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Intracellular cholesterol transporters and modulation of hepatic lipid metabolism: implications for diabetic dyslipidaemia and steatosis

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Abstract

Aims/Hypotheses
To examine hepatic expression of cholesterol-trafficking proteins, mitochondrial StarD1 and endosomal StarD3, and their relationship with dyslipidaemia and steatosis in Zucker (fa/fa) genetically obese rats, and to explore their functional role in lipid metabolism in rat McArdle RH-7777 hepatoma cells.

Methods
Expression of StarD1 and StarD3 in rat liver and hepatoma samples were determined by Q-PCR and/or immunoblotting; lipid mass by colorimetric assays; radiolabelled precursors were utilised to measure lipid synthesis and secretion, and lipidation of exogenous apolipoprotein A-I.

Results
Hepatic expression of StarD3 protein was repressed by genetic obesity in (fa/fa) Zucker rats, compared with lean (Fa/?) controls, suggesting a link with storage or export of lipids from the liver. Overexpression of StarD1 and StarD3, and knockdown of StarD3, in rat hepatoma cells, revealed differential effects on lipid metabolism. Overexpression of StarD1 increased utilisation of exogenous (preformed) fatty acids for triacylglycerol synthesis and secretion, but impacted minimally on cholesterol homeostasis. By contrast, overexpression of StarD3 increased lipidation of exogenous apoA-I, and facilitated de novo biosynthetic pathways for neutral lipids, potentiating triacylglycerol accumulation but possibly offering protection against lipotoxicity. Finally, StarD3 overexpression altered expression of genes which impact variably on hepatic insulin resistance, inducing Ppargcla, Cyp2e1, Nrlh4, G6pc and Irs1, and repressing expression of Scl2a1, Igfbp1, Casp3 and Serpine 1.

Conclusions/Interpretation
Targeting StarD3 may increase circulating levels of HDL and protect the liver against lipotoxicity; loss of hepatic expression of this protein, induced by genetic obesity, may contribute to the pathogenesis of dyslipidaemia and steatosis.

Keywords Insulin resistance, lipid metabolism, high density lipoprotein, steatosis, dyslipidaemia, cholesterol trafficking proteins
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<td>ABCA1</td>
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<td>MLN64</td>
<td>Metastatic Liver Node 64; StarD3</td>
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<td>NAFLD</td>
<td>Non-alcoholic Fatty Liver Disease</td>
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<td>NASH</td>
<td>Non-alcoholic Steatohepatitis</td>
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<td>PGC-1α</td>
<td>Peroxisome Proliferator Activated Receptor Gamma Coactivator 1-alpha</td>
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<td>Rplp1</td>
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<td>StAR</td>
<td>Steroidogenic acute regulatory protein (StarD1)</td>
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<td>StarD</td>
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<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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Introduction

Hepatic insulin resistance triggers an atherogenic dyslipidaemia, characterised by high levels of triglyceride-rich very low density lipoproteins (VLDL) and their cholesteryl ester-rich remnant particles, low levels of high density lipoprotein (HDL), and small dense low density lipoproteins (LDL) [1-4]. Overproduction of large VLDL is driven by increased flux of non-esterified fatty acids from adipose tissue, upregulation of sterol regulatory element binding protein-1c (SREBP-1c), chronic stimulation of de novo lipogenesis, and reduced fatty acid oxidation in the liver [1-4]. Circulating HDL levels are maintained by hepatic ABCA1 [5], insulin resistance causes loss of hepatic ABCA1 protein expression [5-9] and a strong reciprocal relationship exists between ABCA1 expression and hepatic VLDL output [10-13], integrating triglyceride and cholesterol lipoprotein metabolism. Dysregulated hepatic lipid metabolism also contributes to non-alcoholic fatty liver disease (NAFLD), present in 70-80% of diabetic and obese patients [14], and a condition which ranges from simple steatosis (SS) to steatosis combined with necrosis and inflammation (non-alcoholic steatohepatitis; NASH) which can progress to hepatic fibrosis and cirrhosis [14-18]. Crucially, hepatic accumulation of free cholesterol exacerbates development of NASH and fibrosis, while correction of cholesterol overload reduces the severity of liver disease [16-18]. Since NASH confers a 1.8-fold higher mortality, and itself increases risk of type 2 diabetes and cardiovascular disease [14,15], it is clear that effective treatments for diabetic dyslipidaemia and NAFLD are urgently needed.

One approach could be to sustain, or improve, the efficiency of intracellular cholesterol transport within the liver, particularly when challenged by exposure to elevated levels of glucose, fatty acids and atherogenic lipoprotein remnants. Non-vesicular trafficking of lipids is mediated, in part, by the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain family. The START domain is a ~210 amino acid α/β helix-grip fold, found in 15 distinct mammalian proteins (STARD1-D15), providing an adaptable hydrophobic lipid binding or sensing site for sterols, bile acids, phospholipids, ceramides and small drug molecules [19-21]. The prototypic member of this family, steroidogenic acute regulatory protein (StAR; StarD1) is a mitochondrial cholesterol trafficking protein primarily involved in steroidogenesis [19-21]. In the liver, StAR supplies cholesterol to the cytochrome P450 enzyme, CYP27A1, located on the inner mitochondrial membrane, facilitating metabolism of this sterol via the alternative bile acid pathway [22-25]. The other member of the StarD1 subfamily, StarD3 (Metastatic Lymph Node 64), is a 54 kDa endosomal cholesterol trafficking protein, variously reported to facilitate trafficking of cholesterol to the endoplasmic reticulum, mitochondria and the plasma membrane [26-29]. Certainly,
overexpression of either StarD1 or StarD3 enhances bile acid synthesis via CYP27A1 in primary rat hepatocytes [23].

Importantly, our previous work demonstrated that expression of both StarD1 and StarD3 are regulated by lipid-responsive transcription factors [30], and that overexpression of StarD1, or StarD3, in macrophages enhances expression of ABCA1, and cholesterol efflux to acceptor (apo)lipoproteins [31, 32]. This led to dual hypotheses: that loss of these proteins may be implicated in diabetic dyslipidaemia or steatosis, and that targeting these proteins may help to resolve the problems of low HDL and/or overproduction of VLDL by diabetic liver. Thus, we examined expression of StarD1 and StarD3, members of the StarD1 subfamily of lipid trafficking proteins, and their relationship with dyslipidaemia and hepatic steatosis, in the Zucker (fa/fa) rodent model of genetic obesity [33], and explored their functional role in lipid synthesis and secretion by genetically manipulating expression of these proteins in rat McArdle RH7777 hepatoma cells.

**Methods**

**Materials**

Apolipoprotein A-I was purchased from Athens Research and Technology (Georgia, USA). All other materials were from UK companies, or UK suppliers for the companies indicated. Radiochemicals were purchased from Perkin Elmer, Tri-Fast from PeqLab and cDNA synthesis kits from Bioline. Rabbit polyclonal antibodies to Cyp27A1, Gapdh and StarD3 were purchased from AbCAM, and to StarD1 from Santa Cruz. Sodium pentobarbital was supplied by J.M. Loveridge plc, (Southampton), tissue culture reagents from Lonza, and sterile tissue culture plastics from Greiner. Silencing RNA was purchased from Integrated DNA Technologies, and StarD1 and StarD3 rodent clones (pCMV) from Origene.com via Cambridge Biosciences. Complete™ protease inhibitor cocktail was purchased from Roche, primers and probes (FAM/TAMRA) from EuroGenTech, and Rat Fatty Liver RT²-PCR arrays from SABiosciences (Qiagen). Chemicals, t.l.c.plates and solvents were purchased from Sigma Aldrich.

**Experimental animals**

Heterozygous Zucker rats (Fa/fa) were purchased from Harlan Laboratories (Bicester, UK) and a breeding colony established. Rats were maintained in group (n=3) cages on sawdust bedding, on a 14h-light/10h-dark cycle with standard chow and water provided *ad libitum*. Male lean (Fa/?; n=10) and obese (fa/fa; n=6), and female lean (n=5) and obese (n=4) rats were terminally anaesthetized at four months, using pentobarbital sodium (100mg kg⁻¹, I.P); blood was collected by cardiac puncture, and plasma levels of glucose and lipids determined, as described [33]. Principles of laboratory animal care (NIHA publication no. 85-23, revised 1985 (http://grants1.nih.gov/grants/olaw/references/phspol.htm) were
followed. The study was approved by the institution’s Animal Ethics and Welfare Committee, and procedures performed according to the UK Animals (Scientific Procedures) Act, 1986.

**Preparation of tissue samples** Samples (100mg) of liver were suspended in Dulbecco’s phosphate buffered saline (1ml), using a Beadbeater (Thistle Scientific, UK), and a Bligh and Dyer (1959) lipid extraction performed [34]. Lipid extracts were dried under N₂, and lipid mass measured, as described [30-32]. Total RNA from liver (60mg) was isolated after homogenisation in 500µl of TriFast™, genomic DNA removed by treatment with DNase, and cDNA prepared by reverse transcription; negative controls, omitting reverse transcriptase enzyme, were included for each sample. Hepatic protein lysates were prepared in RIPA buffer plus Complete™protease inhibitor cocktail.

**Cell culture and experiments** Rat McArdle RH7777 (CRL-1601; ATCC) cells, derived from female *Rattus norvegicus* (Morris hepatoma 7777) were maintained in DMEM (Dulbecco’s Modified Eagle Medium) containing foetal bovine serum (10% v/v), penicillin/streptomycin (50U/ml, 50µg/ml respectively), sodium bicarbonate (NaCO₃ 0.06% w/v), HEPES buffer (10mM) and L-glutamine (2mM), at 37°C in a humidified atmosphere of 95% air and 5% CO₂. McArdle RH7777 cells were selected for this study, as they retain the ability to secrete VLDL₁ and VLDL₂ particles [48], containing both apoB48 and apoB100 [49], which can be stimulated in a dose-dependent manner by exogenous non-esterified fatty acids [50]. Successful transfection studies, manipulating DGAT-1 and ACAT-1/2 activity, have been performed in this cell line, resulting in altered secretion of apoB lipoproteins [51]. However, it should be noted that McArdle hepatoma cells differ somewhat in their nutrient partitioning, compared with primary rat hepatocytes [52]. Hansson *et al* (2004) demonstrated that hepatoma cells had a higher rate of fatty acid uptake (2-fold), and a lower rate of fatty acid oxidation (2-fold), compared with primary hepatocytes. Further, McA-RH7777 cells metabolised glucose to lipids or into oxidation, rather than synthesizing glycogen, unlike primary hepatocytes [52]. It has also been demonstrated that McA-RH7777 cells exhibit impaired utilisation of triglycerides derived from recycling of phospholipids, resulting in VLDL particles with a slightly different triglyceride composition than those produced by primary hepatocytes [53]. Transfection of McArdle cells with 2µg of empty vector (EV; pCMV.Neo) or the same vector encoding full length rodent StarD1 or StarD3 was achieved using Amaxa Nucleofector-II (Kit V, protocol T-028). Stable populations were selected using G418 (400µg ml⁻¹). Transient delivery (48h) of silencing RNA (100nM) was delivered using the same protocol.

Lipidation of apoA-I (10µg ml⁻¹; 24h) was assessed in cells labelled with 0.5µCi ml⁻¹ [³H]cholesterol, as described [29-31]. For lipid synthesis and secretion experiments, cells were cultured in serum-free DMEM in presence of [1-2 ¹⁴C]acetate (1.5µCi/ml; 0.5mM), [1-
14C]oleate (1µCi/ml; 0.7mM complexed 1:1 with BSA) or [2-3H]glycerol (1.18µCi/ml; 0.25mM) for 2h, in the presence or absence of 0.35mM oleate/BSA (1:1) complex; concentrations of acetate (0.5mM) and glycerol (0.25mM) remained the same under all conditions tested.

**Lipid Analyses** Samples of media were extracted according to Bligh and Dyer (1959), and cellular lipids using hexane:isopropanol (3:2, v/v), as detailed [30-32], and extracts dried under N2 before resuspension in isopropanol containing rat liver lipids, and separation by t.l.c. using petroleum ether: diethyl ether: glacial acetic acid (90:30:1 by vol.) as the mobile phase. Lipids were identified by comparison with authentic standards, and dpm determined by scintillation counting (Hidex 300SL).

**Gene and protein analyses** Levels of mRNA encoding StarD1 and StarD3 were measured relative to Gapdh, as described [29-31]; specific sequences for primers and fluorescent probes (FAM/TAMRA) StarD3 sense (5’-TGCCCAGGCTGCTTGTTG-3’), antisense (5’-GAGAGTAGAAGTGCCCTCAGACA-3’), probe (5’-CCGTGGGACCCC TTCTATTCTCAGGT-3’); StarD1 sense (5’-AACCTGAAAGCAACACTCTAGTG-3’), antisense (5’-AGGCCCTTTTGCAATGCTCTCCT-3’) probe (5’-CAGGAGCTGTCT ACATCCAGCAAGGA-3’); GAPDH sense (5’-CACTACATGGTCTACATGGTCCA GTATG-3’), antisense (5’-CTTCCCCATTCTCAGCCTTGGACT-3’), probe (5’-CT CCCACGGCAAGTTCAACGGCA-3’).

Gene expression of Dgat1, Dgat2 and Nrih3, relative to Gapdh, were measured using commercial primers and Sybr Green (Maxima SYBR Green QPCR Master Mix), using the following primer sequences: Dgat1 sense (5’-CGGATAGCTACGTTACGTTCTG-3’), antisense (5’-CATCATACTCCCATCTGCCTGCACTCTC-3’); Dgat2 sense (5’-GCCAGGTGAGAAGGAGATG-3’), antisense (5’-GCAGGACGAGAAAGGAGATG-3’); Nr1h3 sense (5’-GCTTCTTTCTTTGCCGGCTGCTT-3’), antisense (5’-AGTGTGCCTTCCCAGCAAAAA-3’), and Gapdh, sense (5’-GTAACCAGGCGTCCGATAC-3’) and antisense (5’-TCTCTGCTTCCTCCTGGTC-3’).

Results are expressed as ratio to Gapdh, using the 2^(-ΔΔCt) comparison method (Applied Biosystems). Hepatic protein lysates were separated using 10% (w/v) SDS PAGE gels, transferred to nitrocellulose membranes, and probed using rabbit polyclonal antibodies to Cyp27A1 (1:1000), StarD1 (1:1000), StarD3 (1:4000) and Gapdh (1:1000) as previously [30-32], except that fluorescently-labelled secondary antibodies (Licor) were employed and bands quantified using a Licor Odyssey FC and Image Studio software.

**Statistical analysis** All values indicate mean±SEM; n denotes numbers of independent determinations. Significant (p<0.05) differences were determined using Student’s t-test, or Kruskal-Wallis with post-test.

**Results**
Hepatic expression of StarD1 and StarD3 in obese, hyperlipidaemic and steatotic fa/fa rats

Expression of StarD1 and StarD3 were examined in four month old obese Zucker (fa/fa) rats, compared with lean (Fa/?) rats. At this age, fa/fa rats are normoglycaemic, but exhibit weight gain and hyperinsulinaemia [33], hyperlipidaemia and/or hepatic lipid accumulation (Figure 1a) compared with lean controls. Obese (fa/fa) male rats (583±9.0g; n=6) weighed significantly (p<0.001) more than lean controls of the same gender (384±11g; n=10); fa/fa female rodents also weighed significantly (p<0.001) more than lean controls (228±11.5g; n=5. versus 494±9.4g; n=4). Blood glucose levels were 8.3±0.3mmol l\(^{-1}\) and 7.7±0.3 mmol l\(^{-1}\) in obese and lean male rats, and 6.7±0.4 mmol l\(^{-1}\) and 7.4±0.4 mmol l\(^{-1}\) in obese and lean female rats respectively. Serum cholesterol, triacylglycerol and phospholipids were elevated in obese male and female rats compared to lean rats of the same gender (Fig 1a). Obese male Zucker rats accumulated greater quantities of hepatic triacylglycerol and phospholipids than obese female rats, while obese female rats were characterized by a more profound hyperlipidaemia (Fig 1a), providing an interesting model in which to investigate gene expression of cholesterol-binding StarD1 subfamily of lipid trafficking proteins.

Levels of mRNA encoding StarD1 and StarD3, relative to Gapdh, are shown in Figure 1b. No significant changes in expression of StarD1 were noted in hepatic tissues (n=4) from lean and obese rats, of either gender. By contrast, levels of StarD3 (48%; p<0.01; n=4) mRNA decreased in hepatic tissues from obese, compared to lean male rats; a similar trend, albeit non-significant, was noted in hepatic tissues from obese compared with lean female rats.

While levels of StarD1 protein were low and difficult to detect by Western blotting, as previously noted [25], expression of StarD3 protein was markedly regulated, and substantively higher (2.27-fold; p<0.001; n=4 and 11.45-fold; p<0.01; n=4) in female lean and obese rats, compared with male lean and obese rats, respectively (Figure 1c). Equally, the presence of obesity induced marked decreases in hepatic levels of StarD3 protein in male (11.65-fold; p<0.01) and female (2.31-fold; p<0.001) rats, resulting in equivalent levels of StarD3 protein in lean male and obese female rats.

These data implied a relationship between hepatic levels of StarD protein and the ability of the liver to store or export lipids. This possible causal modality was explored by stably overexpressing rodent StarD1 (10-fold; p<0.05) and StarD3 (1.32-fold; p<0.05) in rat McArdle (McA-RH7777) hepatoma cells (Figures 2b and 2d) which retain expression of CYP27A1 (Figure 2a). Transient knockdown (4-fold; p<0.05) of StarD3 was achieved using SiRNA (100nM; 48h), compared with a scrambled SiRNA control. The efflux of \[^{3}H\]cholesterol to exogenous apoA-I (10\(\mu\)g ml\(^{-1}\); 24h), one measure of ABCA1 activity, is shown in Figure 2b and 2d. Overexpression of StarD3, but not StarD1, significantly increased the lipidation of apoA-I by 1.66-fold (p<0.01) (Figure 2e), while knockdown of
StarD3 tended to reduce cholesterol efflux slightly (NS) (Figure 2f). This suggested that increasing hepatic levels of StarD3 could enhance circulating levels of HDL, although this finding requires validation in vivo.

Cholesterol synthesis, esterification and secretion into the medium were also studied in these cell lines, under basal conditions, and following treatment with non-esterified fatty acid to stimulate synthesis and secretion of neutral lipids (Figure 3). The incorporation (2h) of [14C]acetate into synthesized and secreted cholesterol, and cholesteryl ester are shown in Figures 3a to 3d. No changes in either synthesized or secreted [14C]cholesterol were noted in cells overexpressing StarD1 or StarD3, but StarD3 overexpression significantly increased incorporation of [14C]acetate into hepatic cholesteryl esters under basal conditions (1.8-fold; p<0.05) and following incubation with 0.35mM oleate (1.3-fold; p<0.01) (Figure 3c). The total cholesterol mass of EV (0.49 ± 0.06 mg mg⁻¹ protein), StarD1 (0.44 ±0.07 mg mg⁻¹ protein) and StarD3 (0.43 ± 0.07 mg mg⁻¹ protein) cell lines did not change significantly, either in the presence or absence of 0.35mM oleate. The incorporation of 0.7mM [14C]oleate into the cholesteryl ester pool within these hepatocytes did not change, although decreased (45%; p<0.01) secretion of cholesteryl [14C]oleate was noted (Figure 3f).

The impact of StarD1 and StarD3 overexpression on synthesis and secretion of triacylglycerol, the major neutral lipid generated in hepatic cells, is shown in Figure 4. Incorporation of [3H]glycerol into hepatic triacylglycerol (2h) (Figure 4a) was significantly increased by overexpression of StarD1 (2.26-fold; p<0.05) and StarD3 (2.04-fold; p<0.05) in McArdle cells under basal conditions. These effects were essentially lost when the cells were challenged by the presence of 0.35mM oleate, which significantly increased (8-fold; p<0.001) biosynthesis of triacylglycerol. The secretion of triacylglycerol (2h) labelled with [3H]glycerol was also stimulated by the addition of 0.35mM oleate (1.9-fold; p<0.05), but was not changed in cells overexpressing StarD1 or StarD3 (Figure 4). Increased [14C]acetate incorporation (2h) into the cellular triacylglycerol pool was noted in StarD3 (1.8-fold; p<0.05) overexpressing cells; again, this effect was abrogated in the presence of 0.35mM oleate (Figure 4c). No changes in secretion of de novo synthesized [14C]triacylglycerol were noted (Figure 4d). The triacylglycerol mass tended to increase in the presence of 0.35mM oleate (2h) in EV (0.89 ± 0.06 mg mg⁻¹ protein) versus 0.35mM oleate 1.07 ± 0.15 mg mg⁻¹ protein; n=3; NS), and this increase was potentiated by overexpression of StarD1 (0.71 ±0.03 mg mg⁻¹ protein versus 0.35 mM oleate 1.52 ± 0.20 mg mg⁻¹ protein; n=3; NS) and StarD3 (0.69 ±0.04 mg mg⁻¹ protein versus 0.35mM oleate 1.18± 0.09 mg mg⁻¹ protein; n=3; p<0.05). Most notably, when the McArdle cell lines were incubated with 0.7mM [14C]oleate (2h), StarD1 but not StarD3, significantly enhanced incorporation of this fatty acid into synthesized (1.43-fold; p<0.05) and secreted (1.6-fold; p<0.05) triacylglycerol; these trends were also observed in the presence of additional unlabelled oleate (Figure 4e). Taken together, these
data indicate differential effects of StarD1 and StarD3 overexpression on hepatic triacylglycerol synthesis and/or secretion.

Accordingly, we measured expression of the genes encoding DGAT-1 and DGAT-2 in samples of rodent liver (Figures 5a and 5b) and in StarD1 and StarD3 overexpressing cells, compared with empty vector controls (Figures 5c and 5d). While no significant differences in hepatic Dgat1 expression were noted in male or female, lean or obese rats, the expression of Dgat2 was significantly higher in obese female rats, when compared with lean female controls (1.86-fold; p<0.01), and with obese male rats (2.3-fold; p<0.01) (Figure 2b).

Overexpression of StarD1 in McA-RH7777 cells induced a 2.3-fold change in expression of Dgat1, and an almost 3-fold increase in Dgat2 expression, compared with EV control. By contrast, Dgat1 levels were 40% lower in StarD3 overexpressing cells, while Dgat2 levels were elevated by 2.2-fold compared with the EV control.

Hepatic expression of the gene encoding LXRα (Nr1h3) was also significantly higher in lean female rats, compared with lean male rats (1.5-fold; p<0.05), obese male rats (2.2-fold; p<0.01) and obese female rats (2.5-fold; p<0.01) (Figure 5e). Levels of Nr1h3 were low in McA-RH7777 EV controls; a modest repression was seen in StarD1 overexpressing cells (23%), and in StarD3 overexpressing cells (58%). However, we note this latter repression was not seen when this gene was investigated in StarD3 overexpressing cells using commercial Q-PCR arrays (Figure 7b), suggesting that the level of this transcription factor may fluctuate, or that the low level of expression of this gene may preclude accurate quantitation.

The effect of StarD3 knockdown (Figure 2a) on hepatic lipid synthesis and secretion is shown in Figure 6. Incorporation of [14C]acetate into cellular cholesterol and cholesteryl ester decreased by 59% (p<0.05) and 81% (p<0.01) (Figure 6a); a decrease (71%; p<0.01) in secretion of de novo synthesized cholesteryl ester was also noted (Figure 6b). The incorporation of [3H]glycerol and [14C]oleate into synthesized and secreted triacylglycerol were not significantly affected by StarD3 knockdown, but incorporation of [14C]acetate into the cellular triacylglycerol pool declined significantly (58.5%; p<0.05). Thus, the knockdown of StarD3 exerted opposite effects to StarD3 overexpression on incorporation of [14C]acetate into cellular triacylglycerol, and also reduced flux of this isotope into de novo synthesized cholesterol and cholesteryl ester pools.

The impact of StarD3 overexpression on expression of genes involved in insulin signalling, cholesterol metabolism and transport, carbohydrate and lipid metabolism, and inflammation and apoptosis, are shown in Figure 7. Modest, but statistically significant increases in expression of Abca1 (1.5-fold; p<0.001) and Dgat2 (1.4-fold; p<0.001) were seen in StarD3 overexpressing hepatocytes, compared with EV, supporting the data in Figures 2, 4 and 5. However, gene expression changes of ≥2-fold were considered biologically significant. Five
housekeeping genes were monitored between test StarD3/EV control hepatocytes, yielding the following fold-change values (n=3) for Actb (0.97±0.04), B2m (0.96±0.04), Hprt1 (1.33±0.05), Ldha (0.91 ±0.01) and Rplp1 (0.89±0.04). Increases in expression of genes encoding peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; 8.6-fold; p<0.001), glucose 6-phosphatase (5.9-fold; p<0.001), cytochrome P450 2E1 (CYP2E1; 2.8-fold; p<0.001), insulin receptor substrate 1 (2.6-fold; p<0.001) and the bile acid farnesoid X receptor (2.4-fold; p<0.001) were noted in StarD3 overexpressing hepatocytes. By contrast, StarD3 overexpression was associated with down-regulation of serpine 1 (3.8-fold; p<0.001), glucose transporter 1 (GLUT1; 2.8-fold; p<0.01), insulin growth factor binding protein 1 (2.1-fold; p<0.001) and caspase 3 (2.1-fold; p<0.001). Despite one previous report [35], expression of genes encoding proteins mediating inflammatory or apoptotic responses did not increase in StarD3 overexpressing hepatocytes, presumably reflecting the physiological levels of StarD3 utilised in this study.

Discussion

The present study demonstrates hepatic expression of StarD3 protein to be repressed by genetic obesity in both male and female rats. Substantively higher levels of StarD3 protein were also noted in female, compared with male rats, raising the possibility that StarD3 might facilitate lipid export, rather than retention in the liver. Alternatively, it was possible that lack of StarD3 might promote lipid accumulation within the liver and/or impair export in obese male rats. Hepatic levels of StarD1, the other member of this subfamily, were detectable by Q-PCR, but not Western blotting, as previously reported [24], and also tended to decline somewhat in genetic obesity. Overexpression of rodent StarD1 and StarD3, and knockdown of StarD3, in McArdle hepatoma cells, allowed evaluation of the causal relationship between these proteins, and lipid synthesis and secretion by the liver. In interpreting these data, it is important to recognise that differences exist between McA-RH7777 cells and primary rat hepatocytes: hepatoma cells preferentially utilise fatty acids rather than glucose, which may reflect the demand for lipid substrates to sustain membrane biogenesis in proliferating cells [48]. Dissection of cholesterol homeostasis in StarD1 overexpressing cells did not yield any significant differences, when compared to the EV; by contrast, overexpression of StarD3 increased the flux of [14C]acetate into the cholesteryl ester pool, and stimulated cholesterol efflux to exogenous apoA-I. The latter finding agrees well with previous studies demonstrating increased expression of ABCA1 and efflux of cholesterol to apoA-I in human macrophages [32], and with the suggestion that STARD3 can access a pool of cholesterol in ‘early’ late endosomes, readily amenable to recycling to the plasma membrane [27]. However, the FFAT motif within an acidic tract in StarD3 interacts with ER-anchored VAP proteins to increase the formation of inter-organelle membrane contact sites between late
endosomes and the ER [26], and StarD3 can also deliver cholesterol to mitochondria [23, 28, 29, 36, 37]. Indeed, enhanced flux of $[^{14}\text{C}]$acetate into the cholesteryl ester pool suggests increased total cholesterol biosynthesis and, therefore, efficient transport of cholesterol away from the ER. Knockdown of StarD3 decreased de novo synthesis of hepatic cholesterol and cholesteryl esters, which implies accumulation of cholesterol at the ER, and may reflect loss of cholesterol delivery from endosomes to the plasma membrane or mitochondria. This conclusion is supported by the modest increases in cholesteryl ester mass noted in mice in which the START domain of StarD3 was deleted [38]. Analysis of the synthesis of triacylglycerol from differing precursors revealed differential effects of StarD1 and StarD3: StarD1 overexpression tended to preferentially facilitate the incorporation of exogenously derived oleate into the triacylglycerol pool, while StarD3 overexpression increased formation of de novo synthesized triacylglycerol. Knockdown of StarD3 blocked incorporation of $[^{14}\text{C}]$acetate into the hepatic triacylglycerol pool, without reducing incorporation of $[^{3}\text{H}]$glycerol. Basal levels of triacylglycerol synthesis were not, therefore, altered by StarD3 knockdown; it is possible that enhanced uptake of glucose via GLUT1 (Figure 6) could have diluted the pool of radiolabelled acetyl CoA available for incorporation into lipid pools.

Diacylglycerol acyl transferase-2 (DGAT-2) utilises glycerol-3-phosphate and de novo synthesized fatty acids as substrates to form de novo synthesized triacylglycerol [39]. A small amount of this triacylglycerol is sequestered for secretion with apoB, and some is stored as cytosolic lipid droplets; however, the majority undergoes hydrolysis to yield partial glycerides which can then be reesterified by DGAT-1, which utilises exogenously derived fatty acids. The activity of DGAT1 on the luminal side of the endoplasmic reticulum membrane generates triacylglycerol used to lipidate nascent VLDL, prior to secretion [39]. Invoking this paradigm, it is possible that overexpression of StarD3 may preferentially promote the activity of DGAT-2, while StarD1 selectively enhances the activity of DGAT-1.

Certainly, gene expression levels of Dgat2 appear to be with linked with protein levels of StarD3: hepatic levels of StarD3 are highest in lean female rats which express Dgat2 at higher levels than male lean and obese rats. Protein levels of StarD3 decline in obese female rats, and this is mirrored by a decline in Dgat2 expression, while overexpression of StarD3 enhances the expression of Dgat2 while repressing expression of Dgat1. By contrast, overexpression of StarD1 appears to enhance the expression of both Dgat1 and Dgat2, in agreement with previous findings indicating that both pathways are required to potentiate increased synthesis of triacylglycerol from preformed oleate [39] (Figures 4a and 4e).

At present, it is only possible to speculate as to the mechanisms by which these outcomes are achieved. Changes in endoplasmic reticulum lipid composition, implied by data in
Figures 3 and 5, suggest activation of SREBPs which induce the expression of lipogenic enzymes. Alternatively, enhanced transfer of cholesterol to mitochondria can enhance the generation of 27-hydroxycholesterol, activating Liver X receptors which stimulate expression of ABCA1, but also Srebp-1c, triggering lipogenic responses [31]. It is evident that lean female rats express higher hepatic levels of the gene encoding LXRα than lean and obese male animals, and that LXRα expression also declines with obesity in female rats (Figure 5e).

Certainly, StarD3 overexpression induces marked changes in gene expression in McArdle hepatoma cells, which may variously influence insulin resistance in the liver. For example, induction of PGC1α [40], insulin receptor substrate 1 [41] and the farnesoid X receptor [42] have been associated with improved mitochondrial function, metabolic responses and/or sensitisation of the liver to insulin. However, increased expression of glucose 6-phosphatase, an established target of the transcriptional coactivator function of PGC-1α, suggests StarD3 overexpression could exacerbate glycaemia in the fasting state [43]; moreover, induction of CYP2E1 is thought to enhance oxidative stress, another pathogenic trigger for onset of fatty liver disease [44]. Perhaps most intriguingly, StarD3 overexpression markedly repressed the expression of serpine 1 (plasminogen activator inhibitor 1; PAI1), elevation of which was recently identified as a link between obesity, diabetes and cardiovascular disease [45].

In summary, the mitochondrial and endosomal cholesterol-trafficking proteins, StarD1 and StarD3, appear to exert differing effects on hepatic lipid metabolism in McArdle hepatoma cells. Mitochondrial StarD1 increases the utilisation of exogenous (preformed) fatty acids for triacylglycerol synthesis and secretion, but impacts minimally on cholesterol homeostasis. By contrast, endosomal cholesterol-trafficking protein, StarD3, appears to impact predominantly on cholesterol efflux to apoA-I, rather than the secretory pathway, and facilitates de novo biosynthesis of neutral lipids. The former suggests one way of improving circulating levels of HDL, while in the latter context, it should be noted that it is not the accumulation of triacylglycerol per se that is damaging to the liver [46], but rather build-up of biologically active metabolites such as long-chain acyl CoAs, diacylglycerols and ceramides, which can impair insulin signalling and promote inflammatory responses [39]. Indeed, it has been suggested that triacylglycerol accumulation protects against fatty acid-induced lipotoxicity [47]; no evidence for increased inflammation or apoptosis was noted in StarD3 overexpressing cells. However, the link between StarD3 and DGAT-2 may potentiate the triglyceridaemia associated with Type 2 diabetes. Further, since StarD3 overexpression also induced expression of a number of genes which impact variously on hepatic insulin resistance, further studies on the role of this cholesterol transport protein in diabetic murine...
models are clearly warranted. Thus, targeting StarD3 may increase circulating levels of HDL and protect the liver against lipotoxicity, while loss of expression of this protein may be part of the pathogenesis associated with hepatic steatosis.

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Figure Legends

Figure 1
Serum and liver levels of cholesterol, triacylglycerol and choline-containing phospholipids from lean male (LM; n=10), obese male (OM n=6), lean female (LF; n=5) and obese female (OF; n=4) Zucker rats (1a). Levels of StarD1 and StarD3 mRNA, relative to Gapdh, are in Figure 1b, and expression of StarD3 protein in Figure 1c; n=4. *p<0.05, **p<0.01 compared with lean controls; §§ p<0.01 and §§§ p<0.001 for obese female compared with obese male animals.

Figure 2
Expression of CYP27A1 in wild type McArdle cells, relative to Gapdh, is shown in Figure 2a. Expression of StarD1 and StarD3 proteins, relative to Gapdh, in McArdle hepatoma cells, stably transfected with empty vector (EV; pCMV.Neo), or the same vector encoding each Gene of Interest (GOI) are shown in Figure 2b, with quantitation in three experiments shown in Figure 2d. Transient knockdown of StarD3, relative to Gapdh, in wild type McArdle cells, by SiRNA (100nM; 48h), compared with siRNA control (100nM) is shown in Figure 2c, with quantitation in three experiments shown in Figure 2e. Lipidation of apoA-I (10μg ml⁻¹; 24h) with [³H]cholesterol by StarD1 and StarD3 overexpressing cell lines, compared with empty vector control, is shown in Figure 2e, and following StarD3 knockdown is shown in Figure 2f. **p<0.01 compared with EV; n=3.

Figure 3
Incorporations of 0.5mM [¹⁴C]acetate into cellular (3a) and secreted (3b) cholesterol ± 0.35mM unlabelled (cold) oleate, from EV, StarD1 and StarD3 overexpressing hepatocytes; incorporations of this radiolabel into cellular (3c) and secreted (3d) cholesteryl esters, and incorporation of 0.7mM [¹⁴C]oleate ± 0.35mM unlabelled oleate, into cellular (3e) and secreted (3f) cholesteryl esters. *p<0.05 and **p<0.01 compared with EV; n=3.

Figure 4
Incorporations of 0.25mM [³H]glycerol into cellular (4a) and secreted (4b) triacylglycerol ± 0.35mM unlabelled (cold) oleate, from EV, StarD1 and StarD3 overexpressing hepatocytes; incorporations of 0.5mM [¹⁴C]acetate into the same pools, under the same conditions, are in Figures 4c and 4d; incorporations of 0.7mM [¹⁴C]oleate into cellular (4e) and secreted (4f) triacylglycerol. *p<0.05 and ***p<0.001 compared with EV; n=3.

Figure 5
Expression of Dgat1, Dgat2 and Nr1h3, relative to Gapdh, in liver samples from lean and obese, male and female rats are shown in Figure 5a, 5b and 5e, respectively; n=4, **p<0.01, ***p<0.001 compared with lean controls; §§ p<0.01 for obese female compared with obese male animals. Levels of Dgat1, Dgat2 and Nr1h3 in EV, StarD1 and StarD3 overexpressing
hepatoma cells are shown in Figure 5c, 5d and 5f; **p<0.01, ***p<0.001 for gene expression differing from the EV control; n=4.

**Figure 6**
Effect of transient StarD3 knockdown (100nM SiRNA; 48h) on incorporation of 0.5mM \[^{14}\text{C}\]\text{acetate} into cholesterol and cholesteryl ester pools, and incorporation of 0.7mM \[^{14}\text{C}\]\text{oleate} into cholesteryl esters, for cellular (5a) and secreted (5b) lipids, respectively, compared with SiRNA control (100nM). Incorporations of 0.25mM \[^{3}\text{H}\]\text{glycerol}, 0.5mM \[^{14}\text{C}\]\text{acetate} and 0.7mM \[^{14}\text{C}\]\text{oleate} into cellular (5c) and secreted (5d) triacylglycerol, following StarD3 knockdown. Nine independent transfections were performed; *p<0.05, **p<0.01 compared with SiRNA control.

**Figure 7**
Effect of overexpression of StarD3 (test)/EV(control) on expression of genes involved in insulin signalling (6a) cholesterol metabolism and transport (6b), carbohydrate and fat metabolism (6c) and inflammation and apoptosis (6d). **p<0.01 and ***p<0.001 for genes exhibiting ≥2-fold up- or down-regulation; n.d.: ‘not detected’; n=3.