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Developmental regulation of oligosialylation in zebrafish

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Abstract Zebrafish appears as a relevant model for the functional study of glycoconjugates along vertebrate's development. Indeed, as a prelude to such studies, we have previously identified a vast array of potentially stage-specific glycoconjugates, which structures are reminiscent of glycosylation pathways common to all vertebrates. In the present study, we have focused on the identification and regulation of major protein and lipids associated α2-8-linked oligosialic acids motifs in the early development of zebrafish. By a combination of partial hydrolysis, anion exchange HPLC-FD and mass spectrometry, we demonstrated that glycoproteins and glycolipids differed by the extent and the nature of their substituting oligosialylated sequences. Furthermore, relative quantifications showed that α2-8-linked sialylation was differentially regulated in both families of glycoconjugates along development. Accordingly, we established that α2,8-sialyltransferase mRNA levels was directly correlated with changes of α2,8-sialylation status of glycolipids, but independent of those observed on major glycoproteins that appear to originate from the mother.

Keywords α2,8-sialylation · α2,8-sialyltransferases · Neu5Ac/Neu5Gc · Zebrafish · Early development · Glycolipids · Glycoproteins

Introduction

Sialic acids constitute a vast family of heterogeneous monosaccharides that are distributed throughout most of the living organisms, from bacteria to vertebrates. About 40 different naturally occurring members have been described so far [1]. Most of them occur in bound forms to glycoproteins, glycolipids and free oligosaccharides as terminal monosaccharide units. Internal α2-8-linked sialic acids in the diasialosyl motif have been early recognized as a common constituent of gangliosides and then of glycoproteins [2–6]. α2-8-polySia chain with a degree of polymerization (DP) >7 was first identified in bacteria soluble colominic acid prepared from culture medium [7]. Not until 1980 was it identified in animal kingdom associated with a major sialoglycoprotein, the so called polysialoglycoprotein (PSGP), isolated from the eggs of rainbow trout [8]. Although ubiquitously identified in all fish species examined so far, α2-8-polySia chains exhibited a remarkable species specificity associated with differential sequences, nature of constituent sialic acids and degree of *O*-acetyl and *O*-lactyl substitutions [9]. Subsequently, α2-8-

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polySia chain were shown to be associated with multiple glycoproteins in eukaryotic organisms, including neural cell adhesion molecule (N-CAM) [10], sodium channels [11], CD-36 from human milk [12] and neuropilin [13].

The cellular sialic acid content is metabolically regulated mainly by sialyltransferases and sialidases. α2,8-sialyltransferases (ST8Sia) are biosynthetic enzymes catalyzing the transfer of sialic acid residues either to sialoglycoproteins or sialoglycolipids (reviewed in [14–16]), while sialidases or neuraminidases are glycohydrolytic enzymes that remove sialic acid residues during sialoglycoconjugate degradation [17]. Sialylated glycoconjugates are known to be tightly regulated developmentally. In particular, gangliosides are exclusively expressed in mice embryos from 7 days, when neural crest appears, in agreement with their major localisation in central nervous system [18]. Then along brain development, gangliosides pattern show drastic modifications, including a decrease of GM3 and GD3 concomitant to an increase of GD1a and GT1b from mid-embryonic development onward [19]. Similarly in rat, ganglioside expression pattern was shown to shift from simple b-series to complex gangliosides along brain development [20]. Polysialylated N-CAM exhibits also an exquisite spatio-temporal regulation in the developing brain. Indeed, shortly after its appearance in mouse brain at 8 days, N-CAM become polysialylated with a peak expression of α2-8-polySia during perinatal phase and a complete clearing within the three weeks of post-natal brain development, with the exception of sites of neuronal plasticity [21, 22]. In fish, polysialylation profile of PSGP is regulated along oocytogenesis in ovary. Indeed, it was shown to shift from a diSia-PSGP in earlier stages of oogenesis to a α2-8-polySia-PSGP in later stages, based on the temporal regulated expression of the α2,8-sialyltransferases ST8Sia II and ST8Sia IV [23, 24]. However, with the exception of N-CAM, little is known about the regulation and functions of polySia sequences of various glycoconjugates along embryogenesis in vertebrates.

The zebrafish, *Danio rerio*, has emerged in recent years as an excellent model system to study the genetic underpinnings of vertebrate development. Recent profiling of zebrafish embryos glycosylation established that this organism synthesizes a wide variety of sialylated glycoconjugates, the expression of which is potentially regulated along development [25]. Collected data has poised zebrafish as an ideal model to study the role of sialylation in embryogenesis. The present report focuses on the identification and regulation of α2-8-linked sialylated glycoconjugates in the early stages of zebrafish development through a concerted approach combining structural, biochemical and molecular biology analyses. In a first step, we established with different structures of oligoSia sequences associated to different types of glycoconjugates. Then, we evaluated the implication of different glycogenes, including α2,8-sialyltransferases (ST8Sia) and sialidases in the early developmental regulation of the expression of α2-8-linked sialic acids.

Materials and methods

Materials

The molecular biology kits RNeasy Midi and Plasmid extraction were obtained from Qiagen (Chatsworth, CA, U. S.A.), the TOPO TA cloning kit was from Invitrogen (Cergy Pontoise, France), the NucleoSpin® RNA II kit was from Macherey-Nagel (Düren, Germany). The oligonucleotides were synthesized and purified by Eurogentec (Seraing, Belgium), Sybr Green Brilliant Q-PCR master mix, eight-well strip tubes and the MX-4000 Quantitative PCR System were from Stratagene (La Jolla, CA, USA). The first strand cDNA synthesis kit was from Amersham Pharmacia Biotech (Little Chalfont, U.K.). The cDNA Kidney library was kindly provided by L. Zon (ZFIN, Oregon). The experion ARN Std Sens Analysis kit was from Biorad (Marnes-la Coquette, France). Taq polymerase, 4-methylumbelliferone (4-MU) and 2'-(4methylumbelliferyl)-α-d-*N*-acetylneuraminic acid (4-MU-Neu5Ac) were from Sigma (St Louis, MO, USA).

Sample collection

Zebrafish (*D. rerio*) were maintained at 28°C on a 14 h-light/10 h-dark cycle. Embryos were incubated at 28°C and different developmental stages were determined according to the description in the Zebrafish Book [26].

Extraction and preparation of glycoconjugates

Embryos were suspended in 200 µl of water and homogenised by sonication on ice. The resulting material was dried and then sequentially extracted three times by chloroform/methanol (2:1, *v*/*v*) and chloroform/methanol (1:2, *v*/*v*). Supernatants from the extractions were pooled, dried and subjected to a mild saponification in 0.1 M sodium hydroxide in methanol at 37°C for 3 h, and then evaporated to dryness. Sample was reconstituted in methanol/water (1:1, *v*/*v*) and applied to a C18 Sep-Pak cartridge (Waters) equilibrated in the same solvent system. After washing with five volumes of methanol/water (1:1, *v*/*v*), glycosylceramides were eluted by five volumes of methanol and five volumes of chloroform/methanol (2:1, *v*/*v*).

Delipidated pellet from chloroform/methanol/water extraction was re-suspended in a solution of 6 M guanidinium chloride and 5 mM EDTA in 0.1 M Tris/HCl, pH 8, and agitated for 4 h at 4°C. Dithiothreitol was then added to a final concentration of 20 mM and incubated for 5 h at 37°C, followed by addition of iodoacetamide to a final concentration of 50 mM and further incubated overnight in the dark at room temperature. Reduced/alkylated sample was dialysed against water at 4°C for 3 days and lyophilized. The recovered protein samples were then sequentially digested by TPCK treated trypsin for 5 h and chymotrypsin overnight at 37°C, in 50 mM ammonium bicarbonate buffer, pH 8.4. Crude peptide fraction was separated from hydrophilic components on a C18 Sep-Pak cartridge (Waters) equilibrated in 5% acetic acid by extensive washing in the same solvent and eluted with a step gradient of 20, 40 and 60% propan-1-ol in 5% acetic acid. Pooled propan-1-ol fraction was dried and subjected to *N*-glycosidase F (Roche) digestion in 50 mM ammonium bicarbonate buffer pH 8.4, overnight at 37°C. The released *N*-glycans were separated from peptides using the same C18 Sep-Pak procedure as described above. To liberate *O*-glycans, retained peptide fraction from C18 Sep-Pak was submitted to alkaline reductive elimination in 100 mM NaOH containing 1.0 M sodium borohydride at 37°C for 72 h. The reaction was stopped by addition of Dowex 50× 8 cation-exchange resin (25–50 mesh, H^+ form) at 4°C until pH 6.5 and, after evaporation to dryness, boric acid was distilled as methyl ester in the presence of methanol. Total material was then submitted to cation-exchange chromatography on a Dowex 50×2 column (200–400 mesh, H^+ form) to remove residual peptides.

Chemical derivatization and MS analyses

Monosaccharide compositions were determined by gas chromatography (GC)-mass spectrometry (MS) analysis as alditol acetate derivatives. Briefly, glycan samples were hydrolysed in 4 M trifluoroacetic acid (TFA) for 4 h at 100°C and then reduced with sodium borohydride in 0.05 M NaOH for 4 h. Reduction was stopped by drop wise addition of acetic acid until pH 6 was reached and borate salts were co-distilled by repetitive evaporation in dry methanol. Peracetylation was performed in acetic anhydride at 100°C for 2 h.

For MALDI-MS analyses, the glycan samples were permethylated by NaOH in dimethyl sulfoxide, and then extracted in chloroform and repeatedly washed with water. MALDI-MS and MS/MS data were acquired on either a Q-TOF Ultima MALDI instrument (Micromass) or a MALDI-TOF/TOF system, the ABI 4700 Proteomic Analyzer, exactly as described [27].

Analysis of oligo-sialylated sequences

In order to minimize internal fragmentation of polysialylated sequences, sialylated glycan samples were directly coupled to 1,2-diamino-4,5-methylenedioxybenzene (DMB) without prior mild hydrolysis [28]. Samples were incubated for 2.5 h at 50°C in 50 μl of a DMB reagent solution (2.7 mM DMB, 9 mM sodium hydrosulfite, and 0.5 mM β-mercapto-ethanol in 20 mM TFA). 10 μl of 1 M NaOH was then added and the reaction mixtures further incubated in the dark at room temperature for 1 h. Samples were stored at 4°C before analysis.

DMB-derivatized sialic acid oligomers were separated on a HPLC apparatus fitted with either an anion exchanger column, mono-Q (Amersham-Biosciences), or a CarboPac PA-100 column (Dionex). For mono-Q column, the sample was loaded and eluted with a flow rate of 0.5 ml/min with 20 mM Tris–HCl (pH 8.0), followed by a NaCl gradient (0–10 min, 0 M; 10–60 min, 0 to 0.6 M; 60–65 min, 0.80 M) in 20 mM Tris–HCl (pH 8.0). CarboPac column was eluted at 1 ml/min with a concentration gradient of 2 to 32% of 1 M NaNO3 in water. In both systems, elution was monitored by an on line fluorescence detector set at wavelengths of 373 nm for excitation and 448 nm for emission. Periodate oxidation and C7/C9 analyses for oligosialyl linkage determination were performed essentially as described by Sato *et al.* (1998) [33]. Briefly, samples were dissolved in a mixture of 25 μl of 40 mM sodium acetate buffer (pH 5.5) and 2 μl of 0.25 M sodium metaperiodate and left at 0°C for 45 min in the dark. Five microliters of 5% glycerol was then added and allowed to react for another 40 min at 0°C, followed by 32 μl of 0.2 M sodium borohydride in 0.2 M sodium borate buffer (pH 8.0) and left overnight at 0°C. Finally, TFA was added to a final concentration of 1 M and incubated at 80°C for 1 h before subjected to DMB derivatization. To determine the chemical nature of sialic acids, intact sialic acids were liberated directly by mild hydrolysis in 0.01 N TFA at 50°C and reacted with a volume of DMB reagent at 50°C for 2 h 30 min. The monomeric DMB-sialic acid derivatives were separated isocratically on a C18 reverse phase HPLC column (250×4.6 mm, 5 μm, Vydac) by a solvent mixture of acetonitrile/methanol/ water (7:9:84) and identified by referring to the elution positions of standard Neu5Ac and Neu5Gc derivatives. For additional MS analysis, the DMB-derivatives were separated instead with a gradient of acetonitrile/methanol/water (7:9:84) mixture in water (0–10 min, 10%; 10–40 min, 10 to 100%). Fluorescence-detected peaks were individually collected on ice and immediately freeze dried. Samples were then reconstituted in 10 μl of water and analysed by nanoESI-MS and MS/MS on an LCQ DK XP+ ion trap (Thermo Finnigan).

RNA extraction and cDNA synthesis

D. rerio unfertilized eggs and embryos (0, 6, 14, 24 and 36 hpf) kindly provided by the Thisses' Lab were sampled (200 embryos) and snap frozen in liquid nitrogen. Total cellular RNA was extracted from embryos at various developmental stages using the RNeasy Midi kit according to the manufacturer's instructions. Total RNA purity was established by calculating the ratio of the absorbance readings at 260 and 280 nm and quantified using the NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). The integrity and purity of the extracted RNA was also analyzed by means of gel electrophoresis on a bioanalyzer (Experion, Biorad). Total RNA (1.2 µg) was reverse transcribed using the first strand cDNA synthesis kit in 33 µl following the manufacturer's instruction. RNA samples were tested for genomic DNA contamination by PCR amplification of the zebrafish β-actin (GenBank accession number AF025305; [29], using oligonucleotide primers designed in two distinct exons (Sup. Table 1) and aerosol contamination by including no template controls (NTC). Another set of developmental stages cDNAs were kindly prepared by H. Ahmed and G. Vasta according to [30].

Real-time PCR of ST8Sia genes during development in zebrafish

Primers used for quantitative Q-PCR (Sup. Table 1) were designed in the coding region of previously identified zebrafish ST8Sia genes [14] using the Primer Premier version 31.1 software (Primer Premier, Biosoft International, Palo Alto, USA). Each primer pair was carefully selected so to give rise to an amplified DNA fragment of about 300 bp and such that their Tm values were very close (around 51°C). The suitability of the primers for their uniqueness to amplify a single PCR product was assured by regular end-point PCR (Denaturation step at 94°C for 2 min followed by 38 cycles at 95°C 1 min; 50°C 1 min; 72°C 1 min and an elongation step at 72°C for 10 min) using cDNA kidney library provided by L. Zon. The amplified products were subsequently run on an agarose gel, subcloned in TOPO TA cloning vector and finally, fully sequenced (Genoscreen, Lille). The TOPO plasmids containing the amplified regions of the targeted genes were amplified, purified and quantified by nanodrop and used for the establishment of a standard curve for absolute quantification. Efficiency of target amplification for each primer set (ST8Sia I, ST8Sia II, ST8Sia III, ST8Sia IV, ST8Sia V, ST8Sia VI, Sup. Table 1) was optimized by real-time PCR performed in a Stratagene MX4000 by trialing several final primer concentrations. Each 25 µl Q-PCR master mix contained 12.5 µl 2X Master Mix (Brilliant® SYBR® Green Q-PCR Master Mix (Stratagene, CA)), 150 nM of each primer, and 5 µl of diluted cDNA (equivalent to 100 ng total RNA) extracted from 0, 6, 14, 24 hpf embryos and the real-time quantitative PCR where the thermal cycling program consisted of 10 min at 95°C followed by 45 cycles of 30 s at 95°C, 1 min at 50°C and 30 s at 72°C and this was followed by a melting step consisting of heating from 50°C to 95°C at an increment of 1°C per 30 s to check the specificity of the amplified product. PCR for all the samples were carried out in triplicate in eight-well strip tubes and data were expressed as means +/− SD. The reactions were quantified by selecting the amplification cycle when the PCR product of interest was detected (threshold cycle, Ct). Calibration curves were generated by ten-fold serial dilution of Hind III linearized TOPO plasmids containing the amplified regions of the targeted genes (from 2×10 5 copies to 2×10 1 copies). The same PCR master mix and thermocycler conditions as described above were used and plasmid standard curve equations were used to calculate the absolute copy number of each gene. The amplification efficiencies of each calibrator were found to be between 95.9% and 100.5%. We used absolute quantification relying on the serial diluted DNA fragment with known concentration, called calibrators, which were amplified from cDNA of 24-hpf embryos with the same primers.

Sialidases assays

D. rerio embryos (0, 8, 24 and 48 hpf) and unfertilized eggs were sampled (500 embryos) and snap frozen in liquid nitrogen. The eggs were homogenized in 500 µl of water, then different amount of total cell lysate corresponding to 1 to 60 eggs, were mixed with 0.2 mM 4-MU-Neu5Ac in 50 mM sodium acetate buffer (pH 3, 4, 5, 6 and 7) in a final volume of 250 µl. Protein concentrations used were determined using the micro BCA TM protein assay reagent kit (Thermo Scientific Pierce, Rockford, USA). The incubation was performed at 37°C. At 0.5, 1, 2 and 4 h, 30 µl of the reaction mixture was taken back, and the reaction was quenched by adding 120 µl 0.5 M Na_2CO_3. The released 4-methyl-umbeliferone (MUN) was measured and quantified by fluorescence detector at 360 nm for excitation, 460 nm for excitation. Sialidase activity was calculated according to a MUN standard curve.

Results

Glycans from embryos contain oligosialic acid chains

Oligosialylation on N-glycans

We previously described in zebrafish embryos a family of unusual di- and tri-antennary sialylated *N*-glycans along

with major ubiquitous oligomannosylated *N*-glycans [25]. They are characterized by the presence of Neu5Ac/Neu5Gc monosialylated Lewis x motifs further substituted by a β4-Gal residue. A 10% sialic acid content of the total *N*-glycan fraction (Fig. 1) nonetheless implied that a much larger proportion of sialylated *N*-glycans than that detectable by MS might be present. In fact, after purification of sialylated *N*-glycans by anion exchange chromatography, the proportion of sialic acids in sialylated *N*-glycans increased sharply up to 23% of total monosaccharides, which represents an average of three to four sialic acid residues per *N*-glycan. As demonstrated by reverse phase (RP)-HPLC analysis of sialic acid-DMB derivatives, the Neu5Gc:Neu5Ac ratio, which ranges between 2:1 and 4:1 depending on the sample batch, was also somewhat inconsistent with a prevalence of Neu5Ac over Neu5Gc implicated by MALDI-MS profiling of the *N*-glycans. These discrepancies between the MS and sialic acid composition data indicated that some additional oligosialylated *N*-glycans may be refractory to MALDI-MS detection.

To gain a better picture of the sialylation, the well established DMB-tagging and HPLC analytical method [31] was further employed to identify possible presence of oligo- or polysialyl motifs. We first conducted structural analyses of glycoproteins associated oligosialylation on 1 hpf embryos, then established that oligosialylation was qualitatively identical in other developmental stages. The *N*-glycan sample was incubated in the acidic derivatization reaction mixtures without prior acidic liberation to minimize internal fragmentation of polysialic acid chain. The resulting tagged products were then separated on anion exchange HPLC columns (MonoQ and CarboPac PA-100) according to their degree of polymerization (DP) and detected with a fluorescent detector (FD). Under the experimental conditions employed, a monoQ column permits a ready detection of polymeric sialic acid chains

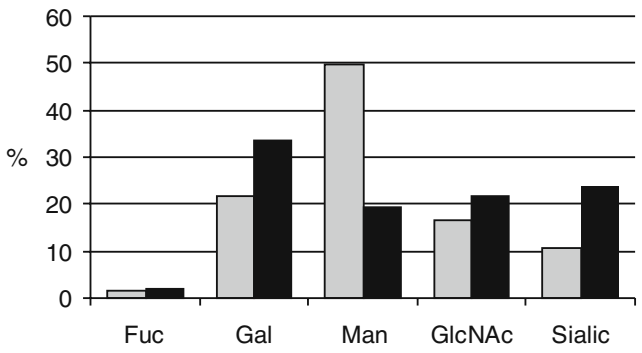

Fig. 1 Relative monosaccharide composition of *N*-glycans. Monosaccharide composition of total (in *grey*) and acidic (in *black*) *N*-glycans liberated from 1 hpf embryos were analysed by gas-chromatography. Results are expressed in percentage of total monosaccharides

from DP 2 up to DP 50 (data not shown), whereas the CarboPac PA-100 column also allows detection of Neu5Ac monomer. Since the FD response per mol of $(Neu5Ac)_n$-DMB remains constant for low DPs [32], integration of peak areas therefore provides a good estimation of the relative abundance of various Sia_n units. On MonoQ column, total *N*-glycans fraction was found to yield at most six peaks with retention times corresponding to $Sia[(\alpha2–8)Sia]_n$-DMB standards of DP 2 to DP 7 (Fig. 2a). Identical results were obtained with acidic *N*-glycans obtained after purification by anion exchange chromatography (data not shown). A sharp drop in the relative intensities of peaks occurred from DP 2 onwards. On PA-100 column, it could be estimated that DP 1 and DP 2 constitute 59% and 38%, respectively, of the total content with higher oligosialyl chains contributing to less than 3% in total (data not shown). As expected, the observed peaks are sensitive to the action of exoneuraminidase (data not shown). Closer examination of the chromatograms showed that standard $[-8)Neu5Ac(\alpha2-]_n$ and $[-8)Neu5Gc(\alpha2-]_n$ exhibited slightly different retention times, in particular for DP 2 and DP 3 (Fig. 3a). Accordingly, chromatographic behaviours of DMB-tagged oligoSia from *N*-glycans suggest that Sia_2 and Sia_3 are exclusively composed of Neu5Gc residues. As shown in Fig. 3c, standard $Neu5Gc_2$ peak co-migrates with DP 2, whereas $Neu5Ac_2$ peak exhibits a clear time shift compared to DP 2 (Fig. 3b).

To ascertain the identity of the major dimeric peak, the same DMB derivative mixtures were subjected to RP-HPLC in order to purify DP 2. The elution position of dimeric sialic acid-DMB was inferred from standard $Neu5Ac_{1–3}$-DMB mixtures. A single major dimeric peak was detected at a retention time similar to that of standard $Neu5Ac_2$-DMB (data not shown) and was collected for MS and MS/MS analyses. As shown in Fig. 4a, ESI-MS analysis of a standard $Neu5Ac_2$-DMB afforded three molecular ion signals in positive ion mode, corresponding to $[M+H]^+$, $[M+Na]^+$ and $[M–H+2Na]^+$ at *m/z* 717, 739 and 761, respectively. Further CID-MS/MS on the mono-sodiated parent ion (Fig. 4b) yielded a major y ion at *m/z* 448 due to facile loss of the non-reducing terminal Neu5Ac residue. In contrast, similar ESI-MS analysis on the collected dimeric peak from the sample gave the corresponding molecular ions at *m/z* 749, 771 and 793 (Fig. 4c), which differ from those afforded by $Neu5Ac_2$-DMB dimer by 32 mass units and are consistent with a $Neu5Gc_2$-DMB composition. This is supported by CID-MS/MS on the candidate mono-sodiated parent ion at *m/z* 771 (Fig. 4d), which afforded a major y ion at *m/z* 464, corresponding to loss of a non-reducing terminal Neu5Gc. Further confirmation was then sought by referring to the CID MS/MS spectrum of an authentic $Neu5Gc_2$-DMB standard which was found to co-elute with $Neu5Ac_2$-DMB

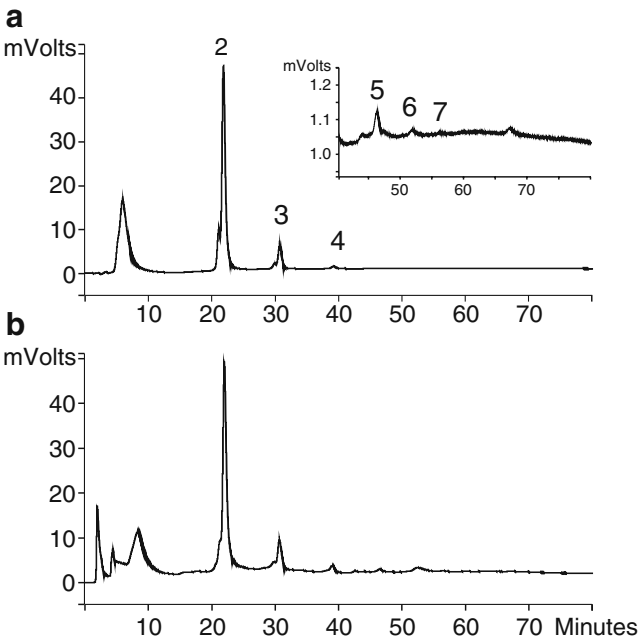

Fig. 2 Profiles of oligosialylated sequences on *O*- and *N*-glycans. OligoSia sequences were released from glycans isolated from 1hpf embryos, tagged with DMB and separated by HPLC-FD on an anion exchange column. OligoSia profiles from (a) *N*-glycans including the *inset in upper panel*, and (b) *O*-glycans. Peaks are labeled according to the DP values as established by comparison with authentic standards

standard at the same retention time under the HPLC conditions employed. In contrast, a putative mono-sodiated $Neu5Ac_1Neu5Gc_1$-DMB peak at *m/z* 755 did not afford either a loss of Neu5Ac or Neu5Gc and was subsequently shown to be a prominent ESI-MS contaminant peak commonly observed, when sample amount was low. Thus, our innovative MS and MS/MS approaches have provided unambiguous evidence for the presence of a Neu5Gc-Neu5Gc dimer, and not a $Neu5Ac_2$ or $Neu5Ac_1Neu5Gc_1$ dimer, as a major oligosialyl motif on the *N*-glycans.

Exclusive presence of Neu5Gc in oligosialylated sequences was further assessed by mild periodate oxidation followed by hydrolysis and DMB-labelling. Applied to the sialylated *N*-glycans, it cleaves the non-substituted side chains of Neu5Ac/Neu5Gc at the C_7–C_8 bond, which are identified by RP-HPLC as DMB-labelling C7/C9 analogues [33]. This demonstrated that all 4 expected products, namely C_9(Neu5Gc)-DMB, C_7(Neu5Gc)-DMB, C_9(Neu5Ac)-DMB and C_7(Neu5Ac)-DMB could be detected at increasing retention time (Fig. 5a), and quantified as representing 28, 51, 2 and 18% of the total sialic acid content, respectively on an *N*-glycan sample with a Neu5Gc to Neu5Ac ratio of 4:1. Assuming the mild periodate oxidation of sialic acid has proceeded to completion and not hampered by any undetected non-saccharide substitution on the side chain, the recovery of intact C_9 Neu5Ac/Neu5Gc-DMB derivatives (about 30% of total) is normally indicative of an internal α2,8-linked sialyl motif since terminal or α2–9 linked sialic acids would be cleaved to C7 analogues. Strikingly, there was 14 fold more α2,8-linked Neu5Gc than Neu5Ac which suggests that where oligosialylation may occur, it preferentially extends from Neu5Gc and not from Neu5Ac. This conclusion is consistent with the observation that Neu5Gc dimer and trimers are the major oligosialyl motifs. Moreover, after subtracting the proportion of terminal Neu5Gc that was α2–8 linked to internal Neu5Gc/Neu5Ac in the dimer, the amount of the remaining C_7-Neu5Gc (21%) was roughly the same as that of C_7-Neu5Ac (18%). This figure is in good agreement with the MALDI-MS analysis which detected complex type *N*-glycans with antenna monosialylated by approximately equal amount of terminal Neu5Gc and Neu5Ac [25].

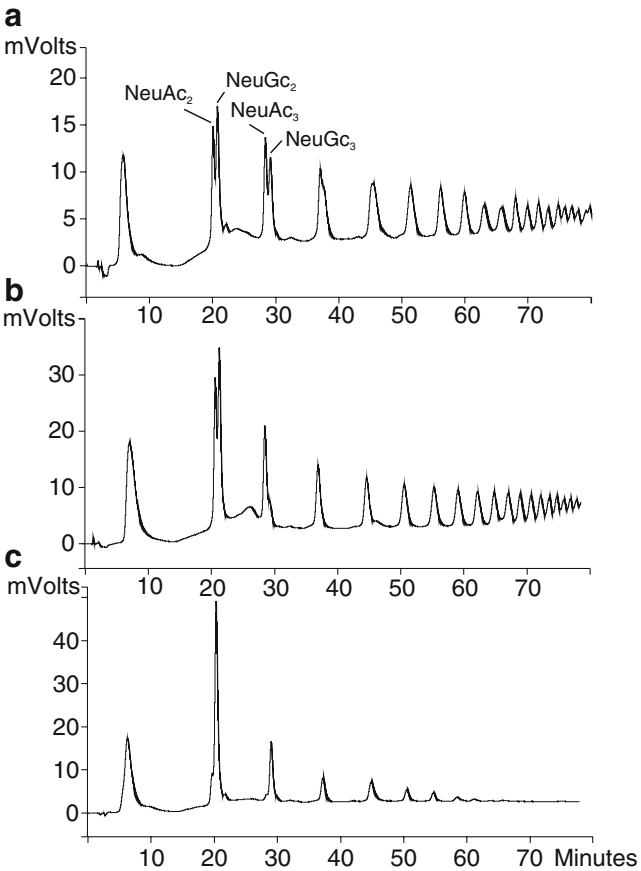

Fig. 3 Identification of oligosialylation on *N*-glycans by anion exchange DMB/HPLC-FD. Chromatographic profiles of co-injected DMB-derivatized **a** [-8)Neu5Ac(α2-]$_n$ and [-8)Neu5Gc(α2-]$_n$ standards, **b** [-8)Neu5Ac(α2-]$_n$ standard and oligo-Sia from zebrafish 1-hpf embryos *N*-glycans, **c** [8)Neu5Gc(α2-]$_n$ standard and oligo-Sia from zebrafish 1 hpf embryos *N*-glycans, showing that the diSia (DP=2) peak from zebrafish *N*-glycans co-migrates exclusively with Neu5Gc (α2–8)Neu5Gc

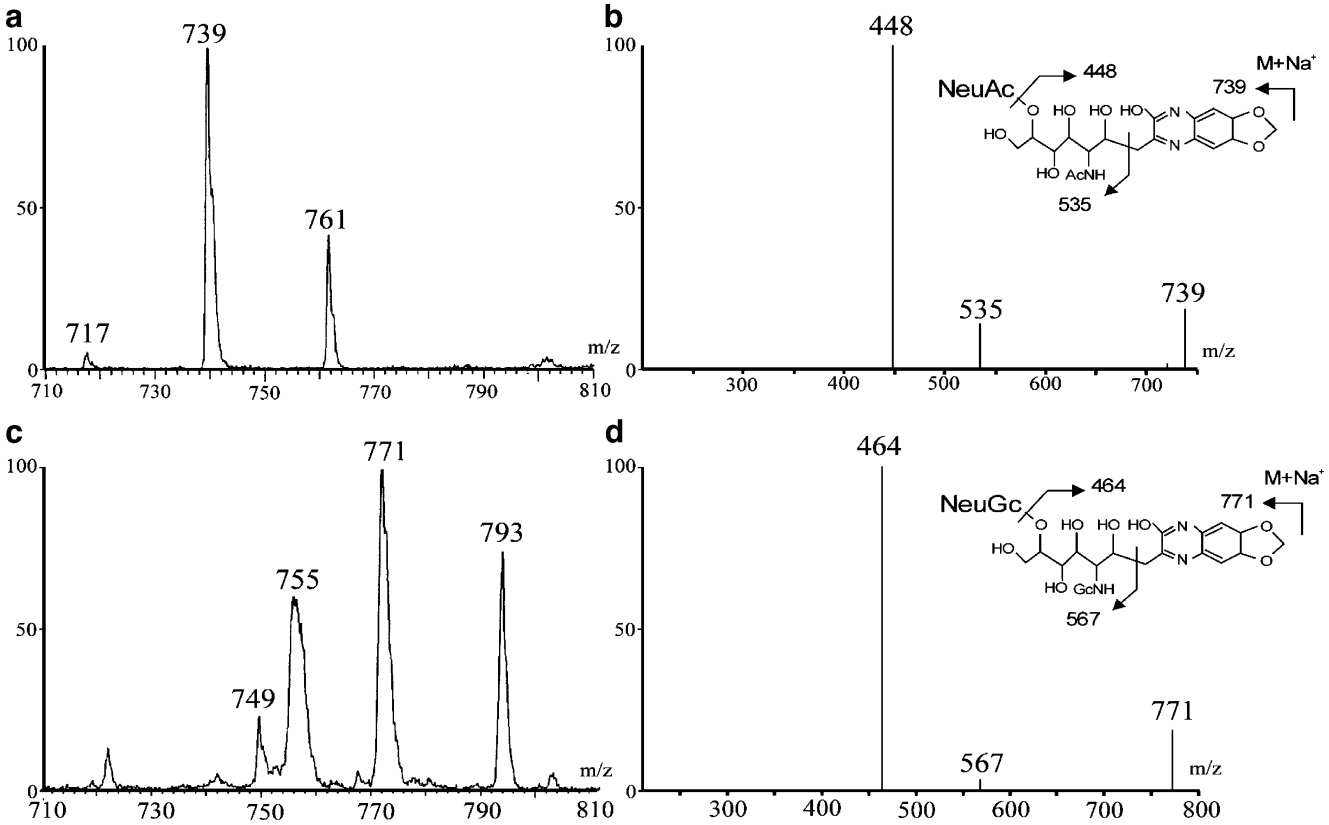

Fig. 4 Structural determination of the disialylated sequences on *N*-glycans by CID-MS/MS of DMB-derivatives. **a** ESI-MS profile of a Neu5Ac2-DMB standard isolated by C18 HPLC fractionation. The most abundant molecular ion at *m/z* 739 corresponds to a monosodiated species which was selected for CID MS/MS sequencing as shown in (**b**). **c** ESI-MS analysis of the putative dimeric sialic acid-DMB peak afforded by zebrafish embryonic *N*-glycans and similarly isolated by C18 HPLC, followed by CID MS/MS on the most abundant molecular ion at *m/z* 771 **d** which established its identity as monosodiated Neu5Gc2-DMB derivative. In both MS/MS, loss of 204 u corresponds to loss of the common DMB moiety through cleavage at C3–C4 of the derivatized, reducing end Neu5Ac/Neu5Gc, as shown schematically

Oligosialylation on O-glycans

Profiling of the glycosylation pattern of zebrafish embryos also demonstrated the presence of prominent sialylated O-linked glycans [25]. In contrast to *N*-glycans, disialylated motifs on *O*-glycans could be directly identified by MS analysis owing to lower molecular mass of *O*-glycans compared with *N*-glycans. In particular, we identified Neu5Gc–Neu5Gc as well as Neu5Ac–Neu5Gc motifs, but could not observe Neu5Gc–Neu5Ac and Neu5Ac–Neu5Ac, suggesting again the existence of exquisite specificity in the synthesis of α2-8-sialylated epitopes. The extent of α2-8-sialylation on *O*-glycans was evaluated using an experimental approach identical to that for *N*-glycans and showed very similar results. *O*-glycans are substituted by oligosialylated motifs including up to seven residues, as determined by DMB/HPLC-FD (Fig. 2b). As observed for *N*-glycans, slight shifts in the retentions times compared with Neu5Ac[(α2–8)Neu5Ac]n-DMB suggest the prevalence of Neu5Gc containing oligoSia over Neu5Ac (Sup. Fig. 1). Separation of periodate oxidised compound by RP-HPLC confirmed also the absence of internal α2-8-linked Neu5Ac residues in the molecule, as observed on *N*-glycans (Fig. 5b). However, *O*-glycans differed from *N*-glycans by a lower C_9(Neu5Gc) to C_7(Neu5Gc) ratio which suggests that the proportion of oligosialylation is lower in *O*-glycans.

Collectively, the data presented show that both *O*- and *N*-glycans are substituted by Neu5Gc containing oligosialylated sequences which exhibit similar overall patterns. Although Neu5Ac has been identified along Neu5Gc in *O*- and *N*-glycans, it seems to be restricted to monosialylated compounds or in non-reducing terminal position of oligosialylated sequences.

Oligosialylation on glycolipids

Direct MALDI-MS-mapping of the acidic glycolipids demonstrated the presence of oligosialylated glycolipids substituted by up to five sialic acids (Fig. 6b). The major tri-sialylated components were previously shown to be substituted by a mixture of Neu5Ac and Neu5Gc residues

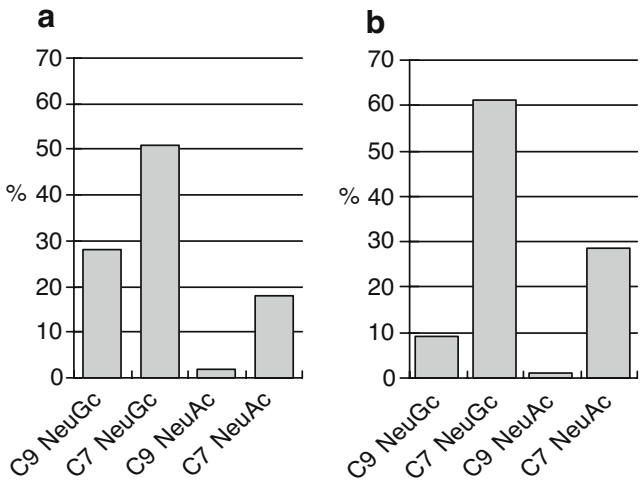

Fig. 5 Relative quantification of internal and external non-reducing sialic acids in glycoproteins oligosialylated motifs. Periodate oxidized Neu5Ac/Neu5Gc-DMB monomers from zebrafish embryonic glycans were resolved on RP-HPLC to distinguish the respective C7/C9 products by referring to the elution positions of authentic standards. Chromatographic profiles of **a** *N*-glycans and **b** *O*-glycans from 1 hpf embryos. Results are expressed in percentage of total oxidized derivatives and are representative of two independent experiments

in all possible combinations [25]. In contrast to *N*- and *O*-glycans, the presence of polymerized Neu5Ac indicates that no restriction seems to prevail in the synthesis of oligosialylated motifs in glycolipids. Accordingly, characterization of sialic acids by DMB/RP-HPLC demonstrated the prevalence of a molar ratio of 1.5:1 for Neu5Ac: Neu5Gc, indicating that sialylation patterns of glycolipids differ from those of glycoproteins in which Neu5Gc prevails over Neu5Ac. In agreement with direct observation of sialylated glycolipids by MS, DMB/HPLC-FD analysis shows the presence of oligosialylated motifs up to DP 6 (data not shown).

The sialic acid content changes along development

The presence of α2-8-sialylation on glycoproteins and glycolipids was assessed along the development time-line from 0 to 48 hpf. For *O*-glycans, presence of di-sialylated glycans could be directly assessed by MS profiling. MALDI-TOF analysis of permethylated glycans after separation of mono- and disialylated compounds shows that previously identified di-sialylated *O*-glycans can be observed exclusively in the earlier stages of developments (0 and 8 hpf) as signals at *m/z* 1706 (Fucα1–3GalNAcβ1–4(Neu5Ac–Neu5Gcα2–3)Galβ1–3GalNAc-itol) and at *m/z* 1736 (Fucα1–3GalNAcβ1–4(Neu5Gc–Neu5Gcα2–3)Galβ1–3GalNAc-itol), but never in the later stages (Fig. 7). Although mass spectrometry does not provide quantitative information, it suggests a disappearance of oligosialylation on *O*-glycans along development. DMB/HPLC-FD analysis provided a semi-quantitative comparison of the oligosialylation content of total *O*-glycan fractions purified from identical numbers of embryos at each development stage. Each fraction presented a very similar pattern of $(Sia)_n$-DMB with $1<n<6$–7, and thus did not show qualitative variation in the extent of α2,8-sialylation. However, a clear decrease in the quantity of each oligomer was observed as shown by integration of chromatographic peaks for di-, tri and tetrasialyl components (Fig. 8a), confirming the rapid decrease of oligosialylated *O*-glycans during embryonic development. Indeed, after 8 hpf, quantity of disialylated motif dropped by more than 60% and less than 5% of the initial di-sialylated

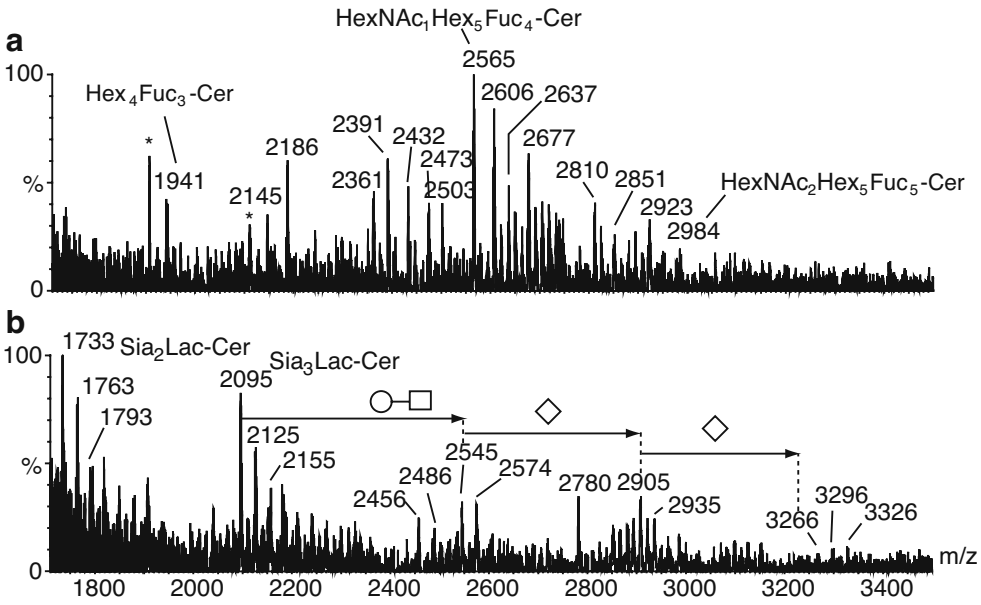

Fig. 6 MALDI-MS analyses of permethylated glycolipids from zebrafish embryos. Glycolipid profiles of high molecular mass glycolipids from **a** 1 hpf and **b** 48 hpf embryos. No sialylated glycolipids were observed at 1 h psf, whereas a complex mixture of oligosialylated glycolipids containing from 2 to 5 sialic acids were detected at the later stages. Symbols used: *circle* Hex, *square* HexNAc, *diamond* sialic acid, *Cer* ceramide

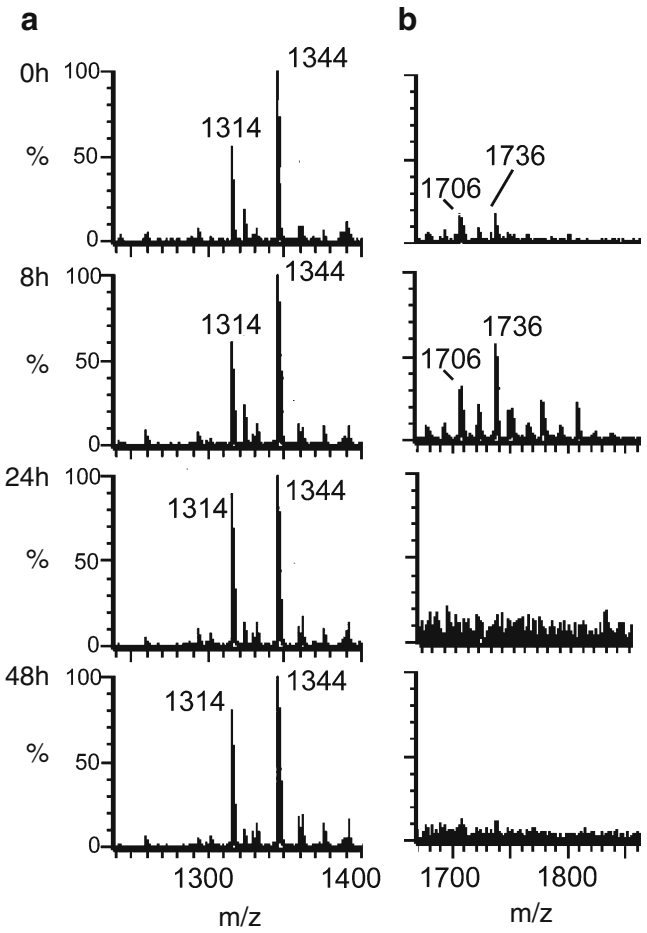

Fig. 7 MALDI-MS profiling of permethylated *O*-glycans from zebrafish embryos. The presence of **a** mono-sialylated *O*-glycans Fucα1–3GalNAcβ1–4(Neu5Acα2–3)Galβ1–3GalNAc-itol at *m/z* 1314 and Fucα1–3GalNAcβ1–4(Neu5Gcα2–3)Galβ1–3GalNAc-itol at *m/z* 1344 and **b** di-sialylated *O*-glycans Fucα1–3GalNAcβ1–4 (Neu5Ac-Neu5Gcα2–3)Galβ1–3GalNAc-itol at *m/z* 1706 and Fucα1–3GalNAcβ1–4(Neu5Gc-Neu5Gcα2–3)Galβ1–3GalNAc-itol at *m/z* 1736 was checked by MALDI-MS analyses of the four embryonic stages from 0 to 48 hpf

motif could be observed prior to hatching (48 hpf). Similarly, the content of tri-sialylated motifs was reduced by more than 20 fold in 48 h. Identical methodology was applied to *N*-glycans and showed a similar trend of rapid clearance of oligosialylation along development (Fig. 8b).

In contrast to glycoprotein glycosylation, several lines of evidence demonstrated that oligosialylation in glycolipids increases along development. First, direct observation of sialylated glycolipids by MS was only possible in later stages of development (24 and 48 hpf) as previously reported. Indeed, MS profiling of total glycolipid from 0 and 8 hpf embryos exclusively showed a complex pattern of neutral fucosylated glycolipids (Table 1), but no sialylated compounds (Fig. 6). Repeated attempts to purify acidic compounds from early stages embryos failed to provide any evidence for their presence. These results were confirmed by comparing endoceramidase treated total glycolipid fractions from embryos at 0 and 48 hpf. Both samples show overall similar profiles characterized by complex mixtures of

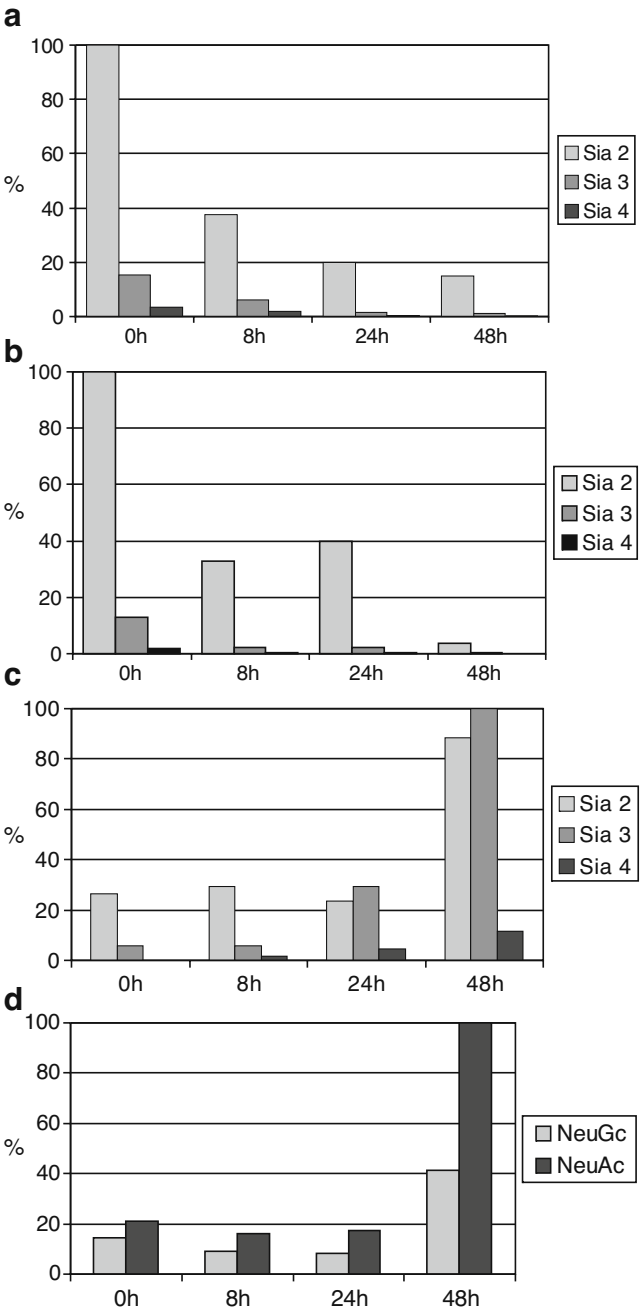

Fig. 8 Relative quantification of oligosialylation along embryos development. Proportions of Sia 2, Sia 3 and Sia 4 associated to **a** *O*-glycans, **b** *N*-glycans and **c** glycolipids were compared from 0 to 48 hpf by anion exchange DMB/HPLC-FD. Relative quantities of Neu5Ac and Neu5Gc from glycolipids were also followed along development by RP-HPLC after total release of sialic acids (**d**). Results are representative of three independent experiments

Table 1 Monosaccharide composition of neutral glycolipids calculated from MALDI-MS analysis of permethyl derivatives

m/z $[M+Na]^+$	Composition		
	HexNAc	Hex	dHex
1,941	0	4	3
2,145	0	5	3
2,186	1	4	3
2,361	1	4	4
2,391	1	5	3
2,432	2	4	3
2,473	3	3	3
2,503	3	4	2
2,565	1	5	4
2,606	2	4	4
2,637	2	5	4
2,677	3	4	3
2,810	2	5	4
2,851	3	4	4
2,923	4	4	3
2,984	2	5	5

identical neutral glycans (Sup. Fig. 2). Later stage sample shows the presence of additional major signals at *m/z* 838.6, 868.6, 1083.8 and 1113.8 attributed to $Neu5Ac_1Hex_2$, $Neu5Gc_1Hex_2$, $Neu5Ac_1Hex_2HN_1$ and $Neu5Gc_1Hex_2HN_1$, respectively. Furthermore, careful analysis of MS spectra reveals the presence of additional minor signals exclusively in later stage at *m/z* 1199.9, 1229.9 and 1259.9 attributed to $Neu5Ac_2Hex_2$, $Neu5Ac_1Neu5Gc_1Hex_2$ and $Neu5Gc_2Hex_2$ as well as *m/z* 1145.0, 1475.1 and 1505.1 attributed to $Neu5Ac_2Hex_2HN_1$, $Neu5Ac_1Neu5Gc_1Hex_2HN_1$ and $Neu5Gc_2Hex_2HN_1$. The chemical nature of oligosialylated motifs in endoceramidase treated glycolipids was confirmed by MS/MS sequencing of their permethylated derivatives. Indeed, fragmentation of molecular ions at *m/z* 1199, 1229 and 1259 showed the presence of $Neu5Ac_2$, $Neu5Ac_1Neu5Gc_1$ and $Neu5Gc_2$ sequences owing to the presence of B/Y ion pairs at *m/z* 760/463, 790/463 and 820/463, respectively (Sup. Fig. 3). Furthermore, presence of both Y ions at *m/z* 825 and 855 established that compound $Neu5Ac_1Neu5Gc_1Hex_2$ at *m/z* 1229 is a mixture of the two isobaric structures Neu5Gc–Neu5Ac–Hex–Hex and Neu5Ac–Neu5Gc–Hex–Hex. In agreement with MS analysis, profiling by DMB/HPLC-FD demonstrated that oligosialylation increased along development, with a sharp increase between 24 and 48 hpf (Fig. 8c). Indeed, as compared with embryos at 0 hpf, quantity of diSia increased by 3.5 fold and triSia by 17 fold in 48 hpf embryos. Accordingly, the total amount of sialic acid at 48 hpf is 4 to 6 higher than in other stage (Fig. 8d).

These data clearly demonstrate that the amount of oligosialylation in embryos varies along development. Surprisingly, comparative profiling of glycoconjugates established that the overall content of sialylation decreases for glycoproteins along embryogenesis, but increases for glycolipids.

Changes of α2-8-sialylation status of glycolipids is directly correlated with α2,8-sialyltransferase mRNA levels, whereas sialidase activities might be responsible glycoproteins

To investigate whether ST8Sia genes may participate in the di-, oligo- and polysialylation of major glycoconjugates during the zebrafish development, we examined the mRNA levels of the α2,8-sialyltransferase genes (ST8Sia I, ST8Sia II, ST8Sia III, ST8Sia IV, ST8Sia V and ST8Sia VI) in different developmental stages by real time PCR. Six human ST8Sia orthologues were previously identified in the zebrafish genome [14] and were molecularly cloned from various zebrafish organs by RT-PCR and sequenced. Since β-actin showed variable level of expression among the various developmental stages with the same amount of total cDNA (data not shown), we have chosen absolute quantification to quantify ST8Sia. Except for ST8Sia II, all the ST8Sia genes showed very low level of expression at 0 hpf (Fig. 9b). They all increase along embryogenesis from 0 to 36 hpf but at very different rates (Fig. 9a). Indeed, expression levels of ST8Sia I and ST8Sia VI sharply increase in the first 14 h of development, whereas other ST8Sia exhibited either more modest or delayed increase (Fig. 9a). Then, ST8Sia I and ST8Sia III reach maximum sustained expression levels at 14 hpf, whereas the others show a gradual increase in expression level up to 36 hpf. Interestingly, ST8Sia I and ST8Sia V genes, the human orthologs of which are known to be involved in glycolipids sialylation, are both dramatically up-regulated along development by a factor of 300 and 165, respectively. This result is in agreement with the observation of an increase of sialylation associated to glycolipids. On the contrary, the modestly increased expression levels of ST8Sia II and ST8Sia IV, which are responsible for the biosynthesis of polysialic acid, and of ST8Sia III also involved in the α2,8-sialylation of glycoproteins are still slightly inconsistent with the decreased oligosialylation status observed on glycoproteins. More surprisingly is the 4000 fold increase of ST8Sia VI gene expression that is not directly correlated with an increase of Sia2 motifs synthesis on glycoproteins.

In order to check whether zebrafish sialidases might be involved in these α2-8-sialoglycoprotein metabolism, we performed sialidase assays using 4-methyl-umbelliferyl-Neu5Ac (4MU-Neu5Ac) as a substrate at various pH for various embryonic developmental stages (0, 8, 24, and 48 hpf) and unfertilized eggs. Our preliminary data showed

a

	ST8Sia I	ST8Sia II	ST8Sia III	ST8Sia IV	ST8Sia V	ST8Sia VI
unf	0.15	6.38	3.91	1.82	2.79	21.65
0h	1.00	1.00	1.00	1.00	1.00	1.00
14h	266.78	0.91	4.51	1.19	0.46	173.39
24h	92.78	0.97	5.77	1.55	13.00	75.18
36h	303.74	6.31	5.53	4.58	165.56	4059.40

b

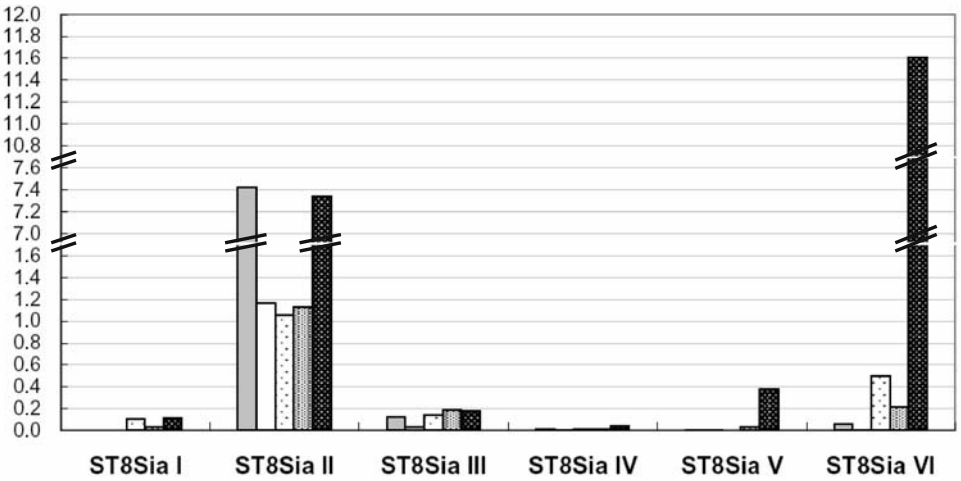

Fig. 9 Absolute quantification of Dre ST8Sia genes expression by real-time PCR. **a** "Fold of increase" represents the relative expression quantity of each ST8Sia in different developmental stages compared with expression at 0 hpf. **b** The different expression levels of all the ST8Sia genes were analyzed with cDNA from 0 hpf *empty square*, 14 hpf ⬚, 24 hpf ▨, 36 hpf ▩ eggs and ovary *filled square* by quantitative real-time PCR. The absolute amount of transcripts was quantified according to the same DNA fragment amplified and cloned in the plasmid. Values are the mean of triplicate points

the existence of intense sialidase activities associated to embryos. Survey of sialidase activities showed dramatic differences of intensities depending on the pH and along development (Sup. Fig. 4). The fact that the evolution of total sialidase activities measured at different pH follows different trends strongly suggest the presence of different enzymes, as recently demonstrated by the identification of several genes coding for potential sialidases in zebrafish [34]. Indeed, the sialidase activities detected at pH 5 sharply decreased along the zebrafish development, whereas sialidase activities detected at pH 4 increased slightly along the zebrafish development. These data demonstrate a tight regulation of sialidase activities along development and suggest a possible involvement of sialidases in the α2-8-sialylation status of sialoglycoproteins, which may explain the discrepancies observed between the regulation of ST8Sia genes involved in the glycoproteins biosynthesis and the actual synthesis of oligosialylated motifs on glycoproteins.

Discussion

A previous study of the glycosylation profiles of zebrafish embryos demonstrated that this organism synthesizes a vast panel of unusual sialylated glycoconjugates. Of particular interest are the β4-galactosylated sialyl Lewis x, Galβ1–4(Siaα2–3)Galβ1–4(Fucα1–3)GlcNAc, motif observed on all complex *N*-glycans and Fucα1-3GalNAcβ1–4(Siaα2–3)Galβ observed on *O*-glycans. The Galβ1–4Galβ1–4GlcNAc motif now appears to be a common feature of several fish glycoproteins, as described in *Oryzias melastigma*, *Tribolodon hakonensis* and *Oryzia latipes* [35, 36], but the α2–3Neu5Ac sialylated Galβ1–4Galβ1–4GlcNAc motif observed in *D. rerio* was additionally identified only in *O. latipes*. However, its Neu5Gc substituted equivalent is specific to *D. rerio* so far. Similarly, the Fucα1–3GalNAcβ1–4(Siaα2–3)Galβ motif has been exclusively identified on the *O*-glycans from *D. rerio* although a wide range of other *O*-glycan structures have been described in fishes [37].

The present study extended further our knowledge of the fine structures of sialylated glycans synthesized by zebrafish along embryonic development by focusing on the α2-8-sialylation pattern (quality and quantity) of glycoproteins and glycolipids. Our data clearly established that both glycoproteins and glycolipids were α2-8-sialylated. Surprisingly, fine structural analysis demonstrated that the major glycolipids and glycoproteins presented different patterns of oligosialylation. Whilst the α2-8-sialylated glycolipids may be substituted by mixtures of oligo (Neu5Ac), oligo(Neu5Gc) and hybrid type oligo(Neu5Ac, Neu5Gc) sequences, glycoproteins are mainly substituted by oligo(Neu5Gc). Indeed, although Neu5Ac and Neu5Gc

are present in similar proportions on mono-sialylated motifs of *N*-glycans [25], we have now demonstrated that only Neu5Gc is further elongated by other sialic acids to form oligo(Neu5Gc) sequences. Identical biosynthetic restrictions seem to prevail during the extension of oligosialic acids associated with *O*-glycans that prevent formation of oligo(Neu5Ac). These differences between glycoproteins and glycolipids were also reflected in their respective content of sialic acids: two to four times more Neu5Gc than Neu5Ac in glycoproteins and about twice as much Neu5Ac than Neu5Gc for glycolipids. As previously reported, polysialic acid structures of fish egg glycoproteins are exquisitely species specific, differing by their extent, composition, acetylation and sequences [9]. However, to our knowledge, zebrafish represents the only documented example of the preferential use of Neu5Gc over Neu5Ac for oligosialic acid elongation found on glycoproteins. This suggests an α2,8-sialyltransferase activity dedicated to α2-8-sialylation of glycoproteins, such as ST8Sia II, ST8Sia III, ST8Sia IV or ST8Sia VI that would exhibit a preference for CMP-Neu5Gc over CMP-Neu5Ac. In mammals, ST8Sia II and ST8Sia IV are known to be involved primarily in the polysialylation of the *N*-glycans of N-CAM, ST8Sia III catalyzes the transfer of one to seven sialic acid residues onto glycoproteins or glycolipids, whereas ST8Sia VI catalyzes the transfer of a unique sialic acid residue on the *O*-glycans of glycoproteins [16, 38]. In this context, it is also interesting to note that the zebrafish ST8Sia IV shows very low capability to transfer Neu5Ac from CMP-Neu5Ac onto N-CAM compared to the zebrafish ST8Sia II [39]. This last observation might reflect a preference of the zebrafish ST8Sia IV for CMP-Neu5Gc over CMP-Neu5Ac. Also, it is noteworthy that the major glycoprotein associated α2-8-sialyl motifs in zebrafish exhibit a significantly lower DP compared to those previously identified on the polysialylated glycoprotein (PSGP) of other fish species. Indeed, whereas salmonids eggs contain polysialyl units with chain length up to 20 residues [8, 37], the major sialylated glycoproteins of zebrafish eggs are mainly substituted by diSia (DP=2) motif as well as minute amounts of oligoSia (3<DP<6).

In addition, expression of oligosialylation on glycolipids and glycoproteins is differentially regulated along embryonic development. Indeed, the extent of α2-8-sialylation on major glycoproteins sharply decreases whereas that of glycolipid increases along development. Surveys of sialylation by MS analyses of intact and endoceramidase treated glycolipids, as well as the quantification of sialic acids and oligosialic acids, all indicated that significant sialylation specifically and reproducibly appears around 24 hpf, which strongly suggests that glycolipid associated α2-8-sialylation is triggered during early development. Accordingly, we failed to detect significant glycolipid associated sialylation in mature ovaries before spawning (data not shown). It is noteworthy that the complex neutral glycolipids observed in all development stages are apparently not the substrates for sialylation events occurring in later developmental stages. MS analyses demonstrated that the sialylated glycolipids of later stages comprised mainly Neu5Ac/Neu5Gc substituted $(Sia)_{1-4}$LacCer, GM2 and GD2 with no sialylated equivalents of the fucosylated neutral glycolipids (Table 1), suggesting that these two families of compounds are independently synthesized.

The appearance of glycolipids associated α2-8-sialylation in later stages of embryonic development positively correlates with the up-regulation of genes coding for α2,8-sialyltransferases ST8Sia I, ST8Sia III and ST8Sia V (Fig. 9). The human recombinant enzymes have been shown to be involved in the biosynthesis of gangliosides GD3, GT3, GD1a, GT1b and GQ1c (reviewed in [16]) [40–46]. The onset expression of these genes starts around 10 hpf and is essentially located in the developing brain (Chang *et al.* 2008, this issue). Up-regulation of oligosialylation along zebrafish development is in agreement with previous observations made on *Xenopus laevis* showing by *in vitro* assays that ST8Sia I (SAT-2) and ST8Sia V (SAT-4) activities were dramatically increased along the early development, with a maximum activity at day 4 [47]. However, whereas quantities of both neutral glycolipids and gangliosides sharply increase in *X. laevis*, only gangliosides appear to be up-regulated in *D. rerio* [48]. In contrast to glycolipids, evolution of glycoproteins associated α2,8-sialylation does not correlate with the temporal expression and the general increase of mRNAs of ST8Sia II, ST8Sia IV and ST8Sia VI from 10 hpf along the early stages of zebrafish development, therefore implicating a different kind of regulation. These latter ST8Sia genes were found to be expressed in the ovaries (Fig. 9), suggesting that the α2-8-sialylated glycoproteins of the zebrafish embryo detected at very early developmental stages well before the onset zygotic expression of these ST8Sia (around 10 hpf), might be inherited from the mother. We hypothesize that these inherited α2-8-sialylated glycoproteins could be subsequently degraded in the embryos by endogenous sialidases.

As a first step towards substantiating this hypothesis, we have essayed the sialidase activities with synthetic substrates at various pH (3, 4, 5, 6, 7) in unfertilized eggs. Since the higher and lower activities were obtained at pH 5 and 4 respectively, we then assayed the sialidase activities at pH 4 and 5 for the various developmental stages of interest. Our preliminary data showed that sialidase activities found at pH 4 increased along zebrafish development, whereas sialidase activities found at pH 5 decreased (Sup. Fig. 4). The correlation between the detected sialidases activities and glycoproteins oligosialylation along

embryonic development therefore remain equivocal and the implicated zebrafish sialidases still need to be clearly identified. A recent study of Manzoni *et al.* [34] reported the identification of seven zebrafish sialidase genes homologous to three of the four known human genes (NEU1, NEU2, NEU3, NEU4) The zebrafish neu3.1, neu3.3 and neu4 were shown to be active towards gangliosides at very low pH (2–3) and the corresponding genes were found to be expressed differentially along the embryonic development. On the other hand, an additional NEU3 orthologous gene named neu3.2 has been described, which started to be expressed at 24 hpf. The corresponding enzyme appears to be soluble in the cytosol and active at higher pH (5.5). However, fine enzymatic characterization of all the zebrafish sialidases still awaits studies.

Such an up-regulation of the ST8Sia II gene expression along embryonic development of zebrafish has been previously reported in the context of an increased synthesis of polysialic acid chains (PSA) on the *N*-glycans of the neural cell adhesion molecule (N-CAM), which reaches a maximum around 27–40 hpf [49]. Our present study focused instead on the global α2-8-sialylation status of the different classes of glycoconjugates along embryonic zebrafish development and has identified a rapid decrease of glycoproteins associated α2-8-sialylation content. This might be explained by the large quantities of PSGP synthesized in the cortical alveoli during oogenesis in fishes [24] compared to the natural low abundance and restricted localization of PSA-N-CAM. It is most probable that the N-CAM polysialylation pattern cannot be discriminated from the one of PSGP or other polysialylated glycoproteins by a global approach.

As a premise to the identification of other oligosialylated glycoproteins in zebrafish fertilized eggs, we have assessed the oligosialylation patterns of glycoproteins in different compartments of the fertilized oocyte: embryo tissue, chorion and perivitelline space. Semi-quantification analysis by DMB-HPLC revealed that about 94% of the total oligoSia in 1 hpf fertilized oocyte was associated with soluble glycoconjugates in the perivitelline space, 5% in the chorion and less than 1% in the embryonic tissue (data not shown). Surprisingly, the extent of oligosialylation distributed within each of these fractions was very different. The perivitelline space associated components show a very short DP distribution dominated by DP 2, reminiscent of the one observed on total glycoprotein fraction, whereas the chorion and embryonic tissues exhibit more evenly distributed patterns with up to DP 10 (Sup. Fig. 5). Each fraction was further differentiated by their sialic acids content, as embryo associated glycoproteins was almost exclusively composed of Neu5Gc (Neu5Gc/Neu5Ac 14:1), whereas perivitelline space associated glycoproteins of Neu5Gc and Neu5Ac in a 4:1 ratio (data not shown).

The presence of large quantities of oligosialylated soluble glycoproteins in perivitelline space of zebrafish is in good agreement with previous reports of the polysialylated peptides originating from a fertilization induced proteolysis of cortical alveoli PSGP in several other species of fish, including trouts, salmonids and medaka fish [9, 24, 50]. Accordingly, we observed large quantities of protein associated oligoSia chains in zebrafish mature ovaries that exhibit a distribution pattern and a composition identical to that of the soluble glycoproteins of fertilized oocytes (data not shown). However, despite the postulated conservation of this phenomenon among fishes and the large quantity of excreted PSGP, the destiny and the function of these compounds are still largely unknown. Altogether, our data established that the vast majority of oligosialylation observed in fertilized oocytes is synthesized prior to embryogenesis in the mother ovary and then degraded along embryonic development for a yet unknown purpose.

The observation of a protein associated oligosialylation in embryonic tissue as early as 1 hpf, thus long before that N-CAM associated PSA appears, strongly suggests the presence of so far undescribed oligosialylated glycoproteins in oocytes. Diversity of polysialylated components in fish embryos increases the possible endogenous substrates for zebrafish α2,8-sialyltransferases. Indeed, ST8Sia II and IV were shown to be involved in the synthesis of polysialic acid chains on both N-CAM *N*-glycans in zebrafish and on PSGP *O*-glycans in rainbow trout, which suggest that these enzymes might have multiple substrates acceptors in various animal species [49, 51]. Altogether, the results presented here establish the structural bases for the investigation of the fine enzymatic specificities of the different ST8Sia identified from zebrafish genome [14]. These studies are actually in progress by using the endogenous molecules as acceptor substrates. Further work is also needed to identify the protein carriers of the oligosialylated motifs and to assess their localization within the fertilized embryo. The collected data will enable a better evaluation of the importance of sialyltransferases as well as sialidases in the regulation of synthesis of sialylated motifs along zebrafish embryogenesis.

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Abbreviations

Sialyltransferases nomenclature is according to Tsuji *et al.* [52], gangliosides nomenclature is according to Svennerholm [53]. hpf|hours post-fertilization;diSia|disialyl motif;triSia|trisalylmotif;DHB|2,5-dihydroxybenzoic acid;DP|degree of polymerisation;GC|gas chromatography;Hex|hexose;HexNAc|*N*-acetyl hexosamine;HexNAcitol|reduced *N*-acetyl hexosaminitol;LacCer|lactosylceramide;MALDI|matrix-assisted laser-desorption ionization;MS|mass spectrometry;TFA|trifluoroacetic acid;TOF|time-of-flight

References

1. Schauer, R., Kamerling, J.: Chemistry, biochemistry and biology of sialic acids. In: Montreuil, J., Vliegenhart, J., Schachter, H. (eds.) Glycoproteins II, pp. 243–372. Elsevier, Amsterdam (1997)
2. Finne, J., Krusius, T., Rauvala, H.: Occurrence of disialosyl groups in glycoproteins. Biochem. Biophys. Res. Commun. **74**, 405–410 (1977). doi:10.1016/0006-291X(77)90318-7
3. Handa, N., Handa, S.: The chemistry of lipids of posthemolytic residue or stroma of erythrocytes. XIV. Chemical structure of glycolipid of cat erythrocyte stroma. Jpn. J. Exp. Med. **35**, 331–341 (1965)
4. Handa, S., Burton, R.M.: Lipids of retina. I. Analysis of gangliosides in beef retina by thin layer chromatography. Lipids **4**, 205–208 (1969). doi:10.1007/BF02532630
5. Krusius, T., Finne, J., Karkkainen, J., Jarnefelt, J.: Neutral and acidic glycopeptides in adult and developing rat brain. Biochim. Biophys. Acta. **365**, 80–92 (1974)
6. Kuhn, R., Wiegandt, H.: Die konstitution der ganglio-n-tetraose und des gangliosids gl. Chem. Ber. **96**, 866–880 (1963). doi:10.1002/cber.19630960329
7. McGuire, E.J., Binkley, S.B.: The structure and chemistry of colominic acid. Biochemistry **3**, 247–251 (1964). doi:10.1021/bi00890a017
8. Inoue, S., Iwasaki, M.: Characterization of a new type of glycoprotein saccharides containing polysialosyl sequence. Biochem. Biophys. Res. Commun. **93**, 162–165 (1980). doi:10.1016/S0006-291X(80)80260-9
9. Sato, C., Kitajima, K., Tazawa, I., Inoue, Y., Inoue, S., Troy 2nd, F.A.: Structural diversity in the alpha 2®8-linked polysialic acid chains in salmonid fish egg glycoproteins. Occurrence of poly (Neu5Ac), poly(Neu5Gc), poly(Neu5Ac, Neu5Gc), poly(KDN), and their partially acetylated forms. J. Biol. Chem. **268**, 23675–23684 (1993)
10. Finne, J.: Occurrence of unique polysialosyl carbohydrate units in glycoproteins of developing brain. J. Biol. Chem. **257**, 11966–11970 (1982)
11. James, W.M., Agnew, W.S.: Multiple oligosaccharide chains in the voltage-sensitive Na channel from electrophorus electricus: evidence for alpha-2,8-linked polysialic acid. Biochem. Biophys. Res. Commun. **148**, 817–826 (1987). doi:10.1016/0006-291X(87)90949-1
12. Yabe, U., Sato, C., Matsuda, T., Kitajima, K.: Polysialic acid in human milk. CD36 is a new member of mammalian polysialic acid-containing glycoprotein. J. Biol. Chem. **278**, 13875–13880 (2003). doi:10.1074/jbc.M300458200
13. Curreli, S., Arany, Z., Gerardy-Schahn, R., Mann, D., Stamatos, N.M.: Polysialylated neuropilin-2 is expressed on the surface of human dendritic cells and modulates dendritic cell-T lymphocyte interactions. J. Biol. Chem. **282**, 30346–30356 (2007). doi:10.1074/jbc.M702965200
14. Harduin-Lepers, A., Mollicone, R., Delannoy, P., Oriol, R.: The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology **15**, 805–817 (2005). doi:10.1093/glycob/cwi063
15. Harduin-Lepers, A., Recchi, M.A., Delannoy, P.: 1994, the year of sialyltransferases. Glycobiology **5**, 741–758 (1995). doi:10.1093/glycob/5.8.741
16. Harduin-Lepers, A., Vallejo-Ruiz, V., Krzewinski-Recchi, M.A., Samyn-Petit, B., Julien, S., Delannoy, P.: The human sialyltransferase family. Biochimie **83**, 727–737 (2001). doi:10.1016/S0300-9084(01)01301-3
17. Saito, M., Yu, R.: Biochemistry and function of sialidases. In: Rosenberg, A. (ed.) Biology of Sialic Acids, pp. 261–313. Plenum, New York (1995)
18. Hakomori, S.I.: Structure and function of glycosphingolipids and sphingolipids: Recollections and future trends, Biochim Biophys Acta (2007).
19. Ngamukote, S., Yanagisawa, M., Ariga, T., Ando, S., Yu, R.K.: Developmental changes of glycosphingolipids and expression of glycogenes in mouse brains. J. Neurochem. **103**, 2327–2341 (2007). doi:10.1111/j.1471-4159.2007.04910.x
20. Yu, R.K., Macala, L.J., Taki, T., Weinfield, H.M., Yu, F.S.: Developmental changes in ganglioside composition and synthesis in embryonic rat brain. J. Neurochem. **50**, 1825–1829 (1988). doi:10.1111/j.1471-4159.1988.tb02484.x
21. Hildebrandt, H., Muhlenhoff, M., Weinhold, B., Gerardy-Schahn, R.: Dissecting polysialic acid and NCAM functions in brain development. J. Neurochem. **103**(Suppl 1), 56–64 (2007). doi:10.1111/j.1471-4159.2007.04716.x
22. Probstmeier, R., Bilz, A., Schneider-Schaulies, J.: Expression of the neural cell adhesion molecule and polysialic acid during early mouse embryogenesis. J. Neurosci. Res. **37**, 324–335 (1994). doi:10.1002/jnr.490370305
23. Asahina, S., Sato, C., Kitajima, K.: Developmental expression of a sialyltransferase responsible for sialylation of cortical alveolus glycoprotein during oogenesis in rainbow trout (*Oncorhynchus mykiss*). J. Biochem. **136**, 189–198 (2004). doi:10.1093/jb/mvh106
24. Kitazume, S., Kitajima, K., Inoue, S., Inoue, Y., Troy 2nd, F.A.: Developmental expression of trout egg polysialoglycoproteins and the prerequisite alpha 2,6-, and alpha 2,8-sialyl and alpha 2,8-polysialyltransferase activities required for their synthesis during oogenesis. J. Biol. Chem. **269**, 10330–10340 (1994)
25. Guérardel, Y., Chang, L.Y., Maes, E., Huang, C.J., Khoo, K.H.: Glycomic survey mapping of zebrafish identifies unique sialylation pattern. Glycobiology **16**, 244–257 (2006). doi:10.1093/glycob/cwj062
26. Westerfield, M.: The zebrafish book. A guide for laboratory use of zebrafish (*Danio rerio*), p. 385. University of Oregon Press, Eugene (1995)
27. Yu, S.Y., Wu, S.W., Khoo, K.H.: Distinctive characteristics of MALDI-Q/TOF and TOF/TOF tandem mass spectrometry for sequencing of permethylated complex type *N*-glycans. Glycoconj. J. **23**, 355–369 (2006). doi:10.1007/s10719-006-8492-3
28. Sato, C., Inoue, S., Matsuda, T., Kitajima, K.: Fluorescent–assisted detection of oligosialyl units in glycoconjugates. Anal. Biochem. **266**, 102–109 (1999). doi:10.1006/abio.1998.2921
29. Chen, W.Y., John, J.A., Lin, C.H., Chang, C.Y.: Molecular cloning and developmental expression of zinc finger transcription factor MTF-1 gene in zebrafish, *Danio rerio*. Biochem. Biophys. Res. Commun. **291**, 798–805 (2002). doi:10.1006/bbrc.2002.6517
30. Ahmed, H., Du, S.J., O'Leary, N., Vasta, G.R.: Biochemical and molecular characterization of galectins from zebrafish (*Danio rerio*): notochord-specific expression of a prototype galectin during early embryogenesis. Glycobiology **14**, 219–232 (2004). doi:10.1093/glycob/cwh032
31. Inoue, S., Inoue, Y.: A challenge to the ultrasensitive chemical method for the analysis of oligo- and polysialic acids at a

nanogram level of colominic acid and a milligram level of brain tissues. Biochimie **83**, 605–613 (2001). doi:10.1016/S0300-9084(01)01307-4

32. Inoue, S., Lin, S.L., Lee, Y.C., Inoue, Y.: An ultrasensitive chemical method for polysialic acid analysis. Glycobiology **11**, 759–767 (2001). doi:10.1093/glycob/11.9.759
33. Sato, C., Inoue, S., Matsuda, T., Kitajima, K.: Development of a highly sensitive chemical method for detecting alpha2®8-linked oligo/polysialic acid residues in glycoproteins blotted on the membrane. Anal. Biochem. **261**, 191–197 (1998). doi:10.1006/abio.1998.2718
34. Manzoni, M., Colombi, P., Papini, N., Rubaga, L., Tiso, N., Preti, A., *et al*.: Molecular cloning and biochemical characterization of sialidases from zebrafish (*Danio rerio*). Biochem. J. **408**, 395–406 (2007). doi:10.1042/BJ20070627
35. Inoue, S., Iwasaki, M., Ishii, K., Kitajima, K., Inoue, Y.: Isolation and structures of glycoprotein-derived free sialooligosaccharides from the unfertilized eggs of *Tribolodon hakonensis*, a dace. Intracellular accumulation of a novel class of biantennary disialooligosaccharides. J. Biol. Chem. **264**, 18520–18526 (1989)
36. Iwasaki, M., Seko, A., Kitajima, K., Inoue, Y., Inoue, S.: Fish egg glycophosphoproteins have species-specific N-linked glycan units previously found in a storage pool of free glycan chains. J. Biol. Chem. **267**, 24287–24296 (1992)
37. Inoue, S., Inoue, Y.: Fish glycoproteins. In: Montreuil, J., Vliegenthart, J., Schachter, H. (eds.) Glycoproteins II, pp. 143–161. Elsevier, Amsterdam (1997)
38. Teintenier-Lelievre, M., Julien, S., Juliant, S., Guerardel, Y., Duonor-Cerutti, M., Delannoy, P., *et al*.: Molecular cloning and expression of a human hST8Sia VI (α2,8-sialyltransferase) responsible for the synthesis of the diSia motif on *O*-glycosylproteins. Biochem. J. **392**, 665–674 (2005). doi:10.1042/BJ20051120
39. Marx, M., Rivera-Milla, E., Stummeyer, K., Gerardy-Schahn, R., Bastmeyer, M.: Divergent evolution of the vertebrate polysialyltransferase Stx and Pst genes revealed by fish-to-mammal comparison. Dev. Biol. **306**, 560–571 (2007). doi:10.1016/j.ydbio.2007.03.032
40. Haraguchi, M., Yamashiro, S., Yamamoto, A., Furukawa, K., Takamiya, K., Lloyd, K.O., *et al*.: Isolation of GD3 synthase gene by expression cloning of GM3 α2,8-sialyltransferase cDNA using anti-GD2 monoclonal antibody. Proc. Natl. Acad. Sci. USA. **91**, 10455–10459 (1994). doi:10.1073/pnas.91.22.10455
41. Nara, K., Watanabe, Y., Maruyama, K., Kasahara, K., Nagai, Y., Sanai, Y.: Expression cloning of a CMP-NeuAc: NeuAcα2–3Galb1–4Glcb1–1¢Cer α2,8-sialyltransferase (GD3 synthase) from human melanoma cells. Proc. Natl. Acad. Sci. USA. **91**, 7952–7956 (1994). doi:10.1073/pnas.91.17.7952
42. Sasaki, K., Kurata, K., Kojima, N., Kurosawa, N., Ohta, S., Hanai, N., *et al*.: Expression cloning of a GM3-specific α2,8-sialyltransferase (GD3 synthase). J. Biol. Chem. **269**, 15950–15956 (1994)
43. Daniotti, J.L., Rosales Fritz, V., Kunda, P., Nishi, T., Maccioni, H.J.: Cloning, characterization and developmental expression of alpha2,8 sialyltransferase (GD3 synthase, ST8Sia I) gene in chick brain and retina. Int. J. Dev. Neurosci. **15**, 767–776 (1997). doi:10.1016/S0736-5748(97)00027-0
44. Lee, Y.C., Kim, Y.J., Lee, K.Y., Kim, K.S., Kim, B.U., Kim, H.N., *et al*.: Cloning and expression of cDNA for a human Sia alpha 2,3Gal beta 1, 4GlcNA:alpha 2,8-sialyltransferase (hST8Sia III). Arch. Biochem. Biophys. **360**, 41–46 (1998). doi:10.1006/abbi.1998.0909
45. Kim, Y.J., Kim, K.S., Do, S., Kim, C.H., Kim, S.K., Lee, Y.C.: Molecular cloning and expression of human alpha2,8-sialyltransferase (hST8Sia V). Biochem. Biophys. Res. Commun. **235**, 327–330 (1997). doi:10.1006/bbrc.1997.6725
46. Kono, M., Yoshida, Y., Kojima, N., Tsuji, S.: Molecular cloning and expression of a fifth type of α2,8-sialyltransferase (ST8Sia V). Its substrate specificity is similar to that of SAT-V/III, which synthesize GD1c, GT1a, GQ1b and GT3. J. Biol. Chem. **271**, 29366–29371 (1996). doi:10.1074/jbc.271.46.29366
47. Gornati, R., Basu, S., Bernardini, G., Rizzo, A.M., Rossi, F., Berra, B.: Activities of glycolipid glycosyltransferases and sialidases during the early development of *Xenopus laevis*. Mol. Cell. Biochem. **166**, 117–124 (1997). doi:10.1023/A:1006891414663
48. Gornati, R., Rizzo, A.M., Tong, X.W., Berra, B., Bernardini, G.: Glycolipid patterns during Xenopus embryo development. Cell Biol. Int. **19**, 183–189 (1995). doi:10.1006/cbir.1995.1061
49. Marx, M., Rutishauser, U., Bastmeyer, M.: Dual function of polysialic acid during zebrafish central nervous system development. Development **128**, 4949–4958 (2001)
50. Taguchi, T., Seko, A., Kitajima, K., Inoue, S., Iwamatsu, T., Khoo, K.H., *et al*.: Structural studies of a novel type of tetraantennary sialoglycan unit in a carbohydrate-rich glycopeptide isolated from the fertilized eggs of Indian Medaka fish, *Oryzias melastigma*. J. Biol. Chem. **268**, 2353–2362 (1993)
51. Asahina, S., Sato, C., Matsuno, M., Matsuda, T., Colley, K., Kitajima, K.: Involvement of the α2,8-polysialyltransferases II/STX and IV/PST in the biosynthesis of polysialic acid chains on the O-linked glycoproteins in rainbow trout ovary. J. Biochem. **140**, 687–701 (2006). doi:10.1093/jb/mvj200
52. Tsuji, S., Datta, A.K., Paulson, J.C.: Systematic nomenclature for sialyltransferases. Glycobiology **6**, v–vii (1996). doi:10.1093/glycob/6.7.647
53. Svennerholm, L.: The gangliosides. J. Lipid Res. **5**, 145–155 (1964)