Reversed phase liquid chromatography

Lipids (10 µl injection) were separated on a Acquity UPLC BEH C_6 column (130 Å, 1 × 100 mm for cellular PL profiling; 2.1 × 30 mm for *in vitro* activity assays, Waters, Milford, MA) using a Acquity^{TM} Ultraperformance LC system (Waters, Milford, MA, USA). Chromatography was performed at a flow rate of 0.1 ml/min (BEH C_6, 1 × 100 mm) or 0.8 ml/min (BEH C_6, 2.1 × 30 mm) at 45°C using a gradient from 80% mobile phase A (20 mM aqueous ammonium bicarbonate)/20% mobile phase B (acetonitrile) to 5% mobile phase A/95% mobile phase B within 20 min (BEH C_6, 1 × 100 mm) or 8.5 min (BEH C_6, 2.1 × 30 mm) followed by isocratic elution with mobile phase A/B = 5/95 for 10 or 1.5 min, respectively. The LC system was controlled with Acquity UPLC Software 1.40 (Waters, Milford, MA, USA).

Mass spectrometry

The LC system described above was coupled to a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an HESI-II electrospray ionization source. The ion spray voltage was set to 3000 kV in the positive ion mode and to –2500 kV in the negative ion mode, the heated capillary temperature to 220°C (BEH C_6, 1 × 100 mm) or 280°C (BEH C_6, 2.1 × 30 mm), the vaporizer temperature to 450°C (BEH C_6, 1 × 100 mm) or 550°C (BEH C_6, 2.1 × 30 mm), the sheath gas (nitrogen) pressure to 50 psi, the auxiliary gas (nitrogen) pressure to 15 psi and the collision gas (argon) pressure to 0.7 mTorr. The other parameters were set according to the manufacturer's recommendations. The scan range of the instrument was set at *m/z* 400–1200. PG, PA and free fatty acids were measured by a full scan in the negative ion mode with a scan time of 1 s. The scan time for precursor ion scans and neutral loss scans was set to 0.8 s. PC was analyzed by

m/z = 189 precursor ion scans (positive ion mode, collision energy: 35 V), PE by *m* = 141.0 neutral loss scans (positive ion mode, collision energy: 25 V), PS by *m* = 87.0 neutral loss scans (negative ion mode, collision energy: 25 V) and PI by *m/z* = 241.0 precursor ion scans (negative ion mode, collision energy: 35 V). To determine the fatty acid composition of the PLs, selective reaction monitoring (collision energy: 40 V) or product ion scans (*m/z* 200-400, collision energy: 40 V, scan time: 1 s) were used. PLs from *in vitro* activity assays were detected by selective ion monitoring (scan time: 0.25 s, scan width: 0.6 *m/z* units).

Assay of lysophospholipid acyltransferase activity

Reaction mixtures contained 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, acyl-CoA (25 μM), lyso-PL (50 μM) and enzyme (100,000 × g pellets) in a total volume of 100 μl. After incubation at 37°C for 30 min, reactions were stopped by the addition of 375 μl chloroform/methanol (1:2, v/v) containing the internal standard 1,2-di-14:0-*sn*-glycero-3-PE (0.8 nmol). The reaction progressed linearly at least for 30 min. Total lipids were extracted using Bligh-Dyer method (25) and subsequently analyzed by LC-MS as described above.

Statistics

Data are presented as mean ± SE. Statistical evaluation of the data was performed by oneway ANOVAs for independent or correlated samples followed by Tukey HSD post hoc tests or by Student's t test for paired and correlated samples. *P* values < 0.05 were considered statistically significant. All statistical calculations were performed using GraphPad InStat 3.0 (GraphPad Software Inc., La Jolla, CA, USA). Kinetic constants were assessed by fitting the data to the Michaelis-Menten equation using SigmaPlot 9.0 (Systat Software Inc., San Jose, USA).

Results

Knockdown of mLPAAT3 in TM4 cells and expression of FLAG-tagged mLPAAT3 in CHO-K1 cells and the effects on cell proliferation

Predictions concerning the cellular function of LPAAT3 and its contribution to PL diversity from i*n vitro* activity studies are only of limited value because the *sn*-2 fatty acid profile of PLs not only depends on *de novo* acyltransferases but also on a subset of remodeling enzymes (19). To investigate the physiological role of LPAAT3 in PL metabolism and fatty acid homeostasis by Sertoli cells, we generated two cellular models with altered LPAAT3 expression, namely mLPAAT3-shRNA-transfected TM4 Sertoli cells and mLPAAT3-overexpressing CHO-K1 cells. Expression of mLPAAT3 mRNA in TM4 cells was first confirmed under resting conditions by quantitative real time PCR (**Fig. 1A**). Then, cells were transfected with control-shRNA ('control'), encoding a scrambled non-matching sequence, or 4 different shRNA sequences ('lot 1-4') with target sites within the mLPAAT3 mRNA. Only the 'lot 3'-shRNA sequence significantly suppressed mLPAAT3 mRNA expression (by approx. 60%) (**Fig. 1B**). Monoclonal cell populations of stable 'lot 3'-shRNA transfected TM4 cells were prepared but failed to markedly exceed the knockdown efficiency of polyclonal cells (unpublished observations). All following experiments, therefore, refer to polyclonal 'lot 3'-shRNA transfected TM4 cells. Compensation affecting mLPAAT1 or -2 transcription was excluded because neither mLPAAT1 nor mLPAAT2 mRNA levels were noteworthy affected in 'lot 3'-shRNA-transfected cells (**Fig. 1C**). We have previously shown that mLPAAT3 efficiently catalyzes the esterification of *sn*-1-18:1-lysophosphatidic acid (LPA) with 20:4-CoA to *sn*-1-18:1-*sn*-2-20:4-PA (23). This acyltransferase activity was significantly decreased in isolated microsomes by the specific interference of 'lot 3'-shRNA with mLPAAT3 mRNA expression (**Fig. 1D**). Taken together, the knockdown of LPAAT3 in

TM4 cells by 'lot 3'-shRNA combines a selective reduction of mLPAAT3 mRNA with functional consequences on cellular acyltransferase activity.

Effects of an increased expression of LPAAT3 on the cellular PL profile were investigated in CHO-K1 cells transfected with an expression vector encoding for an mLPAAT3 fusion protein that contains the FLAG epitope fused in frame to the N-terminus of mLPAAT3 (23). Expression of FLAG-tagged mLPAAT3 was confirmed by Western Blotting using anti-FLAG antibody (**Fig. 1E**).

Changes in the cellular mLPAAT3 expression were associated with small but significant effects on cell proliferation. Knockdown of mLPAAT3 increased the cell number of TM4 cells by approx. 15.9 ± 3.7% (*, n = 21) compared to vector control when seeded at 6×10^5 cells/10 cm dish and incubated for 72 h (37°C, 5% CO_2). Accordingly, expression of mLPAAT3 in CHO-K1 cells significantly decreased the cell count by approx. 13.8 ± 3.3% (*, n = 3) when seeded at 3.0×10^6 cells/10 cm dish, transfected after 24 h with FLAG/mLPAAT3 vector and incubated for another 48 h (37°C, 5% CO_2). The number of dead cells, as determined by trypan blue staining, was comparable to vector controls rather precluding effects of LPAAT3 on cell viability.

Expression of mLPAAT3 specifically affects the cellular glycero-PL composition

Next we compared the glycero-PL profile of mLPAAT3-shRNA-transfected TM4 cells (Table 2) and mLPAAT3-expressing CHO-K1 cells (Table 3) with the profile of respective vector control-transfected cells. PC and PE were extracted from cells by the method of Bligh and Dyer, whereas anionic PLs (PS, PI, PG and PA) were extracted by *n*-butanol and separated from other lipids by anion-exchange chromatography. Then, PLs were separated by reversed phase chromatography and analyzed by electrospray ionization (ESI)-MS in the negative full scan mode (for PG and PA) or by ESI-MS/MS using head-group-specific precursor-ion scans

(PC, PI) or neutral loss scans (PE, PS). The fatty acid composition of each molecular species was confirmed by analyzing the fatty acid anions after fragmentation by selective reaction monitoring or product ion scanning. The relative abundance of each PL species is expressed relative to the sum of all detected species within the PL subclass. Only the relative intensities of mLPAAT3-shRNA transfected TM4 cells in Table 2 are provided as percentage of control because slight constant shifts in the relative PL compositions (for both control- and LPAAT3-shRNA-transfected cells) were observed between the individual data sets. If regioisomeric PLs could not be separated by liquid chromatography, the relative intensity of the combined peak is given. Knockdown of mLPAAT3 in TM4 cells led to a significant decrease of PC species containing 20:4 (16:0-20:4-, 18:0-20:4-, 18:1-20:4/18:0-20:5/16:0-22:5-PC), 20:5 (18:1-20:5) and 22:5 (18:0-22:5-PC) (Table 2). 22:6 was not detectable in PC from murine TM4 cells, but represents the major hPUFA in CHO-K1 cells. Along these lines, expression of mLPAAT3 in CHO-K1 cells significantly increased the abundance of PC containing 20:4 (18:0-20:4-PC) and 22:6 (16:0-22:6-, 18:0-22:6/18:1-22:5-, 18:1-22:6-PC) (Table 3). Expression of mLPAAT3 in TM4 cells also significantly decreased the relative amount of 18:2-containing PE (16:1-18:2/16:0-18:3- and 18:1-18:2-PE), PS (16:0-18:2-, 18:1-18:1/18:0-18:2-PS) and PG (16:0-18:2/16:0-20:3-PG) (Table 2), whereas expression of LPAAT3 in CHO-K1 cells essentially had the opposite effect and significantly increased the relative levels of 18:2-containing PE (16:0-18:2/16:1-18:1- and 16:1-18:2/16:0-18:3-PE) and PS (18:1-18:1/18:0-18:2-PS) (Table 3). Significant effects on saturated PC (14:0-16:0-, 16:0-16:0-PC) and 18:0-20:4-PI were only observed for an increased mLPAAT3 expression (Table 3) but not for the knockdown of mLPAAT3 (Table 2). Surprisingly, although suggested by *in vitro* activity assays (23), the relative amount of detected PA was neither significantly affected by knockdown nor expression of LPAAT3 (except for an increase of 16:0-18:1-PA in Table 2 which is not understood). However, only PA species with saturated, mono- and di-

unsaturated fatty acids could be detected but not species with PUFAs, whose acyl-CoA esters are the preferred substrates of LPAAT3 *in vitro* (23). In summary, a role of LPAAT3 in the formation of specific polyunsaturated PC and 18:2-containing PE and PS was confirmed both by LPAAT3-knockdown and expression studies, whereas the limited effects on PG and PI were dependent on the cellular model used.

To access the total concentrations of cellular PLs, changes in the detected PL intensities were calculated per number of cells, normalized to the added standards and summed up for each PL subclass. The total intensities of PC, PE, PI, PG and PA were neither significantly affected in mLPAAT3-shRNA-transfected TM4 cells nor in mLPAAT3-expressing CHO-K1 cells (Table 4). In contrast, the total intensity of PS was significantly decreased in TM4 cells (approx. by 16%) when mLPAAT3 expression was reduced (Table 4).

The effects of mLPAAT3 on the cellular PL composition are mediated by its LPAAT activity

The specific modulation of the cellular PL pattern by LPAAT3 might either depend on the biosynthesis of PA, its conversion to glycero-PLs and the subsequent specific variation of the *sn*-2 fatty acid profile in the remodeling pathway, or LPAAT3 might possess additional so far unidentified LPLAT activities. To discriminate between these possibilities, we prepared microsomes from mLPAAT3-shRNA-transfected TM4 cells and mLPAAT3-expressing CHO-K1 cells and analyzed their LPLAT activities for different substrate (*sn*-1-LPL/acyl-CoA) combinations. Focus was set on the formation of PL species 1) whose concentration was significantly changed in response to LPAAT3 in the previous cellular assays and 2) for which the respective substrates were commercially available. The 20:4-transferase activity of LPAAT3 for *sn*-1-LPA reported by Yuki et al. (23) could be confirmed for microsomes from mLPAAT3-expressing CHO-K1 cells (**Fig. 2A**). Moreover, we show LPAAT activity of

LPAAT3 for 18:2- and 22:6-CoA (**Fig. 2A**). 22:6-CoA seems to be the preferred substrate of LPAAT3 being even superior to 20:4-CoA (at least according to the relative MS-peak intensities). The opposite trend was essentially found for microsomes from mLPAAT3-shRNA-transfected TM4 cells (**Fig. 3A**), although slight differences were obvious regarding the *sn*-1-LPA specificity (e.g. for 16:0-20:4-PA or 18:0-22:6-PA). Neither lyso-PE- (LPE-), lyso-PS- (LPS-), lyso-PG- (LPG-) nor lyso-PC-acyltransferase (LPCAT) activities were observed for LPAAT3 in any of these models (**Fig. 2B** and **Fig. 3B**), indicating that LPAAT3 primarily modulates the fatty acid composition of PLs via a defined synthesis of PA species.

Kinetic characterization of LPAAT3

Kinetic analysis of mLPAAT3 expressed in CHO-K1 cells showed Michaelis-Menten rate behaviour toward *sn*-1-18:1-LPA in the presence of 22:6-CoA (25 μM) and toward 22:6-CoA in the presence of *sn*-1-18:1-LPA (50 μM) (**Supplemental Fig. 1**). Michaelis-Menten constants K_M (18:1-LPA: K_M = 26.3 μM; 22:6-CoA: K_M = 11.0 μM) and V_{max} (18:1-LPA: V_{max} = 8.5 pmol min^{-1} μg^{-1}; 22:6-CoA: V_{max} = 12.3 pmol min^{-1} μg^{-1}) for the formation of *sn*-1-18:1-*sn*-2-22:6-PA were calculated by fitting the Michaelis-Menten equation to the entire concentration range of *sn*-1-18:1-LPA and 22:6-CoA, respectively. The kinetic constants are in line with values previously reported for the formation of 16:0-20:4-PA (16:0-LPA: K_M = 26.3 μM; 20:4-CoA: K_M = 15.9 μM) (23).

LPAAT3 is required to maintain levels of distinct free hPUFAs

PLs represent a large storage for fatty acids which can be released by specific phospholipases A_2 (27). The accumulation of PUFAs in PC through LPAAT3 might therefore have an impact on the basal availability of respective free fatty acids. In fact, the level of free 22:5 was selectively and significantly decreased in TM4 cells by the knockdown of mLPAAT3

(approx. by 22%) (Table 5). In mLPAAT3-expressing CHO-K1 cells, however, free 22:5 acid was not detectable. Instead, the level of the related eicosapentaenoic acid (n-3, 20:5) significantly increased by 21% (Table 5). Moreover, significant changes in the concentration were found for linolenic acid (18:3) and eicosatrienoic acid (20:3) in mLPAAT3-shRNA-transfected TM4 cells and mLPAAT3-expressing CHO-K1 cells, respectively (Table 5).

Discussion

We found that LPAAT3 essentially contributes to the formation of specific cellular PLs, particularly of polyunsaturated PC (containing 20:4, 20:5, 22:5 and 22:6) and several 18:2-containing PE, PS and PG species. These changes in the PL pattern were mediated through an acyltransferase activity of LPAAT3 with preference for LPA as acyl-acceptor and hPUFA-CoA, arachidonoyl-CoA and to a minor extent linoleoyl-CoA as acyl-donor. Further, we have shown that the abundance of polyunsaturated PC correlates with the availability of free hPUFAs (i.e., 22:5 and 20:5). Although previously suggested from an *in vitro* activity assay showing LPIAT activity for LPAAT3 (23), cellular levels of PI species were neither significantly increased by expression of mLPAAT3 in CHO-K1 cells nor decreased by the knockdown of mLPAAT3 in TM4 cells precluding *in vivo* relevance. *In vitro*, we confirmed a weak 20:4-transferase activity of LPAAT3 for lyso-PI (23) with preference for *sn*-1-16:0-lyso-PI (**Supplemental Fig. 2**). Moreover, this is the first detailed lipidomic study investigating the molecular PL composition of a Sertoli cell line.

Our data suggest that the expression of LPAAT3 determines the cellular abundance of polyunsaturated PC species through regulating the availability of PA precursors. Although low abundant polyunsaturated PA species were neither detectable in TM4 cells nor CHO-K1 cells, we could demonstrate a preference of LPAAT3 for 22:6-CoA and slightly less pronounced for 20:4-CoA (see also (23)) in microsomal LPAAT activity assays. Most likely, also 22:5 (n-6), one of the main hPUFAs in TM4 Sertoli cells, is a preferred substrate of LPAAT3, though an experimental confirmation is still lacking because the respective CoA-ester is commercially not available. The exclusive role of LPAAT3 in PA biosynthesis suggests similar fatty acid profiles for PA-derived PLs (PC, PE, PS, PI, PG). However, our study indicates that polyunsaturated PA is predominantly converted into polyunsaturated PC (but not into other PLs), whereas sn-2-18:2-PA is used as precursor for PE, PS and eventually

PG (but not for PC and PI). The biosynthetic route from PA to PC includes the dephosphorylation of PA to diacylglycerol (the precursor of PC, PE and PS) by PA phosphatase and the introduction of choline by CDP-choline:1,2-diacylglycerol choline-phosphotransferase (28). The unique sn-2 fatty acid pattern of PC can only be mediated by the latter enzyme (in the *de novo* pathway). In fact, human CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT)1 is highest expressed in testis compared to other tissues and shows some preference for 18:1, 20:4 and 22:6 in the sn-2 position of diacylglycerol (29). However, the sn-2 fatty acid selectivity not necessarily depends on enzymes of the *de novo* PL biosynthesis. A slow exchange rate of the sn-2 fatty acid for PC compared to other PLs (in the remodeling pathway (19)) might also explain the selective accumulation of PA-derived hPUFAs in PC. In any case, cell-type specific differences in the fate of LPAAT3-derived PAs cannot be excluded.

Our study supports the hypothesis that hPUFAs, which are efficiently synthesized by Sertoli cells, are supplied to neighbouring germ cells (7, 8, 13, 14). In advantage to previous studies monitoring the metabolism of exogenously added fatty acids (8, 10, 11, 13), our study describes an endogenous modulation of hPUFAs via expression of LPAAT3. Moreover, we give insight into the fate of hPUFAs between their biosynthesis in Sertoli cells and their subsequent release. hPUFAs are seemingly first incorporated into PC by LPAAT3 (at the level of the respective PA precursors) before they are released as free fatty acids, and their availability correlates with the cellular levels of polyunsaturated PC. Because our culture medium contained only low amounts of PUFAs (present in the supplemented fetal sera (30)), acyl-CoA substrates of LPAAT3 most likely derive predominantly from the cellular fatty acid biosynthesis (as previously reported for Sertoli cells (7-12)) rather than from a cellular uptake. *In vivo*, however, distinct metabolic situations (e.g., an essential fatty acid rich diet (31, 32)) can increase the blood levels of hPUFAs and thereby their availability for Sertoli cells. Under

such conditions, LPAAT3 might gain additional importance in mediating the efficient storage of hPUFAs.

An intermediate incorporation of PUFAs into PC would not only guarantee a homeostatic continuous supply of fatty acids to germ cells but would also allow a sustained stimulated release. The kinetic profile of LPAAT3 for acyl-CoAs is in agreement with this hypothesis. Within the physiological concentration range of cellular acyl-CoAs (< 200 nM $<< K_M$) (33), the turnover rate of LPAAT3 is highly responsive to small changes in the acyl-CoA concentration and thus to the metabolic cellular state, allowing an effective membrane incorporation of excess PUFAs. A similar mechanism for regulating fatty acid supply was found for 20:4. Incorporation of 20:4 into membrane PLs is required for its regulated release through cytosolic phospholipase A_2 and subsequent conversion to eicosanoids (27).

One might speculate that also the differences in free fatty acid levels themselves (as shown in Table 3) have an impact on the formation of polyunsaturated PC. However, differences in the acyl-CoA concentrations as driving force for the modification of PL profiles can be excluded because of the specificity of the changes. The modulation of LPAAT3 expression specifically affects the PUFA composition of PC species but not of other PL subclasses. Moreover, these changes were not restricted to PC species containing 22:5 or 20:5, whose levels as free fatty acid were affected, but also include 20:4 and 22:6 containing PC species according to the substrate specificity of LPAAT3 (23).

Whether the fatty acid release of Sertoli cells in testis is regulated by extracellular factors (e.g., hormones) is still elusive. However, a tightly regulated supply of hPUFAs to germ cells seems to be essential for fertility (34-36). Elimination of the ability of mice to synthesize PUFAs by disruption of the Δ6-desaturase gene (35, 36) or by inactivation of the peroxisomal β-oxidation (34) caused sterility in male mice, and supplementation of 22:6 (but not of 20:4) completely restored fertility and spermatogenesis (37). Most of the testicular hPUFAs

accumulate in germ cells (5, 6) and there particularly in the sperm tail (38), which suggest a role of hPUFAs for the membrane properties of germ cells required for sperm mobility. This is of particular interest because expression of LPAAT3 is strongly upregulated in mouse testis during puberty when spermatogenesis first occurs and then remains at a high basal level (23), thereby supporting a potential role of LPAAT3 in male fertility.

Opposite effects on fertility would be expected from the anti-proliferative effects of LPAAT3 on TM4 Sertoli cells because the number of Sertoli cells directly correlates with the rate and quality of spermatogenesis (1). The growth-suppressing properties of LPAAT3 might either be related to hPUFAs, which have been shown to impair cell growth and proliferation (39-41), or the LPAAT3-mediated increase of the absolute PS concentration. PS is not only an early marker of apoptosis but also induces apoptosis in several adherent cell lines at high concentrations (42). Changes in the mLPAAT3 expression only affected total PS levels in TM4 Sertoli cells but were without effect in CHO-K1 cells. The lower abundance of 18:2-containing PS species (particularly of 18:1-18:1/18:0-18:2-PS, which is significantly decreased) in CHO-K1 cells compared to TM4 cells might explain the cell-type dependent difference.

In conclusion, we present 1) the molecular glycero-PL profile of a Sertoli cell line, 2) thoroughly characterized the physiological role of LPAAT3 in modulating the cellular PL composition by shRNA and overexpression systems, 3) elucidated LPAAT3 as critical enzyme for the supply of free hPUFAs by Sertoli cells and 4) propose an intermediate storage of hPUFAs in PC of Sertoli cells to be essential for a controlled fatty acid release. Further studies addressing the role of LPAAT3 and hPUFAs for fertility and sperm mobility are required.

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Acknowledgements

We thank Dr. J.-i. Miyazaki (Osaka University) for supplying the expression vector pCXN2.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (T.S.) and a Global COE Program (The University of Tokyo) from Japan Society for Promotion of Sciences (T.S.). T.S. and H.S. were supported by the Center for NanoBio Integration at The University of Tokyo. H.S. was supported by Health and Labour Sciences Research Grants (Research on Allergic Disease and Immunology) of the Ministry of Health, Labour and Grant-in-Aid for Young Scientists (B) from the MEXT of Japan and The Uehara Memorial Foundation and The Cell Science Research Foundation. T.H. was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientist. Andreas Koeberle received a stipend from Takeda Science Foundation.

Tables

Table 1. Primer sequences used in quantitative real-time PCR experiments

gene	sense primer (5'→3')	anti-sense primer (5'→3')
mLPAAT1	CTTCTACAACGGCTGGATCCTCT	TCCCATACAGGTATTTGACGTGG
mLPAAT2	AAGCGTGAGCTAATGTTCACAGG	TTTTTAAAGGGCAACAGGTCCC
mLPAAT3	ACCTATACCGCCGTATCAACTGC	AGTCGATCTCGAAGTTGTGGTTG
GAPDH	TGACAATGAATACGGCTACAGCA	CTCCTGTTATTATGGGGGTCTGG

Table 2. Major diacyl-glycero-PLs from control-shRNA-transfected TM4 cells as relative intensities and from mLPAAT3-shRNA-transfected TM4 cells as percentage of control. Mean values (n = 3-10) and standard error estimates are given; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs. control-shRNA-transfected TM4 cells

fatty acid composition	relative intensity of diacyl-glycero-PLs from control-shRNA-transfected TM4 cells [% of PL subclass intensity]						PL composition after knockdown of mLPAAT3 [% of control shRNA]					
	PC	PE	PS	PI	PG	PA	PC	PE	PS	PI	PG	PA
14:0-16:0	2.4±0.4	n.d.	n.d.	n.d.	n.d.	n.d.	105.8±12.4	n.d.	n.d.	n.d.	n.d.	n.d.
16:0-16:0	4.6±1.0	2.8±0.7	n.d.	n.d.	4.9±0.8	21.0±0.4	100.4±6.1	94.3±9.5	n.d.	n.d.	97.9±9.9	90.6±0.7
16:0-18:0	2.4±0.4	5.8±0.8	n.d.	n.d.	n.d.	n.d.	86.3±4.5	96.4±5.3	n.d.	n.d.	n.d.	n.d.
16:0-16:1/14:0-18:1	6.2±1.2	0.9±0.2	n.d.	n.d.	n.d.	11.2±0.3	112.0±4.0	101.3±11.5	n.d.	n.d.	n.d.	125.0±11.1
16:0-18:1	22.5±1.4	6.2±1.3	7.7±0.6	4.3±1.4	26.1±4.6	29.1±0.5	107.4±2.9	107.8±3.6	114.3±15.4	138.9±13.4	110.4±15.9	115.3±3.8*
18:0-18:1	15.2±2.1	12.7±2.4	36.3±3.0	9.5±3.0	32.6±4.6	n.d.	98.4±5.9	108.0±6.0	131.1±10.3*	117.8±10.2	117.4±13.1	n.d.
16:0-18:2	8.0±0.7	2.7±0.3	1.9±0.5	1.9±0.6	2.9±0.7	21.8±0.3	97.2±4.6	107.8±8.9	56.5±11.9*	105.0±11.1	98.1±14.3	93.7±1.9
16:1-18:2/16:0-18:3	n.d.	0.8±0.1	n.d.	n.d.	n.d.	n.d.	n.d.	72.0±6.5*	n.d.	n.d.	n.d.	n.d.
18:1-18:1/18:0-18:2	24.3±2.5	26.4±2.0	25.2±2.9	16.6±5.2	16.5±1.1	n.d.	104.5±3.8	100.2±2.5	78.7±6.5*	110.4±6.8	108.7±5.5	n.d.
18:1-18:2	7.6±0.5	8.6±1.0	5.7±1.0	6.6±2.1	8.6±0.9	16.9±0.4	91.2±5.8	83.1±3.8*	76.0±8.0	86.3±6.2	68.9±5.1**	91.5±8.1
16:0-20:3	n.d.	n.d.		n.d.			n.d.	n.d.		n.d.		
18:0-20:3	1.7±0.1	7.9±1.1	5.7±0.6	7.6±2.4	4.5±0.9	n.d.	98.7±5.5	104.1±9.3	102.1±13.6	125.1±14.6	88.8±8.6	n.d.
16:0-20:4	0.9±0.2	1.6±0.2	n.d.	2.6±0.8	n.d.	n.d.	79.5±6.0*	101.3±7.7	n.d.	109.4±7.6	n.d.	n.d.
18:0-20:4	0.9±0.1	5.8±1.3	4.6±0.7	22.3±7.1	5.0±0.5	n.d.	76.9±4.2**	106.4±4.3	108.7±17.0	100.6±4.2	94.8±6.5	n.d.
18:1-20:4	2.2±0.3	n.d.	n.d.	n.d.	n.d.	n.d.	80.0±3.1**	n.d.	n.d.	n.d.	n.d.	n.d.
18:0-20:5		8.9±1.7	3.4±0.4	23.4±7.4	n.d.	n.d.		109.2±6.2	98.1±14.6	90.2±7.9	n.d.	n.d.
16:0-22:5		n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.
18:1-20:5	0.5±0.1	1.4±0.2	n.d.	3.7±1.2	n.d.	n.d.	81.5±4.5*	101.7±10.9	n.d.	90.3±5.7	n.d.	n.d.
18:0-22:5	0.5±0.1	4.7±2.2	9.6±2.6	2.5±0.8	n.d.	n.d.	82.0±2.2***	95.2±4.5	98.2±16.2	96.5±10.1	n.d.	n.d.
18:1-22:5	0.3±0.1	n.d.	n.d.	0.9±0.3	n.d.	n.d.	89.1±3.9	n.d.	n.d.	94.1±0.2	n.d.	n.d.
18:0-22:6	n.d.	2.8±0.6	n.d.	n.d.	n.d.	n.d.	n.d.	96.6±6.6	n.d.	n.d.	n.d.	n.d.

n.d. = not detectable