REGULATION OF GLYCAN STRUCTURES IN MURINE EMBRYONIC STEM CELLS: COMBINED TRANSCRIPT PROFILING OF GLYCAN-RELATED GENES AND GLYCAN STRUCTURAL ANALYSIS*

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Background: Glycans contribute to vertebrate development, but regulatory mechanisms are unknown.
Results: Glycans and transcripts encoding the glycosylation machinery were profiled during stem cell differentiation.
Conclusion: Changes in glycans frequently correlated with changes in transcripts, supporting a significant role for transcriptional regulation.
Significance: Knowledge of the mechanisms that regulate glycan expression provides insight into the roles of glycosylation in development.

The abundance and structural diversity of glycans on glycoproteins and glycolipids are highly regulated and play important roles during vertebrate development. Because of the challenges associated with studying glycan regulation in vertebrate embryos, we have chosen to study mouse embryonic stem (ES) cells as they differentiate into embryoid bodies (EBs) or into extraembryonic endodermal (ExE) cells as a model for cellular differentiation. We profiled N- and O-glycan structures isolated from these cell populations and examined transcripts encoding the corresponding enzymatic machinery for glycan biosynthesis in an effort to probe the mechanisms that drive the regulation of glycan diversity. During differentiation from mouse ES cells to either EBs or ExE cells, general trends were detected. The predominance of high mannose N-glycans in ES cells shifted to an equal abundance of complex and high mannose structures, increased sialylation, and increased α-Gal termination in the differentiated cell populations. While Core 1 O-glycan structures predominated in all three cell populations, increased sialylation and increased core diversity characterized the O-glycans of both differentiated cell types. Increased polysialylation was also found in both differentiated cell types. Differences between the two differentiated cell types included greater sialylation of N-glycans in EBs, while α-Gal capped structures were more prevalent in ExE cells. Changes in glycan structures generally, but not uniformly, correlated with alterations in transcript abundance for the corresponding biosynthetic enzymes suggesting transcriptional regulation contributes significantly to the regulation of glycan expression. Knowledge of glycan structural diversity and transcript regulation should provide greater understanding of the roles of protein glycosylation in vertebrate development.

Cell surface glycan structures can contribute to interactions with the extracellular environment that influence cellular physiology, pathology, and recognition (1-3). These interactions can include...
recognition by multivalent lectins that modulate cell adhesion versus migration during embryogenesis (4-6) or generation of lectin lattices that influence cell surface receptor half-life and intracellular signaling (7). Carbohydrate structures can also enhance or block protein-protein interactions in developmental contexts to influence cell signaling and migration during early embryonic development (8). The essential roles and contributions of protein and lipid glycosylation in embryogenesis have also been demonstrated through studies on defects in individual glycosylation steps (9,10), revealing the impact of aberrant glycan modifications on vertebrate development and human genetic disease (9). In contrast, the mechanisms controlling glycan structural diversity are generally poorly understood.

Glycans attached to proteins and lipids have been shown to vary greatly among adult vertebrate tissues and are highly regulated in abundance during development (11-13). Early studies that examined changes in glycoprotein or glycolipid structural diversity during embryogenesis largely focused on developmentally regulated cell surface epitopes (14,15) using classical methods for glycan separation and structural analysis by mass spectrometry. In recent years broad-based analyses of glycan structures have been enhanced by improvements in separations and mass spectrometry methods that provide increased breadth, depth, and sensitivity to the analysis (16-19). MSn approaches can now resolve subtle differences in glycan isoforms and fine details of oligosaccharide structural diversity, greatly enriching glycan abundance measurements even for minor glycan structures which frequently play important roles in animal development (17,20,21).

While methods to analyze complex glycan structural profiles have greatly improved, the underlying mechanisms that regulate glycan diversity have been more difficult to study. In mammals, glycan structures are elaborated by ~700 enzymes involved in glycan extension, modification, recognition, and catabolism (12,13,22,23). In addition, glycan biosynthesis is not directly template-driven like the synthesis of peptide structures from genome-derived transcripts. Numerous additional factors contribute to glycan structural diversity, including enzyme abundance, specificity, localization, potential enzyme competition for substrate glycans, accessibility to glycan modification sites, abundance of the respective protein or lipid acceptors, and availability of activated sugar nucleotide precursors (3,11-13). In an initial effort to characterize the mechanisms that regulate cellular glycosylation, we previously examined adult mouse tissues for transcript abundance encoding the enzymatic machinery for glycan biosynthesis and compared these data with glycan structural profiles derived from the corresponding tissues (13). Several correlations were identified suggesting that global regulation of cellular glycosylation results, at least in part, from alterations in transcript expression of the corresponding biosynthetic enzymes. However, some glycan structures did not correlate with transcript levels for the glycosylation machinery indicating that regulation may also occur at a post-transcriptional level (13).

Difficulties in the analysis of glycan structural changes in mammalian embryonic tissues result from the small amounts of material and mixed cell populations in early embryos (15). As an alternative model system, we have chosen to study mouse embryonic stem (ES) cell differentiation as a model for early mouse embryogenesis (11,24). Differentiation of ES cells has been used extensively as an in vitro model for the study of numerous aspects of early embryonic development (25-28) including late pre-implantation and early post-implantation events and formation of the three germ layers. Selected differentiation into mixed cell populations, such as embryoid bodies (EBs) that contain cell types representing all three germ layers (29,30), or more focused induction of cell types such as extraembryonic endodermal (ExE) cell induced by treatment with retinoic acid (31), allow access to cell populations in sufficient numbers for biochemical study that are not available when studying vertebrate embryos. Numerous studies have previously analyzed the focused changes in glycan structures in ES cell populations, including stage-specific embryonic antigens (Le°/SSEA-1 antigens, SSEA-3 and SSEA-4) (14,32) or tumor-rejection antigens (TRA-1-60 and TRA-1-81) (33). Broad changes in protein (34), glycoprotein (35), and N-glycan (36,37) abundance have been recently profiled
during mouse and human ES cell differentiation. Previous studies from our group have also examined changes in proteoglycans (11) and glycosphingolipid (24) biosynthesis in mouse embryonic stem (ES) cell differentiation into EBs or ExE cells and noted the correlations between glycan and lipid structural changes and the transcripts encoding the corresponding enzymatic machinery.

In the present study, we have expanded our analysis of mouse ES cell differentiation into EBs and ExE cells through the characterization of N- and O-glycan structures by MS^n approaches and correlated changes in glycan abundance with transcript profiles of the glycan biosynthetic machinery. Significant changes in glycan structures were revealed and comparison with transcript abundance for the enzymatic machinery responsible for elaboration of N- and O-linked glycans identified numerous correlations and some instances where changes in glycan structures did not correlate with corresponding transcript abundance. The comparison of these two data sets provide insights into regulation of glycan diversity during ES cell differentiation and provide hypotheses for mechanisms that drive glycan changes in the developing vertebrate embryo.

EXPERIMENTAL PROCEDURES

Mouse stem cell growth and differentiation: Mouse ES cells were grown and differentiation was induced by treatment with either retinoic acid (for ExEs) or removal of LIF (for EBs) as previously described (11,24). Briefly, mouse ES cells were grown on 0.2% gelatin coated tissue culture plates in culture media containing high glucose DMEM (Cellgro), 10% FBS (Atlanta Biologicals), 10% Knockout™ Serum Replacement (Millipore), 1X MEM amino acids (Cellgro), 1mM sodium pyruvate (Cellgro). EBs were generated in 6 days on agarose coated Petri dishes in mES media without Knockout™ Serum Replacement and ESGRO®. ExE cells were generated in 6 days on gelatin coated tissue culture plates in mouse ES cell media without ESGRO® and addition of 4 µM retinoic acid (Sigma). Media was replaced daily. Cell populations of ES cells, EBs, and ExE cells were characterized by flow cytometry using the lineage-specific marker CD9 (11,38), by immunofluorescence staining and by transcript analysis using qRT-PCR targeting key differentiation markers (supplemental Fig. 1) (11,24) prior to further glycomic or glycotranscriptomic analysis.

Gene selection: A collection of glycan-related genes were organized into biosynthetic pathways for the purpose of investigating transcript changes that may occur and alter the synthesis of specific glycan structures (13). For this study, genes involved in the biosynthesis of N- and O-linked glycans were selected for pairing of transcript analysis data with glycan structural data (supplemental Table 1).

Primer design: Primers were designed within a coding exon of a given gene sequence and primer pairs were validated using genomic DNA as a template to ensure single product formation and linear amplification over several orders of magnitude of template dilutions (11,13,24,39) (see supplemental Table 1 for primer sequences).

RNA isolation and cDNA synthesis: Harvested and flash-frozen murine ES cells, EBs, and ExE cell pellets (~10^6 cells each) were stored at -80°C until used. RNA was isolated using Trizol or the RNeasy Plus Mini RNA isolation kit (Qiagen) as described in detail previously (39). Isolated RNA samples were checked for the presence of contaminating genomic DNA using a cDNA synthesis reaction without reverse transcriptase. Template cDNA was prepared using the SuperScript III First Strand Synthesis kit (Invitrogen) as previously described (39). Briefly, 1 µg of total RNA was used in a 20 µl cDNA synthesis reaction containing an equal mixture of oligo-dT and random hexamer primers. Reactions were diluted 1:10 prior to addition to individual qRT-PCR reactions.

qRT-PCR reactions: A detailed description of the qRT-PCR analysis methods can be found in our recent publication (39). Reactions are set up in a 96-well plate format using either a 20 µl reaction volume (Bio-Rad MyIQ) or a 5 µl reaction volume (RealPlex² Mastercycler, Eppendorf), consisting of 50% iQ™ SYBR® Green Supermix (BioRad), 25% diluted cDNA template and 25% primer pair mix (500 nM each primer, 125 nM final concentration, Eurofins MWG Operon).
Transcript abundance for each primer pair was analyzed in technical triplicate on four biological replicate samples and a normalization control gene was included on each plate. Relative transcript abundance was calculated using the ΔΔCt method (40) using Rpl4 as the normalization gene as previously described (39).

Preparation of N- and O-linked glycans: Cell pellets (~10⁷ cells each) were delipidated and total protein was prepared as previously described (16). Briefly, cell pellets were homogenized in 4:8:3 (chloroform:methanol:water, v/v/v) and centrifuged to produce a proteinaceous pellet and a clarified lipid extract. The protein pellets were washed with acetone and dried to form a fine powder that was stored at -20°C until analysis. For the preparation of N-linked glycans, 2 mg of protein powder was subjected to trypsin digestion as previously described (16). Following clean-up and enrichment for glycopeptides, N-linked glycans were released by digestion with PNGaseF (ProZyme, San Leandro, CA) and separated from residual peptide as previously described (16). For the preparation of O-linked glycans, 2 mg of protein powder was subjected to reductive β-elimination as previously described (17). The resulting oligosaccharide alditols were separated from residual peptide by C18 column as previously described (17). For both N-linked and O-linked glycans, final preparations of released oligosaccharides were permethylated (41) for subsequent analysis by mass spectrometry.

Mass spectrometry of N- and O-linked glycans: Permethylated glycans were dissolved in 1 mM sodium hydroxide in 50% methanol, and directly infused into a linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific) using nanospray ionization (NSI). MS analysis was performed in positive ion mode. The total ion mapping (TIM) function of the Xcalibur software package (Thermo Fisher) was utilized to detect and quantify the prevalence of individual glycans in the total glycan profile; peaks were considered quantifiable if they were two-fold or greater above background. The generation of TIM profiles includes automated acquisition of MS/MS spectra across user-defined mass ranges. All of these fragmentation spectra (as many as 900 per sample) were manually reviewed in order to identify glycan structural features and to highlight the presence of glycans at particular m/z values for subsequent analysis by manual fragmentation (MSⁿ) to more completely resolve structural ambiguities.

Glycan annotation: Structural assignments for the glycans detected at the reported m/z values were based on the compositions predicted by the mass of the intact molecule, the presence of diagnostic MS/MS fragments that report specific N-glycan features, and the limitations imposed on structural diversity by known glycan biosynthetic pathways. Key structural features that were used to assign glycan topologies included the detection of b-ion fragments and their y-ion neutral loss counterparts (42) corresponding to terminal LacNAc (Hex-HexNAc, assumed to be Gal-GlcNAc; fragment at m/z = 486.2, [m+Na]+), internal LacNAc (Hex-HexNAc, fragment at m/z = 472.2, [m+Na]+) sialic acid (fragment at m/z = 398.2, [m+Na]+), outer arm Fuc (as fucosylated LacNAc; deoxyHex-Hex-HexNAc and/or Hex-(deoxyHex)-HexNAc; fragment at m/z = 660.3, [m+Na]+), terminal Fuc (deoxyHex-Hex; fragment at m/z = 415.2, [m+Na]+); core Fuc (as Fuc-HexNAc at the reducing terminal; fragment at m/z = 474.2, [m+Na]+). It is frequently not possible to unambiguously assign non-reducing terminal modifications to a specific arm of a complex N-linked glycan solely using MS/MS spectra. For consistency of presentation and ease of comparison, outer arm modifications are presented as elaborations on the increasingly complex products of the known branching N-acetylglucosamine transferases (GlcNAcTs) in the following succession: GlcNAcT1, T2, T4, T5. For example, a monosialylated, fully galactosylated triantennary glycan is depicted with a single sialic acid on the LacNAc initiated by GlcNAcT1 (the 3-arm) and the three antennae would be represented as products of GlcNAcT1, 2, and 4 (two GlcNAc residues on the 3-arm and 1 on the 6-arm). The disialylated form of the same triantennary glycan would be depicted with the second sialic acid added to the LacNAc initiated by GlcNAcT2 (the 6-arm). Structural ambiguity is also annotated by brackets, which are meant to indicate unresolved sites for elongation. The MS-based methods utilized in this study do not efficiently capture large molecular weight or
highly anionic polymeric glycans such as glycosaminoglycans, polysialic acid, or polyactosamine repeat structures with more than 4 disaccharide repeats. These structures are not characterized here; we did not detect any glycans with molecular weight greater than 4500 daltons by MS. At the other end of the size range, we were able to detect O-linked HexNAc, but cannot distinguish between O-linked GalNAc (the Tn antigen) and O-linked GlcNAc (a nucleocytoplasmic protein modification).

**Data Analysis:** For transcript analysis, average relative abundances were determined for each gene as described previously (13,39) and plotted as a histogram with error bars indicating the standard error of the mean (SEM). Data was plotted on a log10 scale due to the wide dynamic range of the results. For glycan analysis, the prevalence of each glycan was calculated by normalizing its signal intensity to the total signal intensity for all detected glycans and is expressed as “% Total Profile” for each glycan (16). The fold change in transcript abundance or glycan prevalence was calculated by dividing the relative transcript abundance for a given gene or the prevalence of a given glycan in a differentiated cell type (EBs or ExE cells) by the value from the corresponding undifferentiated pluripotent mouse ES cells. Fold-change values were plotted on a linear scale with error bars indicating the standard error of the mean (SEM). For genes or glycans where the ratio was <1, meaning that there was a higher transcript abundance or glycan prevalence in the undifferentiated cells, values were presented as the negative reciprocal to indicate a decrease in abundance.

**Statistical Analysis:** Transcripts from four separate biological samples were analyzed in technical triplicate for each cell type. Variation in technical replicate values were <0.5 Ct units for transcript analysis and <5% for glycan analysis and in general were considerably smaller than biological variation. Glycans from three separate biological samples were analyzed for each cell type. A non-parametric Mann-Whitney test was conducted using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego CA) to generate p-values. A p-value <0.05 was considered significant in these analyses. The associations of transcripts and glycans with cell differentiation status were further queried by hierarchical clustering methods using Euclidean distance calculations as previously described (43-45).

**Immunofluorescence:** Immuno-detection of cell surface polysialic acid and the definitive endodermal differentiation marker, Dab2, were achieved by plating cells on Lab-Tek™ chamber slides, fixation with 4% paraformaldehyde, blocking in PBS-T (PBS, 0.2% Triton X-100) containing 10% donkey serum, and probing with the respective primary antibodies in PBS-T/10% donkey serum. The OL-28 monoclonal antibody (IgM) recognizing α2,8 polysialic acid with DP>4 (46) was used at 1:200 dilution (kind gift of Dr. Karen Colley, University of Illinois School of Medicine). The S2-566 monoclonal antibody (IgM) recognizing the Neu5αc2,8 Neu5acα2,3-Gal sequence (46) was used at 1:200 dilution (kind gift of Dr. Ken Kitajima, Nagoya University). The Dab2 antibody (IgG) was from BD Biosciences. Following washes with PBS-T buffer, appropriate Alexa Fluor® secondary antibodies (Invitrogen) were incubated with the cells at a 1:400 dilution in PBS before mounting coverslip using ProLong® Gold Antifade Reagent (Invitrogen). Cells were visualized and photographed using a Leica DM6000 microscope equipped with epifluorescence illumination.

**RESULTS**

In order to determine the changes in N- and O-linked glycosylation that occur during murine stem cell differentiation, we chose to compare transcripts and glycans from pluripotent mouse ES cells with those from EB and ExE cell types. EBs are formed when LIF is removed from the culture media resulting in the formation of a ball-shaped aggregate that contains cells of mixed endodermal, mesodermal and ectodermal cell lineages (supplemental Fig. 1), as well as a residual undifferentiated cell population. Modest reductions were observed for the transcript abundance of the pluripotency markers Zfp24, Gbx2, Nanog, and Oct4, while the mesendoderm markers (T and Gsc), ectoderm markers (Zic1), visceral endoderm marker (Afp) and endodermal markers (Sox17, Gata4, and Gata6) were elevated...
(supplemental Fig. 1) consistent with the morphological changes and the presence of all three germ layers. In contrast, addition of retinoic acid to undifferentiated mouse ES cells generates a relatively homogeneous extraembryonic endodermal cell population with an epidermal-like morphology, which express a ~4-41-fold reduction in the level of pluripotency markers (Zfp24, Gbx2, Nanog, and Oct4), a >10-fold increase in endodermal markers (Afp, Sox17, Gata4, and Gata6), and no significant elevation of mesendoderm or ectodermal markers (T, Gsc, and Zic1), (11,24,39). In addition, immunostaining of the ExE cells (Fig. 6, panels E and F), reveals a significant elevation of the definitive endodermal marker, Dab2 (47).

The strategy for transcript analysis conducted in this study is an extension of the methodology that was developed previously using mouse tissues as a test platform (13). Biosynthetic pathways involved in N- and O-glycosylation were used to select genes for transcript analysis. The structural analysis of N- and O-glycans employs methods similar to those used in prior studies of tissue glycans from various sources (16,17). Correlations were made between the changes in glycan prevalence during differentiation compared with corresponding changes in relative transcript abundance for the enzymes involved in the synthesis of respective glycan structures. A summary of the major correlated changes that were observed is shown in Table 1, along with several cases where changes in glycans and transcript abundance were not correlated.

**N-linked glycan precursor biosynthesis:** Asparagine linked glycans originate through the synthesis of lipid-linked oligosaccharide (LLO) precursors that are co-translationally transferred onto Asn side chains on nascent polypeptides by a multisubunit oligosaccharyltransferase (OST) (supplemental Fig. 2). The majority of the transcripts for the genes involved in the biosynthesis and transfer of the Glc3Man9GlcNAc2 precursor structure were expressed at constitutive levels in all three cell types analyzed. Transcripts for a few genes were slightly (<5-fold) elevated in the ExE cells compared to mouse ES cells. Transcripts for Alg14 were slightly less abundant in EBs compared to ES cells. The largest change in transcript abundance was a ~15-fold increase in transcripts for a second Alg13 homolog, Gh28d2 (supplemental Fig. 2), in ExE cells compared to ES cells, but transcript abundance for this gene was considerably lower (between ~10–10000 fold) than any other transcript involved in LLO synthesis. Minor elevations (~2-4-fold) in transcript abundance were also seen for several Asn-linked glycosylation (Alg) genes (Alg14, Alg2, Alg9, and Alg8) and the OST subunit, Rpn1, in ExE. The effects of these transcript changes on glycan abundance were not determined, since glycan structures of the corresponding LLO precursors were not determined.

**N-glycan trimming:** Following transfer of the Glc,Man,GlcNAc, oligosaccharide to glycoprotein acceptors, the precursor structure is trimmed and extended by several glycosidases and glucosyltransferases in the endoplasmic reticulum (ER) and Golgi complex (Fig. 1). Among the ER enzymes involved in glycan quality control, the re-glycosylating enzyme isoform, Ugg2, was found to increase in abundance by ~6-fold in ExE cells. Transcripts for four of the five Golgi processing α-mannosidases were significantly increased in ExE cells (Manlal, ~17-fold; Manl2a, ~2.5-fold; Man2a1, 2-fold; and Man2a2, 4-fold), although corresponding increases in the α-mannosidase transcripts in EBs were not statistically significantly. Consistent with the increase in transcript abundance for Golgi α-mannosidase genes, the prevalence of Man,GlcNAc, glycan structures was increased and the prevalence of Man,GlcNAc, was decreased in ExE cells (Fig. 2, supplemental Figs 5 and 6, and Table 1). Grouped together, high mannose glycans possessing >5 Man residues were found to be significantly decreased in both EBs and ExE cells by comparison to ES cells (Table 1).

**N-glycan branching and modification:** Other genes whose transcripts were slightly elevated (~5-fold) in ExE cells include a β3-Gal transferase (B3galt1), several β4-Gal transferases (B4galt1, B4galt2, and B4galt3), and three GlcNAc transferases (Mgat2, Mgat4a, and Mgat4b) (Fig. 1). Increased transcript levels of the Golgi mannosidases, GlcNAc transferases, and Gal transferases correlate with a shift toward greater glycan complexity and greater prevalence of LacNAc (Galβ3/4GlcNAc-) terminated complex
glycans in ExE cells (Table 1). Similarly, increased glycan complexity was also detected in EBs (Fig. 2), but not in conjunction with significant changes for the α-mannosidase or GlcNAc transferase transcripts (Fig. 1). Despite increased glycan complexity in EBs and ExE cells, transcript levels for the enzyme responsible for initiating the synthesis of complex glycans, GlcNAc transferase I (Mgat1), was unchanged. Likewise, the immediate products of Mgat1 activity, hybrid glycans such as GlcNAc2Man5GlcNAc2, were not increased as a group. However, consistent with increased β4-Gal transferase and sialyltransferase transcript levels (see below), the total pool of hybrid glycans was redistributed towards full extension on the single GlcNAc transferred by Mgat1 (Fig. 2 and supplemental Figs. 5 and 6). Although transcripts for the bisecting GlcNAc transferase (Mgat3) were unchanged in ES and ExE cells, a statistically significant increase in bisected glycans was detected in ExE cells (Fig. 2 and Table 1). Overall, however, the prevalence of bisected glycans was low in all three cell populations.

Significant decreases in transcript abundance were observed in EBs (~43-fold) and ExE (~3-fold) cells for Mgat4c (Fig. 1), a gene sequence related to both human GlcNAc transferase IV and chicken GlcNAc transferase VI (48). Prior studies have not resolved the glycosyltransferase specificity for this gene product (48,49), but the overall abundance of the Mgat4c transcripts was low even in ES cells. If Mgat4c encodes either GlcNAc transferase IV- or VI-like activities, then the enzymatic products would likely be represented within the tetra-antennary and penta-antennary classes of N-linked glycans. Penta-antennary structures where not detected in our glycomic analysis and total tetra-antennary structures were unchanged in EBs and ExE cells (supplemental Figs. 4-6).

Transcripts encoding the core α6-Fuc transferase, Fut8, were increased in EBs and ExE cells although the change was not statistically significant in either population. Nonetheless, the prevalence of core fucosylated glycans was ~1.8-fold greater in EB and ExE cells than in ES cells (Fig. 2). The latter increase in core fucosylated structures generally reflects the increase in complex type glycan structures, since both bi-antennary and tri-antennary structures increased in abundance and both classes of structures were predominately core fucosylated. However, there was a striking asymmetry of core fucosylated structures among distinct classes of complex and hybrid structures (see Discussion).

Transcripts for the γ subunit of lysosomal enzyme GlcNAc-1-phosphotransferase also showed a small, but significant increase in both EBs and ExE cells compared to ES cells (Fig. 1). Glycans containing terminal phosphomono- or diesters were not detected in the glycomic analysis, since transient lysosomal enzyme transport intermediates are likely to reflect a minor fraction of the total cellular glycoprotein pool.

**Initiation and branching of mucin type O-linked glycans:** Initiation of mucin-type O-linked glycans occurs through the transfer of GalNAc residues to protein Ser or Thr hydroxyl groups by the action of a family of polypeptide GalNAc transferases (ppGalNTs (50)). This initiating glycan can be branched and extended into a wide variety of structures (Fig. 3). Transcripts encoding several of the ppGalNT family members were significantly increased (Galnt1, Galnt3, Galnt7, Galnt14 and GalntL6) or decreased (Galnt4, Galnt6 and GalntL1) during differentiation from ES cells to EBs or ExE cells (Fig. 3).

The initiating O-linked GalNAc can be extended by the core 1 β3-Gal transferase (C1galt1) to form the Galβ1,3GalNAc structure (51), termed the T antigen, and transcripts encoding this enzyme showed a small, but significant increase in abundance in ExE cells compared to ES cells (Fig. 3). However, transcripts encoding the C1galt1-specific chaperone, Cosmc (52), were not significantly elevated.

Sialyltransferases that modify core 1 structures, include St3gal1, which produces NeuAcα2,3Galβ1,3GalNAc-Ser/Thr (53), and three members of the GalNAc α2,6-sialyltransferase family (St6galnac1, St6galnac2, and St6galnac4 (54,55)). Two of the latter enzymes (St6galnac1, St6galnac2) sialylate unmodified GalNAc-Ser/Thr residues to produce NeuAcα2,6GalNAc-Ser/Thr as well as acting on
core 1 (Galβ1,3GalNAc-Ser/Thr) and sialylated core 1 (NeuAcα2,3Galβ1,3GalNAc-Ser/Thr) structures (54). *St6galnac4* prefers extended sialylated core 1 structures as substrates (54,55). The transcript levels for *St3gal1* were significantly increased (6-fold) in both EBs and ExE cells relative to ES cells, but transcripts levels for the three *St6galnac* family members were unchanged between the ES cells and the two differentiated EB and ExE cell populations (Figs. 3 and 4). Glycomic analysis indicated that sialylated core 1 structures with and without additional glycan extensions comprised a majority of the O-linked glycan structures in all three cell populations, but their abundance as a class changed only slightly in EBs and ExE cells (Fig. 5). The major change in sialylated O-linked glycans corresponded with increases in terminal NeuAcα2,3Gal linkages on core 1 structures with a major shift from the unmodified core 1 disaccharide or mono-sialyl core 1 structures (Galβ1,3[NeuAcα1,6]GalNAc-Ser/Thr) to mono-sialyl NeuAcα2,3Galβ1,3GalNAc-Ser/Thr and di-sialyl core 1 (NeuAcα2,3Galβ1,3[NeuAcα1,6]GalNAc-Ser/Thr) structures (Fig. 5 and supplemental Figs. 7-9) consistent with the increased transcript levels for *St3gal1* (Fig. 3 and Table 1).

Branching of core 1 glycans to form core 2 O-glycan structures, by the action of a family of β6-GlcNAc transferases, was reduced 1.6-fold in ExE cells relative to ES cells and EBs (Fig. 5 and supplemental Figs. 7-9). Of the two core 2 β6-GlcNAc transferase isoforms (*Gcnt1* and *Gcnt3*), transcript levels showed modest increases in abundance in ExE cells (Fig. 3). However, sialylation of core 1 glycans by *St3gal1* competes with branching by the core 2 β6-GlcNAc transferases (56) suggesting that elevated sialylation could contribute to reduced abundance of core 2 structures in ExE cells (Fig. 5).

Core 3 structures were shown to increase significantly in EB and ExE in contrast to the exceptionally low abundance in ES cells (Fig. 5). However, transcripts encoding the core 3 β3-GlcNAc branching enzyme, *B3gnt6*, were in very low abundance and showed no significant change in EB or ExE. Transcripts encoding some of the GlcNAc and Gal transferases that add to core 3 structures (*B3gnt1*, *B3gnt4*, and *B4galnt1*) showed significant increases (Fig. 3) and could potentially account for the increased flux toward extended core 3 structures in EB and ExEs.

Three minor O-linked glycans (a single core 1 and two core 2 structures) were found to be modified by the addition of a HexNAc branch onto the Gal residue linked β3 to the core GalNAc (O-linked structures 7, 16, and 21, see supplemental Figs. 7 and 8). All three of these glycans were increased in EBs and ExE cells relative to ES cells. MSn characterization of these structures unambiguously detected the presence of non-reducing terminal HexNAc as well as the other structural features proposed for these novel glycans. It is unclear what enzyme or enzymes might generate this branching residue, but none of the known βGlcNAcTs (B3gnt3, Gcnt1, Gcnt2, Gcnt3) nor a known α4-GalNAc transferase (*A4gnt*) were increased in EBs or ExE cells. The terminal trisaccharide (NeuAcα2,3[GalNAcβ1,4]Gal-) shared by the branched structures 7 and 16 is also a component of the Sda antigen, although it is generally not linked to a reducing terminal GalNAc in that epitope (57). One enzyme capable of generating the Sda antigen (*B4galnt2*) (58,59) was significantly increased in ExE cells (Fig. 4), while corresponding increases in EBs did not reach statistical significance.

**Lactosamine extension of glycans:** Non-reducing terminal GlcNAc residues on N-linked glycans, O-linked glycans, or glycolipids can be extended by a collection of murine glycosyltransferases to create the array of complex termini (Fig. 4). The first sugar that is added to the GlcNAc residue will classify them as either Lac di-NAc (B4-GalNAc addition), Type I (Neo-lactosamine, β3-Gal addition) or Type II (Lactosamine, β4-Gal addition) glycans. Transcripts encoding the Lac di-NAc GalNAc transferases, *B4galnt3* and *B4galnt4*, were unchanged, with the exception of a minor reduction of *B4galnt3* in ExE cells (Fig. 4) consistent with the low and unchanged abundance of Lac-diNAc glycan structures between the ES EB and ExE cell populations (Figs. 2 and 5).

There are three β3-Gal transferase isoforms that can create and extend Type I glycan structures. The transcripts for only one of these isoforms, *B3gal1*, were significantly increased following
Glucosamine transferases (seven members depending on glycan type) and the sequential and repeated action of several polylactosamine repeat structures occur through the concerted action of the B3galt isoforms in conjunction with B3gnt3. Transcripts for the latter gene product have a minor, but significant reduction in EB and ExE suggesting that extended Type I structures may be reduced in the differentiated cell populations (Fig. 4).

In contrast, Type II structures on N- and O-glycans can be initiated by six β4-Gal transferase isoforms (B4galt1-6 depending on glycan type) (60-62) and transcripts for three genes (B4galt1-3) were significantly increased in ExE cells compared to ES cells, while B4galt4-6 were not significantly changed (Fig. 4). Extension of Type II glycans to form (-GlcNAc three of the seven branching is initiated by a family of depending on glycan type) significantly changed (Fig. 4). Extension of Type I chains as compared to ES cells, while were significantly increased in ExE cells (60-62) and transcripts for three genes (isoforms (glycans can be initiated by six β4-Gal transferase isoform (B4galt1-3) were significantly increased in ExE cells, EBs, and ExE cells (Fig. 4). Transcript abundance for ExE cells. Extension of Type I structures may be reduced in the differentiated cell populations (Fig. 4).

Peripheral fucosylation: Fucosylation of glycans plays a role in the formation of several blood group structures. We evaluated the transcript abundances for both the α2- and α3-Fuc transferase gene families involved in the synthesis of these structures. No significant changes were observed in the transcript levels for the three α1,2 fucosyltransferases (Fut1, Fut2, and Sec1) between ES cells, EBs and ExE cells (Fig. 4). Of the five α1,3-fucosyltransferase genes analyzed, only Fut4 (~26-fold) and Fut11 (~5-fold) transcripts were significantly elevated in ExE cells compared to ES cells (Fig. 2). Externally fucosylated structures (α2- and α3-linked Fuc) were detected as minor percentages of the total N- and O-glycan profiles and a slight increase in O-linked external fucose residues was seen for EB cells while a minor increase was seen on N-glycans in both EBs and ExE cells compared to ES cells (Figs. 2 and 5 and supplemental Figs. 6 and 9).

Other glycan modifications: Other genes involved in the formation of blood group structures include the ABO transferase, sulfotransferases and sialyltransferases. No change in transcript abundance was observed for the ABO transferase (Abo) in the three cell types analyzed (Fig. 4). Transcripts encoding the sulfotransferase responsible for the synthesis of 3′sulfoLea and 3′sulfoLeb (Gal3st1) were present at similar levels in ES cells, EBs and ExE cells (Fig. 4). Enzyme products from two genes, Chst2 and Chst4, are responsible for the formation of 6-sulfoLea. Transcripts were unchanged for Chst4, but a significant increase in transcript abundance was observed for Chst2 (~8-fold) in ExE cells compared to ES cells. No sulfated glycans were detected among the N- or O-glycans from the three cell populations.

Glycan structures containing the terminal Galα1,3Gal- xenotransplantation antigen were found to be significantly increased in N-glycan, but not O-glycan structures, particularly in ExE cells, but to a lesser extent in EBs (Figs. 2 and 5 and Table 1). An increase in transcript abundance
for *Ggtal* (>5-fold) was also observed in ExE cells in correlation with increases in N-linked α3-Gal structures, while no significant increases in *Ggtal* transcripts were found in EBs (Fig. 4).

**Sialylation of N- and O-glycans:** Transcript levels encoding the 20 members of the sialyltransferase gene family (CAZy GT29 (22,23,54,55)) were determined and grouped by the type of linkage produced and the acceptor sugar for sialic acid transfer (Fig. 4). Transcript abundances for five of the six α2,3-sialyltransferases were significantly increased in ExE samples (*St3gal1, St3gal2, St3gal3, St3gal5, St3gal6*) compared to ES cells, with fold increases ranging from ~3-fold for *St3gal3* to ~15-fold for *St3gal6* (Fig. 4), while transcripts for *St3gal4* were slightly reduced in EB and ExE cells. Elevated *St3gal3* transcripts suggests a possible increase in sialylation of Type I lactosamine N-glycan termini (54,66) while reduction in *St3gal4* transcripts suggests a decrease in sialylation of Type II lactosamine N-termi (54,67). Sialic acid can also be added to terminal N-glycan Gal residues in an α2,6 linkage by *St6gal1* and *St6gal2* (66). The transcript abundance for *St6gal1* was significantly increased (~8-fold) in EB cells and transcript levels for *St6gal2* were significantly increased in both EB (4-fold) and ExE (26-fold) compared to ES cells (Fig. 4). Transcripts encoding the GalNAc α2,6-sialyltransferases that transfer to O-glycans and glycolipids did not show significant changes in abundance with the exception of *St6galnac5*, an enzyme that prefers glycolipid substrates over O-glycans (54,68), which was significantly increased (~17-fold) in ExE cells compared to ES cells (Fig. 4). The abundance of sialylated structures isolated from the three cell populations revealed a higher prevalence of total sialylation in EBs and ExE cells than in ES cells (Figs. 2 and 5). The glycan structural analysis did not distinguish the linkage position for the various sialylated structures.

Finally, sialyltransferases that add α2,8-sialic acid to terminal glycan sialic acids as an acceptor can be divided into two groups (54,55), those that add single sialic acid residues (*St8sia1, St8sia5* and *St8sia6*) to glycolipids and O-glycans and polysialyltransferases that can add many sialic acid residues (*St8sia2* (also known as STX), *St8sia4* (also known as PST), and *St8sia3*). *St8sia2* and *St8sia4* extend polysialic acid predominately on N-glycans (69) while *St8sia3* extends polysialic acid on glycolipids (70). Interestingly, transcripts for the genes involved in single sialic acid addition were decreased in abundance (or not detected) in EBs and ExE cells compared to ES cells (Fig. 4). In contrast, transcript abundance for *St8sia2* increased in EBs in ExE cells (5-fold and 43-fold, respectively) and transcripts for *St8sia3* increased in both EBs and ExE cells (8-fold and 4-fold, respectively), while no changes were found for *St8sia4* (Figs. 4 and 6). Since the MS methods we employed are not able to detect polysialylated structures, we used two antibodies specific for polysialic acid to probe for cell surface polysialylation in the three cell populations. A significant increase in cell surface antibody staining was apparent in both EBs and ExE cells compared to the background level of staining in ES cells (Fig. 6). Both *St8sia2* and *St8sia4* are known to polysialylate N-glycan structures on the cell surface adhesion molecule, NCAM1, and a small collection of other proteins (69). Transcript levels for *Ncam1* were high in all three ES cell derived populations, but decreased 7-fold in EBs and increased 2.6-fold in ExE indicating that elevated *St8sia2* expression and likely not *Ncam1* expression is driving the appearance of polysialic acid on the surface of the differentiated cell populations.

**Non-mucin type O-linked glycans:** There are several classes of O-linked glycans in addition to those that are initiated by a GalNAc-Ser/Thr (mucin type) linkage (Fig. 7) (summarized recently in (3)). Among these are two distinct classes of protein-O-Fuc glycans found on either the EGF repeats of proteins such as Notch (initiated by *Pofut1*) or on thrombospondin type 1 repeats of proteins such as members of the ADAMTS superfamily, (initiated by *Pofut2*). Transcripts encoding both α-Fuc transferases were unchanged in EBs, but increased in ExE cells compared to ES cells (Fig. 7). Extension of the O-Fuc glycans on EGF repeats occurs through the addition of a β3-GlcNAc by the fringe genes (*Lfng, Mfng, Rfng*), and a significant change for only one isoform, *Mfng*, was observed as a decrease (~10-fold) in transcript abundance in ExE cells compared to ES cells (Fig. 7). Extension of this structure with β4-Gal is presumably catalyzed by
Both α2,3- and α2,6-sialylated forms of the O-Fuc tetrasaccharide have been observed (71-73) indicating that sialylation can likely be catalyzed by St6gal1, St6gal2, St3gal4, or possibly St3gal1. Transcript abundance for four of the five genes (B4galt1, St6gal1, St6gal2, and St3gal1) increased in EB and ExE cell samples compared to ES cells (Fig. 7) while St3gal4 was unchanged in abundance. A single Notch-type O-linked fucose structure was identified in the glycan profiles of all three cell types at very low abundance and the prevalence was slightly higher in EBs and ExE cells compared to ES cells (supplemental Figs. 7-9). O-Fuc glycans on TSR repeats are extended with a single β1,3-Glc residue and transcripts encoding this activity (B3galt1) were modestly increased only in ExE cells (Fig. 7). Extended O-Fuc glycans of this type were not observed in the Fuc glycans of this type were not observed in the MS structural profile s of the three cell types.

Addition of O-linked mannose residues have also been shown to be present on α-dystroglycan mucin type repeats and these glycan structures are initiated by two protein O-Man transferases, Pomt1 and Pomt2. The transcript abundance for Pomt1 was modestly reduced in EB cells while the abundance for Pomt2 was modestly increased in ExE cells compared to ES cell samples (Fig. 7). Two O-Man structures were detected in ES cells, EBs and ExE cells and both of these structures were less prevalent in EBs than in ES and ExE cells (supplemental Fig. 8). Extension of O-Man can occur by the addition of β2- or β6-GlcNAc residues by Pomgnt1 and Mgat5b, respectively, but no significant changes in transcripts were observed for either enzyme between the three cell populations analyzed (Fig. 7). As mentioned for the O-Fuc glycans, O-Man structures are further extended through the addition of β4-Gal residues by B4galt1 and sialic acid, presumably in α2,3-linkage based on the O-Man structures of bovine α-dystroglycan (74), by St3gal4 or St3gal1. Transcripts for B4galt1 and St3Gal1 were significantly more abundant in ExE cells than ES cells and EB (Fig. 7) while St3gal4 transcripts were unchanged in abundance. Modification of the O-Man glycans by α3-Fuc addition can be accomplished by several α3-Fuc transferases and two (Fut4 or Fut11) showed increased abundances in ExE cells (~26-fold and ~5-fold, respectively, Fig. 7). Formation of an HNK-1 epitope on O-Man structures requires the addition of a β3-GlcA residue followed by sulfation of the uronic acid. Transcript abundance for one of the two β3-GlcA transferase genes, B3gat2, was increased ~5-fold in ExE, but no significant change in transcript abundance was found for B3gat1 or the sulfotransferase responsible for addition of the terminal sulfate group (Chst10) (Fig. 7). Two O-Man glycans were detected at low abundance in the glycan structural profile differing only by the presence of a terminal sialic acid residue (supplemental Figs. 7 and 8). The sialylated structure was more abundant in all three cell types and the overall abundance of the O-Man structures was reduced in EBs (supplemental Fig. 9) consistent with reduced transcripts for Pomt1 in these cells but not in ExE cells.

EGF domains are also acceptors for protein-linked O-Glc addition to Ser/Thr residues and extension with α3-Xyl residues. The O-linked glucose is generated by a protein O-Glc transferase (Poglt) and transcripts for this gene were slightly decreased (<5-fold) in abundance in EB cells compared to ES cells (Fig. 7). Xyl addition to the O-linked glucose is accomplished by two α3-Xyl transferase isoforms, Gxylt1 and Gxylt2. Transcript abundance of Gxylt1 was increased in ExE cells (~5-fold) while the abundance of Gxylt2 was decreased >10-fold in EBs compared to ES cells (Fig. 7). A single O-Glc structure with two Xyl residues was detected at similar levels for ES, EB and ExE cell types in the glycan structural analysis (supplemental Figs. 7 and 8).

**Cytosolic O-GlcNAc addition:** Transcript abundance for the cytosolic enzyme required for O-linked GlcNAc addition (Ogt) was slightly increased in ExE cells (<5-fold) compared to ES cells while transcripts for Mgea5, the gene that is responsible for removal of the O-GlcNAc residue, were present at similar levels in ES cells, EB and ExE cells (Fig. 7). Protein O-linked HexNAc was detected by the glycomic analysis, but the MS methods were unable to discriminate protein-linked O-GlcNAc from O-GalNAc residues that arise from unextended mucin-type structures (not shown).
Nucleotide sugar biosynthesis: Another potential point of regulation for protein glycosylation is through regulation of sugar nucleotide precursor levels. Enzymes involved the de novo synthesis, salvage, and interconversion of sugar nucleotides (supplemental Fig. 3) were not significantly altered in transcript abundance (<5-fold), which suggests that nucleotide sugar synthesis is a fairly constitutive process. A large fold-increase in transcript abundance was observed in ExE cells for Cmah (~35-fold), the CMP-NeuAc hydroxylase responsible for the synthesis of CMP-NeuGc, but no change was found for the corresponding reductase (Cyb5r3). However, the overall transcript abundance for Cmah was extremely low and the large fold-change is due to the lack of detectable transcript in either ES cells or EBs. Overall, NeuGc accounted for 6.7±2.4%, 4.7±3%, and 8.1±0.2% of the total sialic acid in ES cells, EBs, and ExE cells, respectively, based on MS analysis.

Significant changes in transcripts and glycans segregate cell types: In addition to general correlations between glycan structural changes and the corresponding changes in transcript abundance for the glycan biosynthetic machinery (Table 1), we compared the global differences in glycan structures and transcripts in the three cell populations. Hierarchical clustering of the changes in the prevalence of glycan classes or transcript abundance of glycosylation enzymes demonstrates that each cell type possesses overlapping, but characteristic, sets of glycan and transcript abundances (Fig. 8). Patterns of change in glycan prevalence among biological replicates demonstrate that ES cells cluster distinctly from EBs and ExE cells in transcript abundance, as well as N-and O-glycan prevalence (Fig. 8). The clustering of transcripts and glycans between the biological replicates was much closer for both EBs and ExE cells than for the corresponding ES cell replicate samples. These data indicate that the ES cell population was more heterogeneous in glycan and transcript expression, but that the abundances were altered and become more focused following differentiation. Surprisingly, glycan and transcript abundances in both EBs and ExE cells were more similar to each other than to the ES cell population despite the diversity of cell types found in the EBs. For both glycan profiles and transcript abundance, relatively large changes in only a small number of glycans and transcripts drove the segregation of cell types.

DISCUSSION

Carbohydrate structures attached to cell surface and secreted proteins or cellular glycolipids play critical roles in fertilization and embryogenesis in all multicellular organisms (1,2,8,75). Cell surface carbohydrates play critical roles in gamete development (76) as well as sperm-egg interactions (77-79) and blockage of polyspermy (80-82). Cell surface “stage-specific carbohydrate antigens” have historically been used as markers during embryogenesis (14,83-90) where they correlate with cellular interactions during developmental transitions in pre- and post-implantation embryos (8,75,91,92). Glycans also influence numerous cellular functions such as modulating cell signals and regulating the balance between cell adhesion versus migration during embryogenesis (8,93,94). Cell movements during gastrulation are influenced by cell surface glycan structures including glycoproteins (2,3,7,8), proteoglycans (95-97) and glycolipids (98,99). Glycoprotein and glycolipid modifications by polysialic acid or sulfation to form HNK-1 epitopes in neural crest cells regulate adhesion and neurite outgrowth from various neural cell types (100-104). Distinct glycosylation of the dystrophin glycoprotein complex at the neuromuscular junction has also been shown to contribute a critical bridge between the extracellular matrix, the plasma membrane, and the cytoskeleton (105). The regulated expression of glycan structures also plays critical roles in numerous stages of organogenesis and hematopoiesis (75,106-109).

Despite their contributions to these diverse aspects of vertebrate development, it has been a challenge to determine the varied patterns of glycan expression in distinct cell lineages in vivo because of the inability to gain access to sufficient numbers of pure cell populations for analysis. We have chosen to employ the use of a standard in vitro stem cell differentiation model to mimic the cellular transitions that occur during embryogenesis and development in order to gain access to large, homogeneous, defined cell populations necessary for profiling changes in glycan structures and gene expression.
In the present study, we have chosen to examine the well documented differentiation of mouse ES cells into EBs, the mixed cell population comprised of all three germ layers induced by removal of LIF from the culture media (25,29,30), or ExE cells, generated by the addition of retinoic acid to the culture medium (31,47,110). In prior studies using this differentiation model, we profiled the distinct changes in proteoglycan elaboration, including alterations in the glycan biosynthetic machinery and core protein expression (11), as well as changes in glycolipid and lipid backbone structures (24). In our present work, we have examined the changes in N- and O-glycan structures and transcripts encoding the corresponding biosynthetic machinery. The goals are to determine whether changes in glycan profiles would implicate either transcriptional or post-transcriptional control in the biosynthesis of these structures.

The complexity revealed in N- and O-glycan structural diversity and the intricate biosynthetic pathways for elaboration of these structures in ES-derived cells make it a challenge to distinguish the overarching trends in glycan regulation during differentiation into EBs or ExEs (seeing the “forest for the trees”). Overall, the profiles indicated significant alterations in both N- and O-glycans following differentiation from ES cells to either EBs or ExE cells. In general, more profound changes in both glycans and transcripts were observed during differentiation to ExE cells by comparison to differentiation from ES cells to EBs (Table 1). This likely reflects the more homogeneous nature of the ExE cell population by comparison to the mixed cell population of the EBs, which also includes residual undifferentiated stem cells. Glycan changes in both differentiation models included a significant conversion from predominately high mannose N-glycan structures (72%) in ES cells to a more equal portion of high mannose structures and complex structures in EBs (54% high mannose) and ExE cells (58% high mannose). The complex type structures (predominately bi- and tri-antennary glycans) were characterized by a greater degree of sialylation (particularly in EBs) or capping by α-Gal structures (particularly in ExEs) and increases in cell surface polysialylation were seen in both differentiated cell populations. For O-glycan structures, both differentiation models revealed increased sialylation (particularly in ExEs) and core 3 branching. Surprisingly, α-Gal terminal structures were not elevated on O-glycans for either differentiated cell type.

While the striking abundance of high mannose N-glycans has been observed previously in human ES cells (111), the conversion to an equal abundance of complex structures following ES cell differentiation to both cell populations was an intriguing observation in the present study. The biochemical basis for this significant conversion in glycan character is unclear. One possibility is that ES cells might inefficiently process N-glycans because of low α-mannosidase or Mgat1 gene expression or possibly have altered intracellular routes for glycoprotein transport where glycan processing may be inefficient. Elevated processing α-mannosidase transcripts were observed in ExEs (2-17-fold), but not in EBs, despite a similar decrease in abundance of high mannose structures. A second possibility is that the endomembrane system of ES cells could be dominated by the endoplasmic reticulum similar to our prior observation in adult mouse liver tissues (13). This latter tissue source was found to contain a strikingly elevated high mannose glycoprotein content (85% prevalence compared with 47-55% for other tissues) that we attributed to the extensive elaboration of the smooth endoplasmic reticulum in liver hepatocytes that provide the enzymatic machinery for drug detoxification and lipid biosynthesis. Little is known regarding the ultrastructure of the endomembrane system in mouse ES cells, but they are generally dominated by a prominent nucleus with little space for an expanded cytoplasmic endomembrane system (112).

The conversion of extended high mannose N-linked glycans to the trimmed Man$_6$GlcNAc$_2$ structure (structure 4, supplemental Fig. 4) generates a precursor for subsequent processing toward all hybrid and complex glycans. Therefore, regulation of processing enzymes and the level of glycan products associated with these crucial steps are worth closer examination. In order to assess the relationship between transcript and glycan changes in the context of biosynthetic pathways,
we have produced graphic representations that combine the fold-changes in transcript abundance and glycan prevalence with their position within a generic glycan processing scheme (Fig. 9). From these representations it is apparent that, in both EBs and ExE cells, the increased levels of ManαGlcNAc2 could arise from increased expression of the α-mannosidases, Man1A1 and Man1A2. While the fold-increase in transcripts for these enzymes was higher in ExE cells than EBs, the enrichment of the ManαGlcNAc2 glycan was higher in EBs than in ExE cells. This reversal could arise from increased abundance in ExE cells of Mgat1, the enzyme responsible for the next step in processing toward the production of complex and hybrid glycans, but Mgat1 transcripts were not significantly changed. Alternatively, most of the enzymes downstream from Mgat1 exhibited higher expression in ExE cells than in EBs, consistent with greater depletion of the Mgat1 product and higher prevalences of many hybrid and complex glycans in ExE cells. The greater changes in glycan prevalence found in ExE cells compared to EBs can be seen in several glycan classes. A clear example of these differences is found for the product of Golgi α-mannosidase II cleavage (GlcNAcManαGlcNAc2, structure 28, supplemental Fig. 4), which was decreased by more than 4-fold in ExE cells, but only 2-fold in EBs, despite an increase in expression of the Man2A1 and Man2A2 transcripts. Therefore, the pull of substrates toward greater complexity through the action of downstream branching GlcNAc transferases or capping Gal transferases, and Sia transferases can obscure the detection of a simple relationship between transcript abundance and glycan prevalence. Furthermore, among the complex glycans, the structural class exhibiting the largest change in EBs and ExE cells was biantennary glycans without core Fuc, which nearly quadrupled in prevalence compared to ES cells, while the fucosylated biantennary glycan class barely doubled (Figs. 2 and 9). Finally, the prevalence of total core fucosylated glycans was higher than total non-fucosylated glycans in all three cell types. However, total non-fucosylated hybrid and sialylated glycans predominate over their core fucosylated forms (supplemental Figs. 5 and 6). Along with the observation that Fut8 transcripts are not elevated in EBs or ExE cells to a statistically significant extent, these examples of divergent glycan core processing indicate that fucosylation may affect subsequent elaboration of the glycan structures to which it is attached and/or alter the trafficking itinerary of the glycoprotein carrier.

Following differentiation into EBs or ExE cells most complex glycan structural features were similar between the two differentiated cell populations (similar elevation in bi- and tri-antennary complex structures, core fucosylation, LacNAc content, peripheral fucosylation). The main differences were the nature and abundance of terminal capping structures. In EBs, the complex N-glycans were predominately capped with sialic acid residues attached α2,3- or α2,6- to Gal residues (18% prevalence) and to a lesser degree Galα1,3Gal linkages (13% prevalence). In ExE cells the trend was reversed, where Galα1,3Gal termini were more abundant (18% prevalence) than terminal sialylation (13% prevalence). At the transcript level, the predominant N-glycan sialyltransferases were either unchanged in both differentiated cell populations (St3gal4) or elevated only for ExE (9-fold for St6gal1). However, the α1,3-Gal transferase (Ggta1) that generates the α-Gal termini was also elevated 5-fold in ExE, but unchanged in EBs. These data suggest that greater abundance of α-Gal terminal structures in ExE cells presumably results from the increased expression of Ggta1 and its ability to compete for modification of glycan structures in this latter cell population.

For O-glycans, the major change in both differentiation model systems was a trend toward
increased sialylation, particularly for ExE cells. While the overall abundance of core 1 structures in all three cell populations was essentially unchanged (~60%), the abundance of mono- and di-sialylated core 1 structures was significantly increased by 20% in ExE cells and to a lesser extent in EBs. This correlates with a 6-10-fold increase in St3gal1 and St6galnac5 in ExE cells to generate the sialylated core 1 structures. In addition to increased sialylation of core 1 structures, there was a decrease in core 2 structures and an increase in core 3 structures in ExE cells. While the loss of core 2 structures could result from a competition between sialylation and core 2 branch synthesis, there was no significant increase in B3gnt6 transcripts encoding the core 3 β3-GlcNAc transferase that can account for the increased abundance of core 3 structures in both EBs and ExEs. Other O-linked glycans were detected in low abundance in the glycomic analyses, including glycans initiated with O-Man, O-Fuc, and O-Glc, and in general these structures were unchanged in abundance.

Some classes of glycan structures are difficult to resolve by MS approaches. For instance, O-linked HexNAc was clearly detectable, but we are unable to distinguish between O-linked GlcNAc, the dynamic nucleocyttoplasmic protein modification, and O-linked GalNAc, the Tn antigen produced in the secretory pathway. In addition, our MS-based methods are not optimal for detecting glycans that are found in extremely low abundance or as high molecular weight polymers, such as polysialylated N- or O-glycans. Transcript analyses indicated an increased expression of the polysialyltransferase, St8sia2, during differentiation from ES cells to both EBs and ExEs. As an alternative to MS analyses, we confirmed that polysialylated structures were induced during differentiation by immunofluorescence studies using established antibody probes for polysialylated glycans. St8sia2 can generate polymers of α2,8-sialic acid on N- or O-glycans associated with a small subset of proteins including the enzyme itself, NCAM1, the α subunit of the voltage dependent sodium channel, CD36, and neuropilin-2 (69). Polysialylation of cell surface NCAM1 has been shown to increase intermembrane repulsion and block homophilic NCAM1 and cadherin adhesion to regulate cell-cell interactions and intermembrane space (113). The increase in polysialylation during mouse ES cell differentiation was accompanied by only a 2.6-fold increase in Ncam1 transcripts in ExE cells and a reduction in EBs. Transcripts encoding a second polysialyltransferase, St8sia4, were low and unchanged during ES cell differentiation. Surprisingly, recent data indicates a similar induction of polysialylated structures during human ES cell differentiation, but in this latter case transcripts encoding ST8SIA4 were induced and ST8SIA2 transcripts were unchanged (manuscript in preparation). Polysialic acid has been observed in all cell lineages in the developing vertebrate embryo and loss of polysialic immunoreactivity generally correlated with later stage differentiation (114). In some neural cell lineages, polysialic immunoreactivity persists postnatally and plays important roles in neural development and plasticity, such as cell survival, cell migration of neural precursors, axon guidance, and synapse formation (115,116). Induction of polysialic acid expression in differentiating ES cells may disrupt tight cell interactions allowing dissociation and migration during the differentiation process. Further studies will be required to determine the time course and consequences of polysialic acid expression during ES cell differentiation.

The overall comparison of glycan structural profiles with corresponding transcript abundance shows a significant degree of positive correlation (Table 1), suggesting that changes in glycan structures are at least in part driven by changes in glycosyltransferase or glycosidase gene expression. These data also indicate that many of the early steps in glycan biosynthetic pathways are constitutive in transcript level (e.g. sugar nucleotide interconversion, N-glycan LLO precursor biosynthesis), as we have seen in our studies on transcript abundance in adult mouse tissues (13). If glycan structures play regulatory and functional roles in biological systems, it is not surprising that the synthesis of the underlying scaffold for glycan elaboration would be constitutive in its expression pattern and a greater degree of regulation would be employed for the synthesis of more distal branching and capping modifications that are accessible for mediating
Differences in glycan profiles during differentiation to EBs or ExE cells correlate with differential transcript expression for terminal capping reactions (sialylation versus α-Gal termini on N-glycans or sialylation versus core 2 O-glycan structures) and are consistent with the roles that these terminal structures play in developmental contexts.

In addition to the positive correlations that we identified between glycan prevalence and transcript abundance, several negative correlations were also observed (Table 1). Some, but not all, of these uncorrelated changes can be explained by the flow of products into subsequent processing steps. Thus, numerous challenges still remain in characterizing the detailed regulation of mammalian glycan diversity. The present studies have established detailed snapshots of glycan structures and transcripts associated with differentiation from pluripotent ES cells to two distinct differentiated states. In order to create a comprehensive view of glycan biosynthesis and catabolism and effectively model its dynamic flux during differentiation, we still require a far more detailed measure of additional parameters, including abundance and catalytic parameters for the respective enzymes, enzyme localization, sugar donor and glycoprotein acceptor levels in discrete secretory pathway compartments, routes and rates of glycoprotein transport, and numerous other parameters, which are presently unattainable for this large array of glycan structures and enzymes in a complex cellular system. However, the present studies clearly point to a focused collection of nodes of regulation where a limited number of enzymes catalyze the major changes in glycan structures during development. Further studies using additional differentiation models will identify additional nodes of regulation in different biological contexts and provide greater insights into the mechanisms that control glycan diversity in animal development.

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The abbreviations used are: ES, embryonic stem cell; EB, embryoid body; ExE, extra embryonic endodermal cell; α-Gal, alpha-linked galactose; Gal, galactose; MS, mass spectrometry; MSn, multidimensional mass spectrometry; Le, Lewis blood group antigen; SSEA, stage-specific embryonic antigen; TRA, tumor-rejection antigen; LIF, leukemia inhibitory factor; qRT-PCR, quantitative real time polymerase chain reaction; PNGaseF, peptide N-glycosidase F; LTQ, linear ion trap mass spectrometer; MSI, nanospray ionization; TIM, total ion mapping; LacNAc, N-acetyllactosamine; Hex, hexose; HexNAc, N-acetylhexosamine; Fuc, fucose; GlcNAc, N-acetylgalactosamine; GlcNAcT, N-acetylgalcosaminyl transferase; SEM, standard error of the mean; PBS-T, Phosphate buffered saline with 0.2% Triton X-100; DAPI, 4',6-diamidino-2-phenylindole; LLO, lipid-linked oligosaccharide; OST, oligosaccharyltransferase; Glc, glucose; Man, mannose; ER, endoplasmic reticulum; ppGalnTs, polypeptide N-acetylgalactosamine transferases; NeuAc, N-acetylneuraminic acid; NeuGe, N-glycoly neuraminic acid; Ser, serine; Thr, threonine; GalNAc, N-acetylgalactosamine; Lac di-NAc, N-acetylgalactosamine β 1-4 N-acetylgalactosamine; CAZY, carbohydrate-active enzymes database; NCAM1, neural cell adhesion molecule 1; EGF, epidermal growth factor; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; TSR, thrombospondin repeat; HNK-1, human natural killer-1; GlcA, glucuronic acid; Xyl, xylose; DP, degree of polymerization; GG, glycans with Gal-Gal termini; Bi, biantennary glycans; Tri, triantennary glycans; Tetra, tetraantennary glycans

**FIGURE LEGENDS**

**Figure 1.** Relative transcript abundance for N-glycan processing steps involving trimming and branching steps in the endoplasmic reticulum and Golgi complex for mouse ES cells, EBs and ExE cells. **Panel A**, schematic representation for N-glycan trimming, branching, and modifications for high mannose, hybrid and complex N-linked oligosaccharides using the glycan schematic nomenclature indicated in the legend and as employed previously (13). Linkages are shown for each step of the biosynthetic pathway and the numbers in the blue ovals designate the pathway steps in **Panel A** that link to the transcript abundance data in the corresponding numbered steps in **Panels B and C**. Relative transcript abundances (**Panel C**) for the three mouse cell types are shown as a set of grouped histograms plotted on a log_{10} scale above the corresponding pathway step number (blue numbered oval) and the corresponding gene name(s). Multiple genes for a given pathway step are listed where multiple distinct subunits contribute to catalysis or where several genes within a common family encode enzymes capable of creating the specified linkage. An asterisk indicates a statistically significant change (p<0.05) in relative transcript abundance for either EBs or ExE cells relative to ES cells. The fold-change in relative transcript abundance for each gene is shown in **Panel B** plotted as a histogram in linear scale for EBs and ExE cells compared to ES cells. Transcripts present at a higher level in EBs or ExE cells are shown as bars above the axis (positive fold-change) and those with higher levels in ES cells relative to the differentiated cells are shown as bars below the axis (negative fold-change). The horizontal black line at 1-fold indicates no change in transcript abundance between ES cells and EBs or ExE cells. Hash marks are shown where values exceed the axes and the resulting value is indicated. Error bars in **Panels B and C** indicate the SEM for four biological replicates.

**Figure 2.** Fold-change and prevalence of N-linked glycan classes. The prevalences of individual N-linked glycans were calculated as % Total Profile as described in Experimental Procedures. Prevalences for glycans that fall into the indicated structural classes were summed and classes that exhibited statistically significant differences between EBs, ExE cells, and ES cells are presented (see supplemental Fig. 6 for all glycan classes and for the description of the class abbreviations). **Panel A**, the fold-change in glycan prevalence is shown for EBs or ExE cells compared to ES cells. Glycans present at a higher level in EB or ExE cells are shown as bars above the axis (positive fold-change) and those with higher levels in ES cells relative to the differentiated cells are shown as bars below the axis (negative fold-change).
change). The horizontal black line at 1-fold indicates no change in glycan prevalence between ES cells and EBs or ExE cells. Panel B, glycan prevalences for each cell type are presented. Asterisks indicate statistically significant changes (p<0.05) for either EBs or ExE cells relative to ES cells. Error bars in Panels A and B indicate the SEM for three biological replicates.

Figure 3. Relative transcript abundance for biosynthetic pathways leading to the synthesis of O-linked (polypeptide-GalNAc core mucin-type) structures in mouse ES cells, EBs and ExE cells. Panel A, schematic representations for the synthesis of the polypeptide-GalNAc linkages and extension into various branched and extended structures using the glycan cartoon nomenclature indicated in the legend and as employed previously (13). The display of relative transcript abundances for O-glycan biosynthetic enzymes from the three mouse cell populations (Panel C) are shown as a set of grouped histograms above the corresponding pathway step number as described in the legend to Fig. 1. The fold-change in relative transcript abundance for each gene is shown in Panel B for EBs and ExE cells compared to ES cells as described in the legend to Fig. 1. Additional capping and extension reactions for Core 2, 3, 4 and 6 O-linked structures are also found in Fig. 4.

Figure 4. Relative transcript abundance for processing steps leading to the extension of complex capping reactions for N-glycan, O-glycan and glycolipid structures in mouse ES cells, EBs and ExE cells. Panel A, schematic representation of complex capping reactions for the N-glycan, O-glycan and glycolipid classes indicated by the boxed structures on the left side of the panel and the branched scheme for glycan processing to various complex termini are indicated using the glycan schematic nomenclature shown in the legend and as employed previously (13). Structures sub-terminal to the nonreducing terminal GlcNAc residues indicated by an “#” in the boxed structures are subsequently depicted as “R” in the remainder of the figure. Individual branches of the complex processing pathways are labeled and shown with colored backgrounds to designate different classes of complex termini. The display of relative transcript abundances for the complex capping reactions from the three mouse cell populations (Panels labeled C) are shown as a set of grouped histograms above the corresponding pathway step number as described in the legend to Fig. 1. The fold-change in relative transcript abundance for each gene is shown in the Panels labeled B for EBs and ExE cells compared to ES cells as described in the legend to Fig. 1.

Figure 5. Fold-change and Prevalence of O-linked glycan classes. The prevalences of individual O-linked glycans were calculated as % Total Profile as described in Experimental Procedures. Prevalences for glycans that fall into the indicated structural classes were summed and classes that exhibited statistically significant differences between EBs, ExE cells, and ES cells are presented (see supplemental Fig. 9 for all glycan classes and for the description of class abbreviations). Panel A, the fold-change in glycan prevalence is shown for EBs and ExE cells compared to ES cells. Glycans present at a higher level in EBs or ExE cells are shown as bars above the axis (positive fold-change) and those with higher levels in ES cells relative to the differentiated cells are shown as bars below the axis (negative fold-change). The horizontal black line at 1-fold indicates no change in glycan prevalence between ES cells and EBs or ExE cells. Panel B, glycan prevalences for each cell type are presented. Asterisks indicate statistically significant changes (p<0.05) for either EB or ExE relative to ES cells. Error bars in Panels A and B indicate the SEM for three biological replicates.

Figure 6. Transcript abundance and immunodetection of cell surface polysialic acid glycan structures on ES cells, EBs and ExE cells. Normalized transcript abundance for St8sta2, St8sta4, and Ncam1 were determined (Panel B) and fold-change in transcript abundance for EBs and ExE cells relative to ES cells are indicated in Panel A. Polysialyltransferase transcripts for St8sta2, but not St8sta4, were elevated in the differentiated cell populations, while Ncam1 transcripts were modestly elevated only in ExE cells. Cell surface staining for polysialic acid was performed using anti-polysialic acid antibodies on fixed preparations of ES cells, EBs or ExE cells. ES cells and EBs were probed with either OL-28 monoclonal antibody specific for α2,8 polysialic with DP>4 (Panel C) or S2-566 monoclonal antibody specific recognizing Neu5cα2,8 Neu5Aαcα2,3-Gal (Panel D) (46). ES cells exhibited weak background antibody staining while EBs displayed robust cell surface staining with both antibodies. ES cells (Panel
were probed with the OL-28 antibody (red) and an antibody to the Dab2 protein (green). Dab2 is a clathrin-associated sorting protein that has been shown to be involved in cell positioning and formation of visceral endoderm during mouse embryogenesis (117) and is an established definitive endodermal marker (47). ES cells, indicated by nuclear staining with DAPI (blue), were negative for OL-28 and Dab2 immunoreactivity (individual staining with OL-28, Dab2, and DAPI as indicated and the merge of three staining patterns in the upper left panel of E). ExE were positive for cell surface staining with OL-28 and the intracellular punctate staining with anti-Dab2 (individual staining with OL-28, Dab2, and DAPI as indicated and the merge of three staining patterns in the upper left panel of F) indicating an upregulation of cell surface polysialic acid structures in the differentiated cell populations. The white bar in each panel represents a scale of 100 μm.

Figure 7. Relative transcript abundance for biosynthetic pathway steps leading to the synthesis of O-linked (non-polypeptide-GalNAc mucin type and non-polypeptide-Xyl proteoglycan-type) structures in mouse ES cells, EB and ExE cells. Panel A, schematic representation of the synthesis of various polypeptide-Fuc, polypeptide-Man, polypeptide-Glc, and polypeptide-GlcNAc linkages with extensions into various linear and branched structures using the glycan cartoon nomenclature indicated in the legend. The display of relative transcript abundances for O-glycan biosynthetic enzymes from the three mouse cell populations (Panel C) are shown as a set of grouped histograms above the corresponding pathway step number as described in the legend to Fig. 1. The fold-change in relative transcript abundance for each gene is shown in Panel B for EBs and ExEs compared to ES cells as described in the legend to Fig. 1.

Figure 8. Hierarchical clustering of N- and O-linked glycans and transcripts encoding N-linked glycosylation enzymes in ES cells, EBs, and ExE cells. Panel A, N-linked glycan classes that exhibited statistically significant differences in prevalence between ES cells and either EBs or ExE cells (see Fig. 2) were used to calculate fold-changes relative to the mean prevalence values measured in ES cells. The fold-changes were converted to log₂ values and used to cluster the three biological replicates of each cell type. Panel B, O-linked glycan classes that exhibited statistically significant differences in prevalence between ES cells and either EBs or ExE cells (see Fig. 5) were used to calculate fold-changes relative to the mean prevalence values measured in ES cells. The fold-changes were converted to log₂ values and used to cluster the three biological replicates of each cell type. Panel C, transcript abundances for N- and O-linked biosynthetic genes that exhibited statistically significant differences between ES cells and either EBs or ExE cells were used to calculate fold-changes relative to the mean abundance value measured for ES cells. The fold-changes were converted to log₂ values and used to cluster the four biological replicates in each cell type. The resulting hierarchical clusters for glycans or transcripts resolved the cell populations into ES cell or differentiated cell types.

Figure 9. Pathway diagram for N-glycan biosynthesis incorporating transcript abundance and glycan prevalence. Panel A, fold-changes in glycan prevalence and transcript abundance comparing EBs to ES cells. The color of each circle within the pathway diagram denotes the fold-change in the indicated glycan structure based on the scale of the heat map shown at the lower right of the figure. Glycans at each node are represented by cartoons and denoted by numbers that refer to the glycan structures and their prevalences shown in supplemental Figs. 4 and 5. The color of each arrow denotes the fold-change in the indicated transcript based on the same heat map scale at the lower right of the figure. Key processing steps that take high mannose precursors towards complex glycans are diagrammed across the equator of the panel. Biosynthetic steps that branch toward the top of the diagram generate core fucosylated glycans and those that branch toward the bottom produce glycans without core Fuc. Interestingly, the changes in the upper and lower branching pathways are not mirror images, indicating that core fucosylation influences or is a consequence of alterations in N-linked glycan processing. The large circles within the grayed areas are pie charts that describe the distribution of complex glycans possessing the indicated structural characteristics in EBs. The individual colors of the pie slices denote the fold-change in the indicated class of glycan between EBs and ES cells, while the area of each pie
segment reflects the prevalence of that glycan class in EBs. The grayscale pie charts to the right of Panel A present the distribution of prevalences for the complex glycan classes in ES cells for reference. Panel B, is the same as Panel A except that it compares ExE cells to ES cells. The general trends are similar for EBs and ExEs, although fold-changes tend to be of greater magnitude for ExE cells. In several instances, the glycan product is decreased for a biosynthetic step catalyzed by an enzyme whose transcript is increased. See Discussion for more details. GG, glycans with Gal-Gal termini; Bi, biantennary glycans; Tri; triantennary glycans; Tetra; tetraantennary glycans.
Table 1. Summary of correlations between transcriptome and glycome changes for statistically significant differences.

| Cell Type | Glycan Type | Transcript or Activity | Fold Change | Glycomic Feature | Fold Change |
|-----------|-------------|------------------------|-------------|-----------------|-------------|
| **Correlated changes:** |
| ExE       | N-linked    | ER and Golgi processing α-mannosidases (*Man1a1, Man1a2, Man2a1, Man2a2*) | ↑ 2-17 | High Man with >5 Man residues | ↓ 1.3-1.7 |
| ExE       | N-linked    | Branching GlcNAcTs and GaIT (*Mgat2, Mgat4a Mgat4b, B3gal1, B4gal1, B4gal2, B4gal3*) | ↑ 1.5–5.9 | Bi- and Triantennary LacNAc extensions | ↑ 2.3- 3.8 |
| EB, ExE   | N-linked    | α2,3 and α 2,6 sialyltransferases (*St3gal4, St6gal1, St6gal2*) | ↑ 3-26 | Sialylated N-linked | ↑ 1.5-2.0 |
| EB, ExE   | N-linked    | α2,3 and α 2,6 sialyltransferases (*St3gal1 and St6galac5*) | ↑ 6-10 | Sialylated O-linked Core 1 with ≥ 2 SA | ↑ 1.3- 2.0 |
| EB, ExE   | O-linked    | Xenotransplantation antigen (*Ggtal, ExE only*) | ↑ 5 | Galα1,3Gal with or without core Fuc (EB and ExE) | ↑ 1.4-2.3 |
| ExE       | O-linked    | Notch-type O-Fuc initiation and extension (*Pofut1, Fut4, Fut11*) | ↑ 2.3-26 | Notch-type O-Fuc tetrasaccharide | ↑ 1.9-2.5 |
| EB        | O-linked    | O-Man initiation (*Pomt1*) | ↓ 1.9 | O-Man trisaccharide and tetrasaccharide | ↓ 2.8 |
| EB, ExE   | N-linked    | α2,8 polysialyltransferases (*St8sia2, but not St3gal4*) | ↑ 5-43 | Polysialylation | ↑ ~10 |
| EB, ExE   | O-linked    | α2,8 sialyltransferases (*St8sia1, St8sia5, St8sia6*) | ↓ 1.9-8.1 | Addition of single α2,8 sialic acid | ↓ 2.9-4.8 |
| **Uncorrelated changes:** |
| EB, ExE   | N-linked    | Branching GlcNAcT1 (*Mgat1*) | nc | Complex | ↑ 1.8-1.7 |
| ExE       | N-linked    | Bisecting GlcNAcTII (*Mgat3*) | nc | Bisected complex | ↑ 5.6 |
| EB, ExE   | N-linked    | Core fucosyltransferase (*Fut8*) | nc | Core fucosylation | ↑ 1.6-1.8 |
| ExE       | O-linked    | Core 2 GlcNAcT (*Gcnt1 and Gcnt3*) | ↑ 1.5-3 | All Core 2 glycans | ↓ 1.6 |
| EB, ExE   | O-linked    | Core 3 branching GlcNAcT (*B3gnt6*) | nc | Core 3 glycans | ↑ 13-23 |
| ExE       | N- and O- linked | Polylactosamine extension (*B3gnt1, B3gnt4, B4gal1*) | ↑ 3.5-37 | Polylactosamine repeats, very minor component in all cells | nc |
| ExE       | O-linked    | Fucosyltransferase (*Fut4, Fut11*) | ↑ 5-26 | External fucosylation | ↓ 1.7 |
| EB        | O-linked    | O-Glc initiation and extension (*Poglet, Gxylt1, Gxylt2*) | ↑ ↓ 5-10 | Xylα1,3Xylα1,3Glc | nc |

*a*not statistically significant

bchange detected by immunofluorescence and western blot

cnc; no change

dincreases and decreases in transcript abundance detected for enzymes in this pathway
Figure 4
Fig 9

Diagram showing the expression levels and fold change of various glycosyltransferases (Mgat1, Mgat2, Fut8, β4GalT1, Ggta1, ST6Gal1) in different cell lines (EB, ES, and ExE). The diagram is divided into two sections, A and B, with the fold change indicated by color gradient from 0.1 to 68.

**A** EB/ES

**B** ExE/ES
Regulation of glycan structures in murine embryonic stem cells: combined transcript profiling of glycan-related genes and glycan structural analysis

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