matrix of cartilage. Williams et al. suggest that future proteomic studies will need to reveal more than just evidence of extracellular matrix degradation and include additional information, such as alteration in the post-translational modifications. Recently, glycomic approaches for *N*-glycans [13], *O*-glycans [47,48] and proteoglycans [49] have also been reported. However, comprehensive identification of proteins carrying the altered glycans in OA has not been performed yet. The significance of protein glycosylations is one of the most important research areas in cartilage biology and related clinical/pharmaceutical fields. In the present study, we demonstrated the importance of combined glycoform-focused reverse proteomics and genomics to discover new differentiation markers, notably the integration of "omics" approach.

In conclusion, we performed quantitative and qualitative analyses of *N*-glycans and glycoproteins during chondrogenic differentiation using glycoblotting method and subsequent glycoform-focused reverse proteomics and genomics. This study showed that levels of high-mannose type *N*-glycans increase during chondrogenic differentiation, suggesting that this glycotype may have key roles in differentiation and/or homeostatic maintenance of chondrocytes. Further, we found 5 candidate chondrogenic differentiation markers. All candidates were cell surface glycoproteins and showed increased expression levels at the earlier stages of differentiation compared with Type II collagen, a well-known chondrogenic differentiation marker. These markers can be detected specifically with much higher sensitivity than Type II collagen in differentiation by immunochemistry or fluorescence-activated cell sorting (FACS). Moreover, these molecules may have key functional roles in chondrogenesis and are also expected to be potential as pharmaceutical targets in OA, though further extensive investigation is needed to assess the practical availability of differentiation markers and pharmaceutical targets. We concluded that glycoform-focused reverse proteomics and genomics, a strategy starting from glycotyping analysis by high throughput glycoblotting method [18,19], is a promising approach that links directly data obtained from different omics methodologies by first obtaining exhaustive quantitative glycomics back to the proteome and gemomics. This approach allows novel classification of proteins with regard to the significance of glycosylation in a way that is not possible with information that can currently be obtained from genomic, transcriptomic, and traditional proteomic analysis alone. This concept will be widely applicable for the identification of diagnostic biomarkers, therapeutic targets, and differentiation markers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.10.027.

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